

Microbial ecology of nitrification in engineered water treatment systems

by

Michelle Marie McKnight

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Examining Committee Membership

The following served on the Examining Committee for this thesis. The decision of the Examining Committee is by majority vote.

External Examiner

Dr. Ameet J. Pinto
Associate Professor
School of Civil and Environmental Engineering
Georgia Institute of Technology, GA, USA

Supervisor

Dr. Josh D. Neufeld
Professor
Department of Biology
University of Waterloo, ON, Canada

Internal Members

Dr. Barbara J. Butler
Continuing Lecturer Emeritus
Department of Biology
University of Waterloo, ON, Canada

Dr. Brendan J. McConkey
Associate Professor
Department of Biology
University of Waterloo, ON, Canada

Internal-external Member

Dr. Wayne J. Parker
Professor
Department of Civil and Environmental Engineering
University of Waterloo, ON, Canada

Author's Declaration

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

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

Statement of Contributions

Michelle M. McKnight was the sole author for **Chapters 1 and 5**, which were written under the supervision of Dr. Josh D. Neufeld. A version of section 1.3.2 (Chapter 1) was also published in the following review, on which I am a co-author:

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This thesis consists in part of three manuscripts written for publication, with the following contributions:

Chapter 2 – Josh Neufeld assisted with sample collection from members of the Kitchener-Waterloo Aquarium Society. Library preparation and high-throughput sequencing of the 16S rRNA gene was done in collaboration with Alexander Umbach. Karoline Faust and Haris Zafeiropoulos provided advice related to network analysis. Nicolette Shaw assisted in writing R scripts used for filtering amplicon sequencing data. A version of this chapter has been published as:

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Chapter 3 – All of the experimental design and sample collection was coordinated by Natasha Szabolcs and was previously published in her MSc thesis, including data analysis for a portion of the collected samples. Under my supervision as a co-op student, Alyssa Graham performed DNA extractions from collected samples, assisted with 16S rRNA gene sequencing, and measured concentrations of ammonia, nitrite, and nitrate from collected water samples. Rachel Beaver performed high-throughput sequencing.

Chapter 4 – Narasimman Lakshminarasimman was responsible for collection of samples from the municipal wastewater treatment plant presented in this chapter, along with providing measurements and calculations associated with plant performance and operational data. Rachel Beaver performed library preparation of extracted DNA samples and high-throughput sequencing.

Abstract

Nitrification is performed primarily by chemolithoautotrophic microorganisms and is important for nitrogen transformation in aquatic environments. The understanding of nitrification has evolved over the years from a previously understood two-step process mediated by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) that perform ammonia oxidation to nitrite, and nitrite-oxidizing bacteria (NOB) that perform nitrite oxidation to nitrate. More recent research has revealed the existence of complete ammonia-oxidizing (“comammox” or CMX) bacteria from the genus *Nitrospira* that are capable of oxidizing ammonia to nitrate. With three groups of ammonia oxidizers often existing in the same environment, research is needed to understand the microbial ecology of nitrifying communities. These ammonia oxidizers play important roles in engineered systems, including wastewater treatment plants (WWTP) and aquarium biofilters, where they transform ammonia waste ($\text{NH}_3/\text{NH}_4^+$) to less toxic nitrate (NO_3^-) via nitrite (NO_2^-).

Prior to the discovery of comammox *Nitrospira*, previous research revealed that AOA dominated over AOB in freshwater aquarium biofilters. In Chapter 2, aquarium biofilter microbial communities were profiled and the abundance of all three known ammonia oxidizers were quantified using 16S rRNA gene sequencing and quantitative PCR (qPCR), respectively. Biofilter and water samples were each collected from representative residential and commercial freshwater and saltwater aquariums. Distinct biofilter microbial communities were associated with freshwater and saltwater biofilters. Comammox *Nitrospira amoA* genes were detected in all 38 freshwater biofilter samples and dominant in 30, whereas AOA were present in 35 freshwater biofilter samples and only dominant in 7 of them. The AOB were at relatively low abundance within biofilters, except for the aquarium with the highest ammonia concentration. For saltwater biofilters, AOA or AOB were differentially abundant, with no comammox *Nitrospira* detected. Additional sequencing of *Nitrospira amoA* genes revealed differential distributions, suggesting niche adaptation based on water chemistry (e.g., ammonia, carbonate hardness, and alkalinity). Network analysis of freshwater microbial communities demonstrated positive correlations between nitrifiers and

heterotrophs, suggesting metabolic and ecological interactions within biofilters. These results indicate that comammox *Nitrospira* play a previously overlooked but important role in home aquarium biofilter nitrification.

Following the identification of comammox *Nitrospira* among dominant ammonia oxidizers in freshwater aquarium biofilters, Chapter 3 monitored microbial community succession and water chemistry for three independent home aquariums during their first 12-weeks after start-up, with weekly collection of biofilter beads and sponge samples. Extracted DNA from biofilter samples was used for 16S rRNA gene sequencing to determine microbial community composition and quantitative PCR (qPCR) to quantify ammonia monooxygenase (*amoA*) genes. Water samples were also collected weekly for measurements of ammonia, nitrite, and nitrate. Biofilter nitrification activity reduced ammonia and nitrite concentrations below detectable limits by week 3 in two of the three aquariums, which showed comparable nitrification activity by the week 8 time point. Detection of ammonia oxidizers by qPCR coincided with ammonia oxidation activity for all systems. The two aquariums with nitrification occurring by week 3 contained live plants, whereas the aquarium with delayed nitrification did not, suggesting that live plants might provide an effective nitrifier inoculation source for aquarium establishment. Additionally, a preference in biofilter material was observed for detected AOA, which were present in higher abundance in bead samples compared to sponge samples. Although the tested aquaria differed in the timing and prevalence of ammonia oxidizers in biofilters during community establishment, samples from all three aquaria were consistently dominated by comammox *Nitrospira* by the end of 12 weeks. Additional metagenomic functional profiling of week 12 biofilter samples confirmed the presence of AOA *amoA* genes and comammox *Nitrospira amoA* genes as detected by qPCR for all aquaria, with nitrite oxidation marker gene *nxrB* for both comammox *Nitrospira* spp. and canonical NOB *Nitrospira* spp. also detected. Although this work sheds light on how ammonia oxidizers establish in residential freshwater aquaria, further research is needed to test factors that impact the establishment of nitrifiers populations, such as inoculation sources, fish loads, and water chemistry.

Novel WWTP technologies, including membrane aerated biofilm reactors (MABR), aim to improve nitrogen removal, reduce energy consumption, and improve nitrification in cold weather conditions. The municipal WWTP in Southern Ontario was upgraded with an MABR system in 2022, which was installed upstream of the existing conventional activated sludge (CAS) system at a municipal scale. This current study evaluates the impact of an MABR upgrade on the CAS and MABR microbial communities, with this MABR system being the largest in North America based on media surface area at the time of its installation. In Chapter 4, the microbial community of the CAS system was characterized before and after the MABR upgrade to evaluate the impact of the upstream MABR installation on the functional potential of the downstream activated sludge. Microbial community characterization was done using 16S rRNA gene amplicon sequencing of the V4-V5 region and additional MABR biofilm samples were characterized using metagenomic analysis to evaluate the functional potential of the biofilm for nitrification and denitrification. Before upgrade, the CAS system included the nitrifiers *Nitrosomonas* (AOB) and *Nitrobacter* (NOB), which exhibited seasonal abundance and activity differences. Following the upgrade to the hybrid MABR-CAS system, seeding effects from the MABR biofilm increased diversity of the CAS, including nitrifiers. Along with AOB, *Nitrospira* NOB and comammox *Nitrospira* were present in the MABR. Metagenomic profiling showed that biofilm microbial communities were well equipped to perform nitrification, denitrification, and phosphate removal. Characterization of the microbial communities in the plant showed that the MABR technology had a positive impact in increasing microbial diversity in this treatment system, along with an increased inventory of nitrifiers with diverse metabolic capabilities.

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Dedication

For my late grandparents, whose paths I am glad to have followed along my doctoral journey.

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List of Abbreviations

AMO	Ammonia monooxygenase
<i>amoA</i>	Subunit A of the ammonia monooxygenase gene
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
AOM	Ammonia-oxidizing microorganism
Anammox	Anaerobic ammonia oxidation
ASV	Amplicon sequencing variant
ATP	Adenosine triphosphate
AXIOME3	Automation, eXtension, and Integration of Microbial Ecology 3
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BNR	Biological nitrogen removal
BOD	Biological oxygen demand
bp	Base pair
<i>Ca.</i>	Candidatus
CaCO ₃	Calcium carbonate
CAS	Conventional activated sludge
CMX	Complete ammonia oxidation (comammox)
CO ₂	Carbon dioxide
DADA2	Divisive Amplicon Denoising Algorithm 2
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
dGH	Degrees of general hardness
dKH	Degrees of carbonate hardness
DNRA	Dissimilatory nitrate reduction to ammonia
dNTP	Deoxynucleotide triphosphate
DO	Dissolved oxygen
DOM	Dissolved organic matter
DPAO	Denitrifying PAO
EtBr	Ethidium bromide
ETC	Electron transport chain

GAO	Glycogen accumulating organism
GTDB	Genome taxonomy database
HAO	Hydroxylamine dehydrogenase
HMM	Hidden Markov model
HS	High sensitivity
$K_{m(app)}$	Apparent half-saturation constant
KWAS	Kitchener-Waterloo Aquarium Society
LED	Light emitting diode
MABR	Membrane aerated biofilm reactor
MAG	Metagenome assembled genome
MLSS	Mixed liquor suspended solids
N_2	Dinitrogen
<i>napA</i>	Subunit A of the periplasmic dissimilatory nitrite reductase gene
<i>narG</i>	Subunit G of the membrane-bound dissimilatory nitrite reductase gene
NCBI	National Center for Biotechnology Information
NH_3	Ammonia
NH_4^+	Ammonium ion
<i>nirK</i>	Subunit K of the copper-containing nitrate reductase gene
<i>nirS</i>	Subunit S of the heme-containing nitrate reductase gene
NO	Nitric oxide
N_2O	Nitrous oxide
NO_2^-	Nitrite ion
NO_3^-	Nitrate ion
NOB	Nitrite-oxidizing bacteria
<i>norB</i>	Subunit B of the cytochrome <i>c</i> -dependent nitric oxide reductase gene
<i>nosZ</i>	Subunit Z of the nitrous oxide reductase gene
NR	Non-redundant
NXR	Nitrite oxidoreductase
<i>nxB</i>	Subunit B of the nitrite oxidoreductase gene
ORF	Open reading frame
OPA	Ortho-phthalaldehyde
OTE	Oxygen transfer efficiency
PAO	Phosphate accumulating organism
PCoA	Principal coordinate analysis

PCR	Polymerase chain reaction
PHB	Polyhydroxybutyrate
PMF	Proton motive force
PNA	Partial nitrification anammox
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
QIIME2	Quantitative Insights Into Microbial Ecology 2
qPCR	Quantitative PCR
RAS	Recirculating aquaculture system
RBC	Rotating biological contactor
RCF	Relative centrifugal force
RNA	Ribonucleic acid
<i>rpoB</i>	Beta subunit of the prokaryotic RNA polymerase gene
rRNA	ribosomal RNA
SD	Standard deviation
SIP	Stable-isotope probing
SNA	Simultaneous nitrification anammox
SND	Simultaneous nitrification and denitrification
spp.	Species
SRA	Sequence Read Archive
SRT	Solids retention time
SSU	Small subunit
TAN	Total ammonia nitrogen
TKN	Total Kjeldahl nitrogen
WWTP	Wastewater treatment plant

“Dans les champs de l’observation, le hasard ne favorise que les esprits préparés.”

– Louis Pasteur

Chapter 1

Introduction to nitrification and the nitrogen cycle

1.1 Nitrification

1.1.1 The nitrogen cycle

Nitrogen plays a crucial role in the existence of life on Earth, comprising the backbone of amino acids, the nitrogenous bases of nucleic acids, and in the structure of essential energy carrying molecules (e.g., adenosine triphosphate; ATP). In addition, nitrogenous compounds, such as ammonia ($\text{NH}_3/\text{NH}_4^+$) and nitrate (NO_3^-), are used by many microorganisms for energy generation as electron donors and acceptors, respectively. Microorganisms are responsible for recycling much of the organic nitrogen from dead biomass back to inorganic ammonia (i.e., mineralization or ammonification), which can be assimilated by microorganisms for the biosynthesis of amino acids (Figure 1.1). Inorganic nitrogen as nitrate can also be assimilated by plants and microorganisms, the latter via assimilatory nitrate reduction pathways to generate ammonia. Overall, biogeochemical cycling of nitrogen has significant implications for Earth's ecosystems because access to nitrogen in the environment is essential for life.

The largest global reservoir of nitrogen exists in the Earth's atmosphere in the form of dinitrogen (N_2) gas (3.9×10^9 Tg of N), inaccessible to the majority of living organisms (Kuypers *et al.*, 2018). To gain access to this inert atmospheric nitrogen reserve, microorganisms have evolved the ability to fix dinitrogen gas into the biologically accessible form of ammonia (Figure 1.1). This energetically expensive process, termed nitrogen fixation, is conducted both by free-living microorganisms and microorganisms living in symbiosis with plants (e.g., legumes). Microbial nitrogen fixation into biologically usable ammonia occurs alongside the abiotic formation of NO_x compounds by atmospheric nitrogen fixation by lightning (Hill *et al.*, 1980), comprising the natural processes responsible for the flux of nitrogen into terrestrial and aquatic ecosystems. Over the last century, human activity has increased the flux of nitrogen

from atmospheric sources with the invention of the Haber-Bosch process that allows for industrial-scale chemical conversion of dinitrogen into ammonia (Haber *et al.*, 1915). The production of ammonia for fertilizers has allowed for increased productivity of agricultural crops, responsible for approximately half of the world's food supply that feeds the current global human population (Smil, 2011).

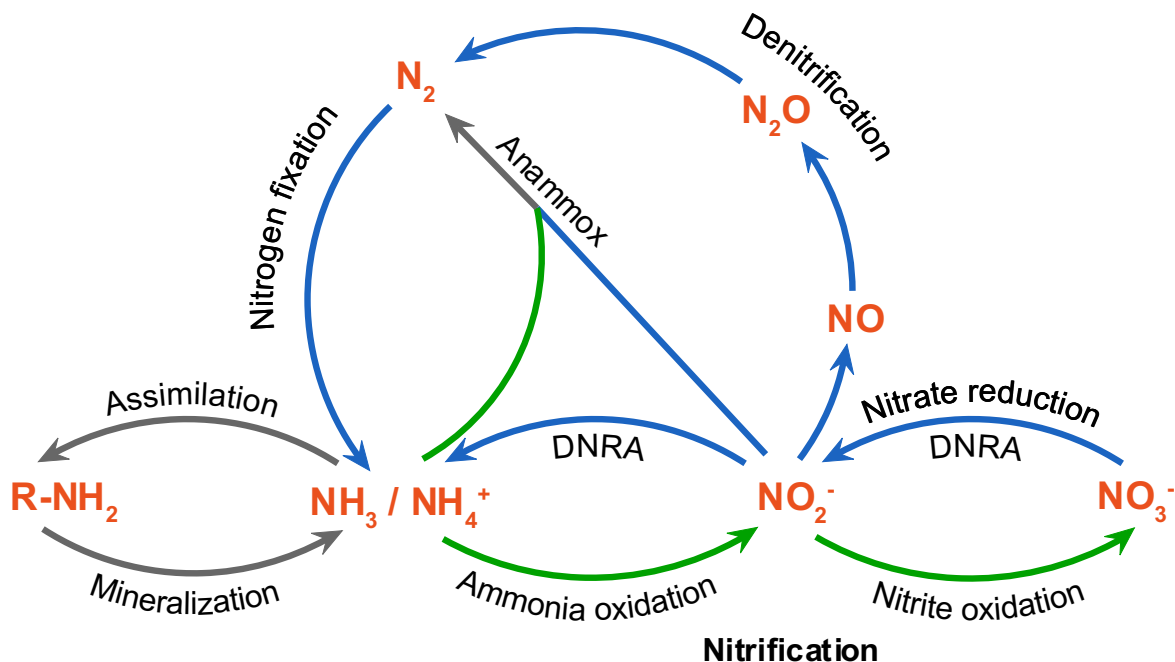


Figure 1.1 The biogeochemical nitrogen cycle. Oxidation reactions are shown with green arrows, reduction reactions are shown with blue arrows, and non-redox reactions are shown with grey arrows. DNRA is dissimilatory nitrite reduction to ammonia, anammox is anaerobic ammonia oxidation. $R-NH_2$ indicates organic molecules containing nitrogen as amine groups (i.e., biomass), NH_3/NH_4^+ indicates the un-ionized and ionized form of ammonia, NO_2^- is nitrite, NO is nitric oxide, NO_3^- is nitrate, N_2O is nitrous oxide, and N_2 is dinitrogen. This figure is adapted from Stein and Klotz (2016) and a modified version is currently published in the review paper by Al-Ajeel *et al.* (2022).

Not all the ammonia fertilizer applied to agricultural fields is fully assimilated by crops because some of the ammonia is converted to nitrite (NO_2^-) and subsequently nitrate (NO_3^-) by nitrifying microorganisms. Although nitrate can also be accessed by plants as a nitrogen source, its mobility in soil is much higher than that of ammonia or ammonium because its negative charge repels the negative charge of soil particles, leading to soil runoff and nitrogen loss. Nitrites and nitrates in the soil produced from nitrification activity can also be used by denitrifying microorganisms. This denitrification results in nitrogen loss to the atmosphere, along with emissions of nitrous oxide (N_2O) when incomplete denitrification occurs (Figure 1.1). Nitrous oxide is a greenhouse gas that is 300 times more potent than CO_2 (IPCC, 2013), making the application of nitrogen fertilizers an indirect source of greenhouse gas emissions.

Despite the negative impacts of nitrification activity for agriculture, this process is nonetheless important for recycling of nitrogenous waste. Humans generate large quantities of wastewater from industrial and municipal sources that contain nitrogenous wastes in both organic and inorganic forms. The treatment of this wastewater is important for the health of both aquatic and terrestrial ecosystems, and for the protection of drinking water supplies. Nitrogenous waste is excreted by animals in various forms, such as ammonia/ammonium ($\text{NH}_3/\text{NH}_4^+$), urea ($\text{CO}(\text{NH}_2)_2$), and uric acid ($\text{C}_5\text{H}_4\text{N}_4\text{O}_3$), which can accumulate to high levels in municipal wastewater and closed water systems like aquariums and aquaculture environments. If untreated, un-ionized ammonia can be toxic to aquatic life and ammonium release into natural ecosystems can lead to eutrophication and the growth of toxic algal blooms and subsequent anoxia (Glibert *et al.*, 2014). Therefore, the process of ammonia removal or transformation is an integral component of wastewater treatment.

Most of the ammonia removal from wastewater during treatment processes occurs via the chemolithoautotrophic process of nitrification, which converts nitrogen from its most reduced form (NH_3) to its most oxidized form (NO_3^-). Ammonia oxidation is the first step in the process of nitrification, which is followed by the oxidation of nitrite to nitrate (Figure 1.1). Although the intermediary product of

nitrification, nitrite, is also toxic to humans and aquatic life, the oxidized form of nitrate has a much lower toxicity (Kroupová *et al.*, 2018). Like in soil environments, both nitrite and nitrate can be used by other microorganisms in aquatic environments to perform the denitrification pathways of the nitrogen cycle, whereby nitrogen is converted back into gaseous forms in the atmosphere via sequential reduction reactions, including nitric oxide (NO), nitrous oxide (N₂O), and dinitrogen (N₂) (Figure 1.1).

Denitrification processes also include the anaerobic ammonia oxidation (anammox) process, in which bacteria use both ammonia and nitrite to form dinitrogen gas for energy generation in the absence of oxygen (Kuenen, 2008). Total nitrogen removal during wastewater treatment is desirable, as nitrate released in treated effluent into the environment can still contribute to eutrophication, as mentioned for agricultural runoff, despite its lower overall toxicity. Considering that nitrification is either desirable or undesirable, depending on the environment, a clear understanding of the microorganisms responsible for this process and the factors influencing nitrification are important to understand.

1.1.2 History of nitrification

For a long time, the process of nitrification was thought to be divided metabolically between two groups of chemolithoautotrophic bacteria that oxidize inorganic N to obtain energy: ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). The AOB, which affiliate with the classes *Betaproteobacteria* and *Gammaproteobacteria*, were first discovered in the late 19th century with the isolation of *Nitrosomonas europaea* (Winogradsky, 1890). For over a century, *N. europaea* and other AOB species were studied in various environments and considered the sole microbial catalysts of ammonia oxidation. However, the discovery of ammonia-oxidizing archaea (AOA) in 2005 with the cultivation of *Nitrosopumilus maritimus* from marine aquarium gravel revealed that some archaeal representatives of mesophilic *Crenarchaeota*, as they were then known, could perform ammonia oxidation (Könneke *et al.*, 2005). All known AOA belong to the phylum *Nitrososphaerota* under NCBI taxonomy classifications (Schoch *et al.*, 2020; Oren and Garrity, 2021) or *Thermoproteota* under GTDB taxonomy, whose members are recognized as chemolithoautotrophs (Hatzenpichler, 2012). The phylum

was formerly known under the name *Thaumarchaeota* (Brochier-Armanet *et al.*, 2008), while GTDB taxonomy had previously classified AOA under the phylum *Crenarchaeota* until release 95 in July 2020, where AOA became reclassified under the novel phylum *Thermoproteota* (Parks *et al.*, 2022).

Phylogenetically, AOA are divided into groups based on *amoA* gene sequence phylogeny, including group I.1a, group I.1b, and group I thermophilic AOA. Surveys demonstrate that AOA are ubiquitous in aquatic and terrestrial ecosystems, indicating their importance in global nitrification (Hatzenpichler, 2012).

To date, only bacteria have been identified as nitrite oxidizers, with the majority of NOB exhibiting chemolithoautotrophic metabolism. Although, phototrophic species of NOB have also been identified (Griffin *et al.*, 2007; Hemp *et al.*, 2016). Since the first isolation of chemolithoautotrophic NOB alongside AOB by Winogradsky (1890), a phylogenetically diverse number of species have been discovered across four different phyla. The NOB belong to seven genera, named with the genus prefix of “*Nitro*”, and are found in the phyla of *Proteobacteria*, *Nitrospinae*, *Chloroflexi*, and *Nitrospirae*. The largest diversity of NOB can be found in the *Nitrospirae* (*Nitrospirota*; GTDB), which includes *Nitrospira* spp. across more than seven different lineages (Daims *et al.*, 2016). As first noted upon their discovery, NOB can be challenging to purify in laboratory cultures, with some culture purifications reported to have taken 12 years (Lebedeva *et al.*, 2008). This makes the use of genetic tools helpful for exploring NOB distributions. Different NOB species are adapted to a wide range of temperatures and environments, including soils, freshwater, marine, geothermal, and engineered water treatment systems (Daims *et al.*, 2016). The NOB maintain a syntrophic partnership with ammonia oxidizers, where their coexistence allows NOB to utilize the nitrite produced by ammonia oxidizers for energy generation (Okabe *et al.*, 2014).

The long-held conventional view of nitrification as being metabolically divided among different populations of bacteria has changed more recently by the discovery of complete ammonia-oxidizing (comammox) bacteria, capable of performing both ammonia and nitrite oxidation. Increased biomass

yields and reduced growth rates associated with theoretically proposed comammox pathways led to the prediction that this process might be associated with low nutrient and high retention environments, such as biofilms, where they could outcompete conventional ammonia oxidizers (Costa *et al.*, 2006). Several years later, comammox bacteria were first reported from a deep oil exploration well and a recirculating aquaculture system (RAS) biofilter, where metagenomic bins associated with the NOB genus *Nitrospira* contained both sets of genes required for ammonia and nitrite oxidation (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Prior to the discovery of comammox, the *Nitrospira* genus was only known to contain strict NOB (Ehrich *et al.*, 1995; Lebedeva *et al.*, 2011).

Since their discovery, other metagenomic surveys have identified comammox *Nitrospira* in a wide range of environments, including drinking and wastewater treatment plants (Chao *et al.*, 2016; Pinto *et al.*, 2016), water distribution systems (Wang *et al.*, 2017), aquaculture biofiltration units (Bartelme *et al.*, 2017), soils (S. Xu *et al.*, 2020), freshwater biofilms (Ghimire-Kafle *et al.*, 2024), and lake sediments (Y. Xu *et al.*, 2020). Based on *amoA* gene phylogeny, two separate clades of comammox bacteria have been identified, clades A and B, both of which belong to lineage II within the genus *Nitrospira* (Daims *et al.*, 2015, 2016). To date, cultured representatives and enrichments of comammox *Nitrospira* belong to clade A, with clade B representatives solely identified from metagenome-assembled genomes (MAGs; Fowler *et al.*, 2018; Orellana *et al.*, 2018). Within *Nitrospira* lineage II, comammox *Nitrospira* do not form a monophyletic group, making it difficult to differentiate between comammox *Nitrospira* and other NOB based on 16S rRNA and nitrite oxidation genes, contributing to the delayed discovery of comammox *Nitrospira* (Pjevac *et al.*, 2017). Currently, there are three known groups of ammonia-oxidizing microorganisms, including AOB, AOA, and comammox *Nitrospira*, which overlap with the NOB in their ability to oxidize nitrite to nitrate. These ammonia-oxidizers are found across many natural and engineered environments, often existing alongside one another (Figure 1.2).

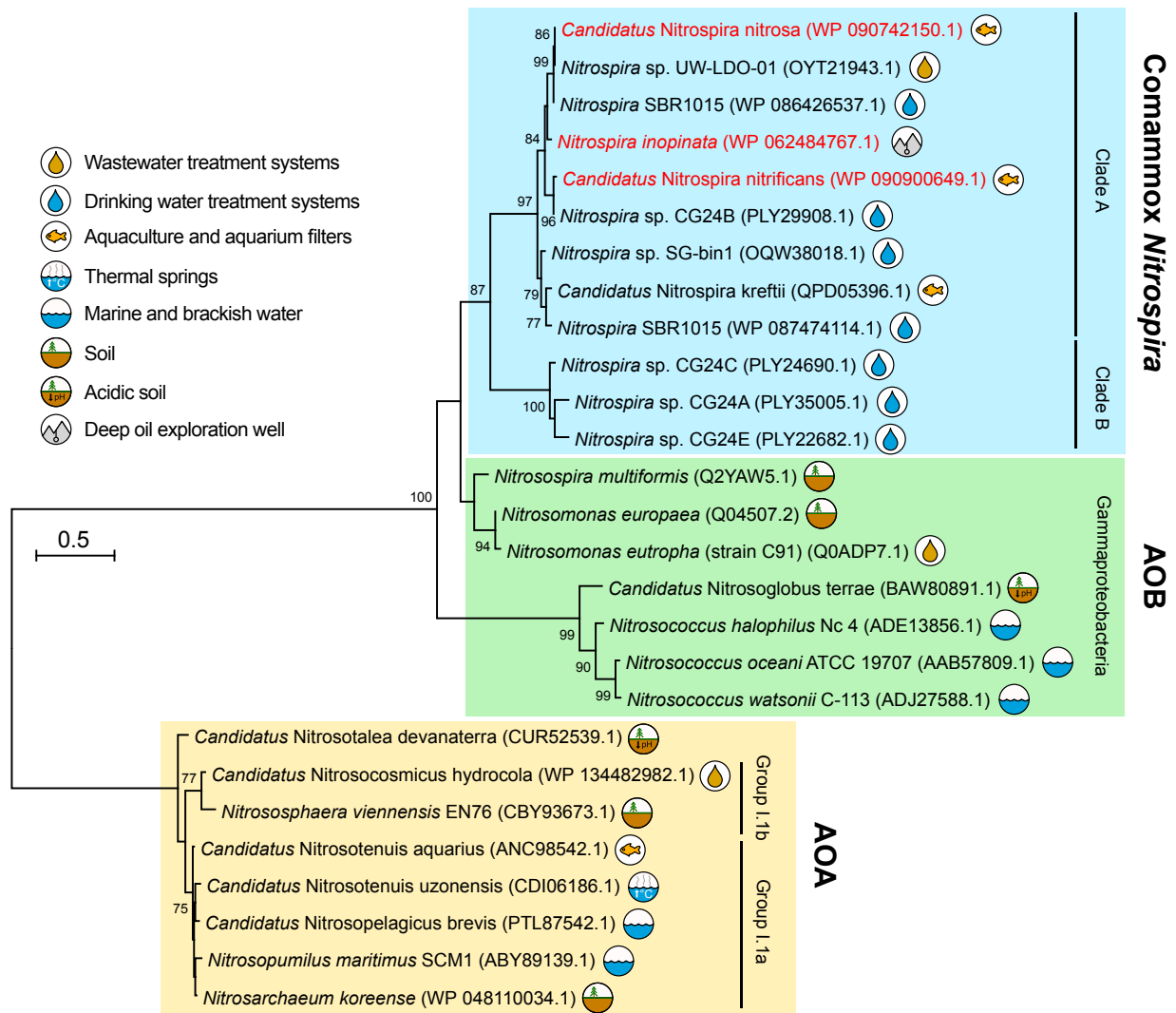


Figure 1.2 Overview of the three groups of ammonia-oxidizing microorganisms, with phylogeny based on the amino acid sequence for subunit A of the ammonia monooxygenase enzyme (AmoA). Environments of origin for each sequence are grouped into major categories and shown to the right of each sequence entry. Comammox *Nitrospira* sequences originate from metagenomic and cultivation studies, with cultivated species shown in red. Sequence alignment was inferred using the Le Gascuel evolutionary model (Le and Gascuel, 2008). The maximum likelihood method was used for tree construction, using a discrete Gamma distribution to model evolutionary rate differences among sites (4 categories (+G, parameter = 2.91). AOA AmoA sequences were used to root the tree. This phylogenetic tree figure was originally published in Al-Ajeel *et al.* (2022).

1.1.3 Biochemistry of nitrification

The first step of aerobic nitrification, ammonia oxidation, occurs via multiple enzymatic steps to produce nitrite, with a total energy yield of -275 kJ mol^{-1} (ΔG°). The first step of ammonia oxidation is catalyzed by a membrane-bound ammonia monooxygenase (AMO) enzyme using both ammonia and oxygen as substrates to produce hydroxylamine (NH_2OH). Ammonia and not ammonium is considered the substrate for AMO in both bacterial and archaeal ammonia oxidizers (Suzuki *et al.*, 1974; Jung *et al.*, 2022). Three subunits for AMO are encoded by the genes *amoA*, *amoB*, and *amoC*, in both bacteria and archaea, although the genes are present in differing copy numbers and arrangements (Koch *et al.*, 2019). Given that it encodes for the catalytic site of ammonia oxidation, the *amoA* gene serves as a functional gene marker for environmental surveys and to determine the phylogenetic relationships among ammonia-oxidizing microorganisms (AOMs; Rotthauwe *et al.*, 1997). The gene sequences encoding the archaeal AMO are divergent from those of AOB, whereas comammox *Nitrospira* AMO genes are related to those of betaproteobacterial AOB, suggesting acquisition through lateral gene transfer (Daims *et al.*, 2015; van Kessel *et al.*, 2015; Fowler *et al.*, 2018). Further differences between bacterial and archaeal ammonia oxidizers include the presence of additional AMO subunits that are unique to both groups. Along with *AmoA*, *AmoB*, and *AmoC*, the *AmoD* and *AmoE* subunits are common among betaproteobacterial AOB and comammox *Nitrospira*, although their functions are not fully understood (Berube and Stahl, 2012; Fowler *et al.*, 2018). The AMO enzyme of AOA includes additional subunits *AmoX*, *AmoY*, and *AmoZ*, unique to the archaeal ammonia monooxygenase that also require further research to determine their functional roles (Hodgskiss *et al.*, 2023).

For AOB and comammox *Nitrospira*, oxidation of hydroxylamine is catalyzed by a periplasmic hydroxylamine dehydrogenase (HAO), formerly called hydroxylamine oxidoreductase. Although it was previously thought that the bacterial HAO was responsible for conversion of hydroxylamine to nitrite, recent studies demonstrate that nitric oxide (NO) is the product, suggesting an additional enzyme is responsible for the oxidation of NO that has not been identified (Caranto and Lancaster, 2017). The HAO

enzyme is genetically encoded by *haoA* and *haoB* genes that are located alongside *cycA* and *cycB* genes for cytochromes *c₅₅₄* and *c_{M552}*, which make up the hydroxylamine-ubiquinone redox module (HURM) involved in electron transport to the terminal oxidase of the electron transport chain (ETC) (Klotz and Stein, 2008). In contrast, there are no known genes encoding HAO in AOA genomes, suggesting the existence of a novel hydroxylamine oxidizing enzyme (Hatzenpichler, 2012). There are hypothetical pathways proposed to model the unknown steps of archaeal ammonia oxidation, both of which help to explain current experimental observations (Kozłowski *et al.*, 2016; Lehtovirta-Morley, 2018). Both proposed pathways involve the production and consumption of NO, which is thought to play a role in archaeal ammonia oxidation because NO scavengers, such as PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide), inhibit AOA (Martens-Habben *et al.*, 2015; Sauder *et al.*, 2016). Overall, further research is needed to better understand the biochemistry of archaeal ammonia oxidation.

The key enzyme responsible for nitrite oxidation to nitrate is nitrite oxidoreductase (NXR), which is associated with the cytoplasmic membrane and consists of three known subunits (Sundermeyer-Klinger *et al.*, 1984; Lückner *et al.*, 2010). Of these subunits, NxrA, encoded for by the *nxrA* gene, is known as the substrate binding subunit of the enzyme, with the NxrB subunit encoded by the *nxrB* gene facilitating electron transfer into the ETC (Vanparys *et al.*, 2007). Amongst NOB, there are two different configurations of NXR in the cytoplasmic membrane. For all *Nitrospira* spp., *Nitrospina*, and *Nitromarina*, NxrA and NxrB are located in the periplasmic space, where the oxidation of nitrite contributes directly to the generation of a proton motive force (PMF) (Spieck *et al.*, 1998; Lückner *et al.*, 2013; Koch *et al.*, 2015; Ngugi *et al.*, 2016). Whereas for NOB genera *Nitrobacter*, *Nitrococcus*, and *Nitrolancea*, NxrA and NxrB are situated in the cytoplasmic space, requiring nitrite to be transported directly across the cytoplasmic membrane for nitrite oxidation via nitrite/nitrate transporters (Watson and Waterbury, 1971; Starkenburg *et al.*, 2006; Sorokin *et al.*, 2012). Phylogenetic analysis suggests that the two types of NXR have different evolutionary origins, with the cytoplasm-oriented NXR having high

similarity to type II enzymes from the DMSO reductase family (Lücker *et al.*, 2010). In contrast, the periplasmic facing NXR has phylogenetic similarity to membrane-bound cytoplasmic nitrate reductase (Lücker *et al.*, 2010). With the low energy yield of nitrite oxidation ($\Delta G^\circ = -74 \text{ kJ mol}^{-1}$) compared to that of ammonia oxidation, it has been hypothesized that the periplasmic NXR is more beneficial for NOB in environments with low nitrite concentrations due to the additional contribution of nitrite oxidation to the PMF. In contrast, NOB with cytoplasmic NXR are better adapted to environments with relatively high nitrogen concentrations (Sorokin *et al.*, 2012). This does reflect the distribution of NOB-associated genera in the environment (Daims *et al.*, 2016).

Following oxidation of ammonia to nitrite, comammox *Nitrospira* are also capable of further oxidizing nitrite into nitrate via nitrite oxidoreductase (NXR), for a total combined energy yield of -349 kJ/mol NH₃ (ΔG°). Genes encoding NXR are also useful functional phylogenetic markers for NOB, and alongside *amoA* genes, can be used to characterize the phylogeny of comammox *Nitrospira* (Pester *et al.*, 2014; Daims *et al.*, 2015; van Kessel *et al.*, 2015). Although comammox *Nitrospira* are capable of growth using only nitrite as an energy and electron source, they still require a supply of additional ammonia for their nitrogen source, which is likely explained by the lack of assimilatory nitrite reductase genes in their genomes, unlike their canonical *Nitrospira* counterparts (Daims *et al.*, 2015; van Kessel *et al.*, 2015; Palomo *et al.*, 2018). Despite their capabilities for complete ammonia oxidation, *Nitrospira inopinata* can show a transient accumulation of nitrite during growth in cultures, which can be attributed to the decreased nitrite affinity observed in this species (Kits *et al.*, 2017). Nitrite affinity is higher in canonical *Nitrospira* and in the more recently cultivated comammox *Ca. N. kryptii* that appears well adapted to highly oligotrophic conditions (Sakoula *et al.*, 2021).

1.2 Niche differentiation and distribution of nitrifiers

Nitrifying microorganisms are found ubiquitously across many ecosystems and the co-occurrence of different ammonia oxidizers is common, with comammox *Nitrospira* frequently present alongside AOA and AOB, in addition to canonical NOB populations (Palomo *et al.*, 2016; Pjevac *et al.*, 2017;

Wang *et al.*, 2017). Of course, with the production of nitrite from ammonia-oxidizers, NOB will live alongside them to obtain the nitrite they produce. In the environment, ammonia-oxidizers compete for the acquisition of ammonia and oxygen. They also compete with heterotrophic microorganisms that have oxygen requirements for aerobic respiration and require ammonia for anabolism. Because of these competitive pressures, nitrifiers have evolved strategies for growth under various environmental conditions.

1.2.1 Ammonia availability and tolerance

Ammonia availability is strongly influenced by the environment's pH. With a pK_a of 9.25, the concentration of ammonia present in its protonated form as ammonium will increase exponentially as the pH decreases below 9.25. Likewise, as the pH increases, the unprotonated ammonia form will increase in relative abundance. Although movement of NH_3 into the cell does not require transporters, because it can diffuse passively across the membrane, transporters are required to move NH_4^+ across membranes. Several different types of ammonium transporters exist, including ammonium transporters (Amts) and Rh-type proteins. Whereas Amts require energy for ammonium transport, Rh-type proteins function as low-affinity ammonia channels that first deprotonate NH_4^+ to facilitate bi-directional flow of NH_3 (Andrade *et al.*, 2005; Wacker *et al.*, 2014). Having ammonium transporters is likely advantageous in nitrogen-limited and low pH environments where access to ammonium or ammonia is a challenge. The genetic inventory of ammonium transporters varies between ammonia oxidizers, with most AOA encoding at least two types of Amt transporters (Lehtovirta-Morley *et al.*, 2016). Amt transporters include both Amt1 and Amt2 types, which are low and high-affinity ammonium transporters, respectively. In oligotrophic environments such as the deep ocean, marine AOA were found to possess only high affinity Amt transporters (Qin *et al.*, 2020), likely as an adaptation to the extremely low concentrations of ammonia. In contrast, about half of the available AOB genomes encode for Rh-type transporters, with the other half not in possession of known ammonium transport genes (Lehtovirta-Morley *et al.*, 2016). For comammox *Nitrospira*, clades A and B exhibit different methods of ammonium acquisition, with clade A

comammox *Nitrospira* in possession of the Rh-type transporters and clade B encoding Amt-type transporters (Palomo *et al.*, 2018). Differences in ammonium acquisition strategies are likely linked to differences in apparent ammonia or ammonium affinities of ammonia oxidizers.

The environmental distribution of AOA and AOB is thought to be primarily influenced by total ammonia concentration ($\text{NH}_3 + \text{NH}_4^+$), where most AOB dominate in relatively high ammonia environments and AOA dominate in low ammonia environments, likely due to their increased substrate affinity for ammonia (AOB *Nitrosomonas europaea*, $K_{m(\text{app})} = 553 \mu\text{M}$ for total NH_3 ; AOA *N. maritimus* $K_{m(\text{app})} = 0.134 \mu\text{M}$ for total NH_3 ; Prosser, 1990; Martens-Habbena and Stahl, 2011). The S-layer present for most AOA may help explain the difference in affinity, with the negatively charged surface acting as a reservoir for NH_4^+ to increase concentrations of ammonia in the periplasmic space (Gorman-Lewis *et al.*, 2014; Li *et al.*, 2018). However, there are exceptions, with AOB species of the *Nitrosomonas* cluster 6a, such as *N. oligotropha* showing adaptation to growth in low ammonia concentration environments (Bollmann and Laanbroek, 2001; Sedlacek *et al.*, 2019). Additionally, AOA have now been characterized with a wide range of ammonia affinities, with members of the genus *Nitrosocosmicus* exhibiting lower ammonia affinities similar to those of AOB (*N. franklandianus*, $K_{m(\text{app})} = 16.3 \mu\text{M}$ for total NH_3 ; Jung *et al.*, 2022). Members of this genus also have tolerance to high environmental ammonia concentrations, unlike other AOA (Lehtovirta-Morley *et al.*, 2016). Cell morphology and size also appears to explain these observed variations in affinities across AOA species, with increased surface area to volume ratios positively correlated to increased ammonia affinities (or low $K_{m(\text{app})}$; Jung *et al.*, 2022).

As described prior to their discovery, microorganisms capable of complete ammonia oxidation were predicted to inhabit oligotrophic environments with low ammonia concentrations and attached surfaces for growth (Costa *et al.*, 2006). Therefore, ammonia availability may be a key factor governing niche differentiation of comammox *Nitrospira*. Kinetic studies of *Nitrospira inopinata* in pure culture reveal that this bacterium displays higher ammonia affinity compared to other AOA or AOB (Kits *et al.*, 2017), making it more competitive in oligotrophic environments. As well, low ammonia environments are

found to have high abundances of comammox *Nitrospira*, compared to AOA and AOB populations (Wang *et al.*, 2017; Spasov *et al.*, 2020). Ammonia concentrations can also inhibit ammonia oxidation of the enriched comammox *Ca. N. kreffii* at concentrations as low as 25 μM , which suggests that the ability of comammox *Nitrospira* to tolerate ammonia concentrations could drive niche differentiation as well (Sakoula *et al.*, 2021). Of course, further characterization of additional comammox *Nitrospira* species is needed to understand ammonia inhibition and tolerance distributions of these bacteria.

1.2.2 Ecophysiology of nitrifiers

In addition to ammonia concentration, other factors that affect niche differentiation of AOA, AOB, and comammox *Nitrospira* include pH, temperature, and oxygen concentration (Hatzenpichler, 2012; Koch *et al.*, 2019). Based on recent insight from genomic and metagenomic data, several metabolism-specific factors may contribute to niche differentiation of ammonia and nitrite oxidizers. Including comammox bacteria, all *Nitrospira* encode genes for the reductive tricarboxylic acid cycle (rTCA) for carbon fixation (Lücker *et al.*, 2010; Camejo *et al.*, 2017). In contrast, AOB fix carbon via the Calvin-Benson-Bassham (CBB) cycle and *Nitrososphaerota* members use the 3-hydroxypropionate-4-hydroxybutyrate (3HP/4HB) cycle for carbon fixation (Berg *et al.*, 2007). Canonical NOB, including *Nitrotoga* and *Nitrobacter*, also fix carbon using the CBB cycle (Starkenbourg *et al.*, 2006; Lücker *et al.*, 2010). The rTCA cycle uses oxygen-sensitive ferredoxin and is typically present in anaerobic/microaerophilic microorganisms, whereas both the CBB and 3HP/4HB cycles are oxygen tolerant (Lücker *et al.*, 2010). This led to the hypothesis that comammox *Nitrospira* may be more adapted to microaerophilic growth, which is also supported by novel cytochrome *bd*-like terminal oxidases found in *Nitrospira* species (Lücker *et al.*, 2010; Lawson and Lücker, 2018). These terminal oxidases may have a higher affinity for oxygen, compared to those of other ammonia oxidizers, which would confer a competitive advantage to comammox *Nitrospira* in environments with relatively low oxygen mixing ratios (Lawson and Lücker, 2018). Since their discovery, comammox *Nitrospira* have been identified in

numerous environments with low concentrations of dissolved oxygen (Roots *et al.*, 2019; Zheng *et al.*, 2022), supporting their success as nitrifiers in low oxygen niches.

When oxygen availability is low, or anoxic conditions are established, AOB have the ability to perform nitrifier-denitrification for survival. Nitrifier-denitrification allows AOB to use alternate electron acceptors such as nitrite in place of oxygen (Wrage *et al.*, 2001). Nitrite is reduced, producing nitric oxide, and further converted to N₂O by nitric oxide reductase (NOR). Additionally, cytochrome P460 in the periplasm of AOB has been shown to produce N₂O directly from hydroxylamine under anoxic conditions (Caranto *et al.*, 2016). The production of N₂O by AOB is a common feature within this group of ammonia oxidizers, although it is not desirable as it causes fertilizer loss from crops and contributes to the release of greenhouse gases into the atmosphere that result from incomplete denitrification (Wrage *et al.*, 2001). Both comammox *Nitrospira* and AOA are incapable of nitrifier-denitrification, with an apparent lack of known NO reductase genes (Prosser *et al.*, 2020). Despite the lack of necessary denitrification genes, both AOA and comammox *Nitrospira* can produce nitrous oxide. The mechanism for N₂O production by AOA is not confirmed, but potential mechanisms have been proposed (Wu *et al.*, 2020). For comammox *Nitrospira* recent work has revealed that any production of N₂O results from the abiotic conversion of hydroxylamine under reduced oxygen conditions (Kits *et al.*, 2019). Overall, current research points toward a higher contribution of AOB to N₂O emissions compared to AOA and comammox *Nitrospira*.

1.2.3 Alternative substrates and metabolisms

Nitrifiers live in environments where they are in competition with both other nitrifiers and heterotrophic microorganisms for resources (i.e., oxygen, nitrogen). In all environments, including aquatic ecosystems and biofilms, nutrient availability and environmental conditions fluctuate, making adaptations to these changing conditions important for survival. Like many microorganisms, nitrifiers have evolved the ability to use different substrates, either as part of their traditional nitrification pathways,

or by relying on different metabolic pathways for energy and carbon acquisition. Having this metabolic versatility and flexibility is important to succeed in complex microbial ecosystems.

Although nitrifiers are generally considered obligate autotrophs, research has shown that several nitrifiers are capable of using organic carbon substrates. Both fructose and pyruvate can support growth of *Nitrosomonas europaea* as the sole carbon source, albeit at a reduced growth rate when compared with autotrophy (Hommes *et al.*, 2003). However, *N. europaea* grown with fructose and pyruvate still requires ammonia as a reductant for energy generation, with most evidence supporting their obligate lithotrophy during aerobic growth (Arp *et al.*, 2007). Under anoxic conditions though, some AOB have been grown using nitrite as an electron acceptor with pyruvate donating electrons for energy generation (Abeliovich and Vonshak, 1992). As for AOA, there is the potential for mixotrophy among this group, but more research is needed to clarify the role of organic carbon in their growth (Wright and Lehtovirta-Morley, 2023). Several organic acids, such as TCA intermediates, have been shown to stimulate the growth of AOA (Spang *et al.*, 2012; Lehtovirta-Morley *et al.*, 2014; Sauder *et al.*, 2017). As well, experiments with marine AOA revealed that these organic acids improved growth by alleviating cellular oxidative stress and were not used as a carbon source (Kim *et al.*, 2016). However, several studies using labelled substrates have shown that AOA, such as *Ca. N. hydrocola*, do not appear to fix CO₂ in wastewater environments, despite their high abundance in the systems, which suggests they may be using organic substrates for carbon acquisition (Mußmann *et al.*, 2011; Sauder *et al.*, 2017). Overall, these results point to a need for future research to further elucidate the role of mixotrophy in AOA.

Along with their ammonia-oxidizing counterparts, NOB also have some mixotrophic tendencies. For both *Nitrospira defluvii* and *Nitrospira marina*, the addition of simple organic compounds was shown to be beneficial to growth in addition to nitrite (Watson *et al.*, 1986; Spieck *et al.*, 2006), while uncultured *Nitrospira* exhibit the ability to uptake pyruvate (Daims *et al.*, 2001). In terms of genetic inventory, *N. defluvii* possess pathways for the assimilation and catabolism of acetate, pyruvate, and formate, along with pathways for hexose sugar metabolism (Lücker *et al.*, 2010). Some *Nitrobacter* genomes possess

complete glycolysis pathways, however, growth on sugars has not been reported (Starkenbourg *et al.*, 2008). Overall, *N. defluvii*, which resides often in wastewater treatment facilities, benefits from the use of organic compounds from sewage in addition to its required nitrite and carbon dioxide for energy and carbon, respectively, but again heterotrophic growth has not been observed. Comammox *Nitrospira* also possess the genetic inventory that suggests the potential for mixotrophic growth, like other *Nitrospira*, including the pathways for glycolysis, gluconeogenesis, and the pentose phosphate pathway (Palomo *et al.*, 2018), but further research is required to assess any mixotrophic growth beyond genetic potential and determine its role in the lifestyle of comammox *Nitrospira*.

Aside from carbon acquisition, nitrifiers possess the ability to use different organic nitrogen compounds to obtain ammonia for respiration. The AOB *Nitrosospira multiformis* is capable of using urea as an ammonia source, by degrading the urea with urease enzymes (Koper *et al.*, 2004). In contrast, *N. europaea* only possess genes for urea transport, but not degradation, and *N. eutropha* has no genetic inventory for urea catabolism (Chain *et al.*, 2003; Arp *et al.*, 2007). The distribution urea of catabolism in AOB appears to be dependent on environment, with the soil inhabiting AOB in possession of urease genes, as the availability of ammonia in the soil can be variable due to lower pH conditions in the soil, and additional competition with plants for ammonia acquisition (Pommerening-Röser and Koops, 2005). In contrast, wastewater environments have more consistent concentrations of ammonia available, perhaps making it less important for AOB to have alternative methods to obtain ammonia and reflecting the absence of urease genes in wastewater AOB like *N. europaea* (Chain *et al.*, 2003).

Comammox *Nitrospira* can use a range of substrates in addition to ammonia, with similar pathways also possessed by their canonical NOB *Nitrospira* relatives who instead would use these pathways for cross-feeding with their ammonia-oxidizing partner (Daims *et al.*, 2016). Based on the presence of genes encoding urease and high-affinity urea transporters, comammox *Nitrospira* have the potential to use urea in the absence of available ammonium, which has been experimentally confirmed in strict nitrite-oxidizing *Nitrospira* (Koch *et al.*, 2015; van Kessel *et al.*, 2015). Additionally, comammox

Nitrospira possess genes for growth on H₂ and formate, although the latter has only been identified in clade B comammox *Nitrospira* (Koch *et al.*, 2019). Some AOA species also possess genes for urea transport and metabolism, along with genes for formate oxidation (Sauder *et al.*, 2017), suggesting the possibility of niche overlap between AOA and comammox *Nitrospira* in specific environments where these organisms are found. At present, *Nitrososphaera gargensis* is the only AOA known to encode cyanase (Palatinszky *et al.*, 2015), although, marine *N. maritimus* can produce ammonia from cyanate using an unknown enzyme (Kitzinger *et al.*, 2019). The ability to use cyanate as an ammonia source was previously thought to only exist in canonical NOB (Palatinszky *et al.*, 2015). However, metagenomes of comammox *Nitrospira* recovered from tertiary treatment biofilm from a wastewater treatment plant (WWTP) encoded genes for cyanate transport and cyanate degradation (Spasov *et al.*, 2020). Most recently, comammox *N. inopinata* was shown capable of growth using guanidine as its sole source of energy, reductant, and nitrogen, further expanding upon the metabolic versatility of comammox *Nitrospira* (Palatinszky *et al.*, 2024). Comammox *Nitrospira* use guanidine in wastewater treatment plant environments given that, along with being a component in plastics manufacturing, guanidine is also excreted as a nitrogenous waste product in human urine (Palatinszky *et al.*, 2024). The metabolic versatility of these ammonia oxidizers suggests that they can adapt to changing environmental conditions and may play complex metabolic roles within microbial communities.

1.2.4 Biofilms and nitrification

Nitrifying communities often form biofilms within aquatic environments (Okabe *et al.*, 1999). In water treatment systems, nitrifying biofilms form on sand, carbon substrates, and sponge surfaces, like those found in aquarium filtration units. The structure of a biofilm can influence the spatial distribution and composition of the nitrifying community (Suarez *et al.*, 2019). Factors such as the depth of the biofilm and location of nitrifiers within the biofilm can influence their access to substrates and oxygen (Okabe *et al.*, 2002). *Nitrospira* reside relatively deep within biofilm communities, where there is a lower concentration of dissolved oxygen (DO; Matsumoto *et al.*, 2010). In contrast, AOB associate with biofilm

surfaces where there are higher concentrations of DO and total ammonia ($\text{NH}_3 + \text{NH}_4^+$; Matsumoto *et al.*, 2010). Biofilm communities also provide the opportunity for cross feeding among microbial populations, and among nitrifiers, such as the syntrophic relationship between ammonia oxidizers and NOB, where NOB rely on the production of nitrite from ammonia oxidizers (Daims *et al.*, 2016).

Because of the attached-growth lifestyle and resultant competition with neighboring microorganisms for substrates, it seems an unfavorable environment for nitrifiers who are not able to compete well for substrates like ammonia and nitrite. However, for those nitrifiers with high ammonia affinities, such as comammox *Nitrospira*, biofilm growth may represent a strategy to dominate over other ammonia oxidizers, which has been observed for attached growth systems of drinking water treatment plants (Zhao *et al.*, 2022). The ability of nitrifiers to use alternative substrates can also be beneficial for survival in a biofilm environment if the preferred substrate is unavailable.

Beyond inhabiting biofilms, nitrifying microorganisms are important for facilitating heterotrophic growth. Studies show that both AOB and NOB in nitrifying biofilms produce soluble microbial products that are used to support growth of neighboring heterotrophic bacteria (Rittmann *et al.*, 1994; Kindaichi *et al.*, 2004). Likewise, research has shown that cultivation of *Nitrospira* alongside heterotrophs stimulated the growth of *Nitrospira* (Murakami *et al.*, 2022). Additionally, interactions with heterotrophs may protect some AOB from the oxidative stress of toxic metabolic intermediates (Sedlacek *et al.*, 2016), and for NOB *Ca. Nitrotoga*, co-culture with *Acidovorax* promoted nitrite consumption by *Ca. Nitrotoga* (Ide *et al.*, 2023).

Cultivation and purification of comammox *Nitrospira* has been challenging, with only one of the characterized strains successfully purified without heterotrophic partners. Because these comammox *Nitrospira* are always enriched alongside additional heterotrophs (Daims *et al.*, 2015; van Kessel *et al.*, 2015), and even canonical NOB (Sakoula *et al.*, 2021), this suggests they maintain strong relationships with microbial partners. The specific nature, benefit, and extent of these partnerships is not fully understood, and with their more recent discovery and culturing challenges, comammox *Nitrospira*

arguably remain the least understood of all nitrifiers. However, an improved understanding of the mutualistic relationships between nitrifiers and heterotrophs may help develop strategies for promoting growth of comammox *Nitrospira* in enrichment cultures.

1.3 Nitrification in water treatment systems

1.3.1 Wastewater treatment systems

In engineered water treatment systems, such as municipal wastewater treatment plants, communities of microorganisms are responsible for nitrogen removal as part of the wastewater treatment process. Municipal wastewater treatment is performed in multiple stages. Primary treatment involves the use of screens, grit chambers, and sedimentation tanks to remove larger objects and particulate matter from the influent. Subsequent secondary treatment processes are crucial for removal of remaining nutrients, organic matter, and contaminants via microbial activity. During secondary treatment, nitrifying microorganisms are active and primarily responsible for removal of ammonia and other organic nitrogen compounds (i.e., urea) via nitrification. Recognizing the harm caused by the release of nitrates in effluent water to the environment, the use of systems facilitating both nitrification and denitrification were implemented in activated sludge treatments in the 1960s to encourage complete nitrogen removal by conversion to dinitrogen gas (Ludzack and Ettinger, 1962; Wuhrmann, 1964). Dependent on plant configuration and requirements, tertiary treatment can be included, which may target removal of specific contaminants with biological or chemical methods. Final disinfection processes, usually via chlorination, will occur before effluent is discharged into the environment.

Secondary treatment is implemented using either suspended-growth or fixed-film engineered systems. Suspended-growth systems include conventional activated sludge (CAS) systems and sequencing batch reactors (SBR), and fixed-film systems include trickling filters, integrated fixed-film activated sludge (IFAS), rapid sand filters, rotating biological contactors (RBC), and moving bed biofilm reactors (MBBR; Machineni, 2019; Narayanan and Narayan, 2019). In these systems, nitrifiers will grow

in microbial communities in response to available nitrogen in the wastewater. However, factors such as temperature, flow rate, solids retention time, hydraulic retention time, available oxygen, and organic matter concentrations can all impact composition and activity of the microbial communities in these systems (Y. Chen *et al.*, 2017).

Both AOB and NOB have been generally recognized as the main aerobic nitrifiers in wastewater treatment systems for a long time, with dominant AOB usually *Nitrosomonas* spp. and *Nitrobacter* as NOB (Okabe *et al.*, 2014). However, other AOB and NOB genera have also been identified in wastewater environments. The consideration of only AOB and NOB driving aerobic nitrification in these systems was challenged as the scientific understanding of nitrification evolved with the discovery of AOA and comammox *Nitrospira*. Since then, research is still working to elucidate the dynamics and presence of all nitrifiers in wastewater treatment systems. As discussed, the theory of niche separation between the groups of ammonia oxidizers was thought to be driven by ammonia concentration, with elevated ammonia concentrations in wastewater likely to favour the growth of AOB over comammox *Nitrospira* and AOA (Martens-Habbena *et al.*, 2009; Schleper, 2010). This niche preference of AOB has been observed in several municipal WWTPs, with some studies showing higher transcriptional activity compared to AOA in the same systems (Wells *et al.*, 2009; Gao *et al.*, 2013, 2014; Fan *et al.*, 2017; Zheng *et al.*, 2021). Likewise, in tertiary treatment RBC systems containing low ammonia concentrations, comammox *Nitrospira* were dominant in the biofilm (Spasov *et al.*, 2020). In addition to ammonia concentrations, it is likely that operational parameters including dissolved oxygen, temperature, solids retention times, and nitrite concentrations, among other conditions may also have contributed to the dominance of AOB in these systems (Al-Ajeel *et al.*, 2022).

In contrast to AOB dominance, several reports also show the presence of AOA at equivalent abundances to AOB in WWTPs (Limpiyakorn *et al.*, 2011; Sonthiphand and Limpiyakorn, 2011; Reddy *et al.*, 2014), and also multiple WWTPs where AOA were the dominant ammonia oxidizers (Limpiyakorn *et al.*, 2011; H. Chen *et al.*, 2017; Pan *et al.*, 2018). In WWTP sludge there have been reports of both

AOB and AOA as active chemolithoautotrophs, showing incorporation of ^{13}C -labelled bicarbonate in the presence of ammonia (Pornkulwat *et al.*, 2018). However, this contrasts with reports that AOA in WWTP biofilm do not show incorporation of bicarbonate despite active ammonia oxidation (Mußmann *et al.*, 2011; Sauder *et al.*, 2017), which suggests the potential use of alternate carbon substrates and mixotrophic lifestyles. Mixotrophic lifestyles for AOA and AOB in WWTPs are supported by studies that show the assimilation of labelled organic substrates by these ammonia oxidizers (Roy *et al.*, 2017; Pan *et al.*, 2018; Srithep *et al.*, 2018), alongside evidence for their contribution to ammonia oxidation activity demonstrated with differential inhibition experiments (Srithep *et al.*, 2018). Overall, it seems that the roles occupied by ammonia oxidizers in WWTPs are complex.

There are several factors that may govern distributions of AOA and AOB in WWTPs. Several AOA species may be better adapted to cold temperatures than AOB, with an increase in *Ca. N. hydrocola* activity in activated sludge during colder months, despite a higher numerical abundance of AOB (Fan *et al.*, 2019). Decreased copper availability due to sequestration by organic compounds is suspected to explain inhibitory effects on AOA in some WWTPs, given that copper is important for the function of ammonia oxidation enzymes (Gwak *et al.*, 2020). Higher concentrations of bioavailable copper have also been associated with dominance of AOA based on both metagenomic and metatranscriptomic analysis (Yang *et al.*, 2020). This positive effect of copper availability has also been observed to increase ammonia removal activity of comammox *Nitrospira* in groundwater fed bioreactors (Koike *et al.*, 2022), further suggesting an important role for copper in influencing the dynamics of ammonia oxidizers in WWTPs.

Since their discovery, comammox *Nitrospira* have been identified in many full-scale WWTP systems (Pjevac *et al.*, 2017; Annavajhala *et al.*, 2018; Yang *et al.*, 2020). Although comammox *Nitrospira* detected in these systems have been associated with clade A, those belonging to clade B have also been identified (Xia *et al.*, 2018). Generally, comammox *Nitrospira* displayed higher levels of strain heterogeneity in several WWTPs studied by Cotto *et al.* (2023), compared to *Nitrosomonas* AOB in the

systems. These comammox *Nitrospira* were associated only with *Ca. N. nitrosa*, suggesting that this species may have adapted specifically to WWTP environments (Cotto *et al.*, 2023).

Although comammox *Nitrospira* associate with low ammonia concentrations within WWTP systems, they are also present in WWTP processes with elevated ammonia. Studies of active sludge secondary treatment stages in WWTPs with elevated ammonium concentrations (12-51 mg/L $\text{NH}_4^+\text{-N}$) revealed detectable comammox *Nitrospira*, although they were not the dominant ammonia oxidizers (Chao *et al.*, 2016; Fan *et al.*, 2017; Pan *et al.*, 2018). Nonetheless, transcriptional analysis of *amoA* genes in activated sludge of several WWTPs in China revealed comammox *Nitrospira* to be the most transcriptionally active ammonia oxidizer (Zheng *et al.*, 2019). In a survey of 14 nitrogen removal systems, comammox *Nitrospira* showed a preference for attached growth systems and long SRT operating conditions (Cotto *et al.*, 2020), coinciding with their predicted niche (Costa *et al.*, 2006). Despite favourable conditions for comammox *Nitrospira* growth, their increase did not result in a decrease of other nitrifiers in the system (Cotto *et al.*, 2020). Overall, questions remain about the importance of each group of AOM to nitrification within WWTP communities and the dynamics among them.

To improve nitrogen removal, the implementation of anammox bacteria in WWTP processes has been explored. By integrating ammonia oxidation (also known as “nitritation” in engineering) with anammox denitrification processes, partial nitritation with anammox (PNA) can be achieved, with potential benefits including improved nitrogen removal and decreased oxygen requirements (Lackner *et al.*, 2014). Since anammox bacteria require both ammonium and nitrite, they must compete with NOB for acquisition of nitrite, making complete nitrification in anammox reactors undesirable. Instead, as anammox still require ammonia oxidation activity to supply them with nitrite, establishing conditions encouraging active ammonia oxidizers (e.g., AOB and AOA), while repressing NOB growth is ideal (Wang *et al.*, 2022). There have been several reports of cooperation between comammox *Nitrospira* and anammox bacteria in wastewater treatment systems capable of performing PNA (Gottshall *et al.*, 2021;

Shao and Wu, 2021; Vilardi *et al.*, 2023; Xu *et al.*, 2023). The mutualistic relationship between comammox and anammox bacteria occurs under conditions with low DO, low total ammonia loading, and additionally low free ammonia ($\text{NH}_3 < \text{NH}_4^+$) (Shao and Wu, 2021). This relationship was recently observed in full-scale attached growth systems (integrated fixed-film activated sludge: IFAS) in a municipal WWTP where lower DO conditions led to increased nitrogen removal rates (Vilardi *et al.*, 2023). In these systems, comammox *Nitrospira* appear to be performing only partial nitrification, likely explained by low DO conditions, releasing nitrite for use by anammox bacteria (Xu *et al.*, 2023).

In addition to advances in PNA methods, new technologies have been developed with the goal of improving the issues faced by traditional WWTP processes. Novel technologies, such as membrane aerated biofilm reactors (MABR), aim to address challenges with conventional activated sludge (CAS) systems. These challenges include expensive and energy consuming aeration requirements, reduced nitrogen removal during cold temperatures, and greenhouse gas emissions. The MABR process promotes both nitrification and denitrification, using passive oxygen diffusion along membrane cords to support a large biomass inventory in the system (Li *et al.*, 2023). Many studies with smaller pilot scale systems show promise for effective nitrogen and phosphorus removal and reduced nitrous oxide emissions (Houweling *et al.*, 2018; He and Daigger, 2023). However, because these technologies are being implemented in full-scale systems, research is needed to evaluate their performance and their associated microbial communities, including roles played by nitrifiers including AOB, AOA, NOB, and comammox *Nitrospira*.

1.3.2 Aquarium biofilters and aquaculture operations

Ammonia, which is excreted as a metabolic waste product by fish and other aquatic organisms, is of particular concern for engineered aquatic ecosystems, such as aquaculture operations and home and commercial aquariums, where ammonia can accumulate to lethal concentrations in the absence of active nitrification. The un-ionized form of ammonia (NH_3) is particularly toxic to fish, with chronic stress and disease associated with concentrations exceeding 0.1 mg L^{-1} in aquarium and aquaculture systems

(Andrews *et al.*, 1988; Parker, 2002). In order to convert toxic ammonia to nitrate, aquarium biofilters are designed to promote the growth of nitrifying populations using the high surface area of filter support material (e.g., sponge, ceramic or polymer) and rapid flow rates of aerated water. Despite their importance to fish health within many industrial biofilters, including in aquaculture systems, little is known of the microorganisms catalyzing nitrification in association with aquarium biofilter support material.

Traditionally, AOB such as *Nitrosomonas* spp., were thought to be solely responsible for ammonia oxidation in aquatic environments, including aquariums. The use of AOB-containing aquarium supplements is widespread for promoting aquarium nitrification. Indeed, evidence suggests that AOB might play a role in aquarium nitrification. For example, Hovanec and DeLong (1996) found that *Nitrosomonas*-like bacteria from the *Betaproteobacteria* were associated with the saltwater aquariums in their study, although they only detected AOB in 2 of 38 freshwater aquariums, despite observing vigorous nitrification rates. This led the authors to conclude that an unknown group of microorganisms was performing ammonia-oxidation in freshwater aquarium biofilters. Subsequent studies determined that *Nitrosomonas* spp. could be enriched from freshwater aquarium biofilters (Burrell *et al.*, 2001), indicating that they may exist in these environments at abundances too low for detection.

Since the discovery of AOA, several studies have investigated AOA in aquarium biofilters. The first isolated AOA representative, *Nitrosopumilus maritimus* SCM1, was obtained from sediment of a marine aquarium (Könneke *et al.*, 2005). The *N. maritimus* genome shares high synteny with contigs obtained from the open ocean (Walker *et al.*, 2010), demonstrating that this isolate from an aquarium may be an important representative of AOA in natural environments. Furthermore, *Ca. Nitrosotenuis aquarius*, cultivated from a freshwater aquarium biofilter, demonstrates *in situ* ammonia oxidation activity, indicating that AOA contribute to ammonia oxidation in aquarium biofilters (Sauder *et al.*, 2018). Other studies have analyzed the diversity of AOA in aquarium biofilters. Urakawa and colleagues (2008) identified the presence of *amoA* genes from AOB and AOA in marine aquarium biofilters from a public

aquarium in Japan and suggested that the diversity of AOA and AOB decreased in low temperature marine aquariums. These studies highlight the overlooked importance of AOA in aquarium biofilters.

Additional studies have detected AOA in marine and freshwater aquariums and have provided evidence for the dominance of AOA in freshwater aquarium biofilters (Sauder *et al.*, 2011; Bagchi *et al.*, 2014; Bik *et al.*, 2019). Sauder *et al.* (2011) examined saltwater and freshwater aquariums, revealing that most AOA *amoA* sequences derived from freshwater and saltwater cluster separately, suggesting niche adaptation of AOA to saltwater or freshwater environments. Additionally, the ratio of AOB to AOA appeared to be largely governed by aquarium ammonia concentrations (Sauder *et al.*, 2011). However, due to their recent discovery, comammox *Nitrospira* were not considered in any of these studies, highlighting the need to reassess the ammonia oxidizers present in aquarium biofilters.

Recirculating aquaculture system (RAS) biofilters use similar strategies to WWTPs to regulate water quality and share many characteristics with aquarium biofilters. Several studies have examined ammonia-oxidizing microbial communities in aquaculture systems. In all compartments, and in sampled tank water of a marine shrimp RAS, AOA *amoA* genes outnumbered bacterial *amoA* genes by orders of magnitude and were phylogenetically related to *N. maritimus* (Brown *et al.*, 2013). Similarly, the abundance of archaeal *amoA* genes was 12-fold higher than those of bacterial *amoA* in a freshwater RAS (Khangembam *et al.*, 2017). In addition to AOA, recent studies have identified comammox *Nitrospira* within RAS biofilters. One study reported that nitrite-oxidizing *Nitrospira* and comammox *Nitrospira* co-exist in a freshwater RAS filter at similar abundances (Bartelme *et al.*, 2017). In the filter, comammox *Nitrospira* were approximately two-fold more abundant than AOA, and AOA were, in turn, more abundant than *Nitrosomonas* AOB. Because *Nitrosomonas* and *Nitrobacter* are commonly used to model RAS capacities, existing models to determine capacities may be inaccurate given their exclusion of abundant nitrifiers.

Similar to RAS biofilters, aquaponics systems also implement a recirculation system that combines both fish and vegetable growth, with potential roles for nitrifying microbial guilds. Comammox

Nitrospira have recently been identified as dominant ammonia oxidizers within a backyard aquaponics system, based on 16S rRNA gene sequencing and metagenomics (Heise *et al.*, 2021). The system operated at a low steady-state ammonia concentration, which corresponds well to the low-ammonia predicted niche of comammox *Nitrospira*. However, the presence of traditional AOB and AOA was very low, which suggested that the *Nitrospira* present were responsible for nitrification in the sampled sediments (Heise *et al.*, 2021). This study did not assess microbial communities present in the grow beds of the system, where nitrification also occurs.

Given the low ammonia concentrations imposed by design constraints and the predicted niche of nitrifying guilds, it is expected that aquaculture systems can favor AOA and comammox *Nitrospira* over AOB (Bartelme *et al.*, 2019). Overall, an improved knowledge of these nitrifiers offers an opportunity to manipulate these systems by providing conditions that favour microorganisms with desired nitrification rates to optimize water treatment and animal production.

1.4 Research objectives

The overarching goal of my thesis research was to explore the ecology of nitrification in engineered water treatment systems, focusing in particular on more recently discovered contributors to this process. Comammox *Nitrospira* went previously undetected due to its genetic similarity with canonical NOB and the challenges in obtaining enrichments and pure cultures, and whether comammox *Nitrospira* are present in systems such as home aquarium biofilters was not yet known. Since their discovery, comammox *Nitrospira* identification in water treatment systems necessitates a re-evaluation of ammonia oxidizers across engineered systems to provide a comprehensive understanding of the microorganisms involved in nitrification, with the possibility of better engineering future water treatment systems.

The detection of ammonia oxidizers in aquarium biofilters has evolved over the years, alongside the discovery of AOB, AOA, and now comammox *Nitrospira*. Preliminary metagenomic data from a local freshwater aquarium revealed a dominance of comammox *Nitrospira* in the biofilter (unpublished

data), suggesting an important role for these bacteria in aquarium nitrification that was previously overlooked. These results highlight the need to re-evaluate ammonia-oxidizing communities in aquarium biofilters to better understand the role of comammox *Nitrospira* in such small-scale water treatment systems. My first research objective was to re-assess the presence of ammonia oxidizers in home aquarium biofilters, exploring distributions of all three groups (AOA, AOB, and comammox *Nitrospira*) and potential factors connected to their abundances for salt and freshwater biofilters (Chapter 2). I used several different detection methods to characterize both the ammonia oxidizers and overall microbial community composition of the aquarium biofilters including 16S rRNA gene sequencing, *amoA* gene sequencing, and qPCR to quantify both the 16S rRNA gene abundance of bacteria and archaea and the *amoA* gene abundance of AOA, AOB, and comammox *Nitrospira*.

With results from Chapter 2 revealing that comammox *Nitrospira* were dominant ammonia oxidizers in most freshwater aquarium biofilters, my second objective was to explore microbial community succession in newly established freshwater home aquarium biofilters, with a focus on all known groups of ammonia oxidizers and nitrifiers (Chapter 3). For this work, I analyzed the microbial community composition of freshwater biofilter samples collected from three home aquariums for the first 12-weeks following aquarium start-up, using 16S rRNA gene sequencing, qPCR to quantify the ammonia oxidizers and total prokaryotes in the biofilter samples, and metagenomics to study the changes in microbial community composition as the biofilms established in the freshwater aquarium biofilters.

The experimental home aquariums were originally part of a project assessing nitrifier establishment in aquarium biofilters before the discovery of comammox *Nitrospira* (Szabolcs, 2014). At that time, AOA were understood as the dominant ammonia oxidizers in home aquarium biofilters with low ammonia concentrations (Sauder *et al.*, 2011). During controlled aquarium experiments exploring the effects of fish biomass on the establishment of AOA and AOB in freshwater aquarium biofilters, there was an apparent lack of AOA present throughout the duration of the experiment, which was hypothesized to potentially be a result of the clean conditions used in the lab environment (Szabolcs, 2014). To assess

whether AOA arrive in and establish in freshwater aquarium biofilters under more “natural” conditions, such as an independently operated home aquarium, this experiment was designed considering three representative freshwater home aquariums to determine when AOA establish (Szabolcs, 2014). In my thesis, I have returned to the samples from this experiment, of which only one third of the samples collected had been analyzed, with the consideration that comammox *Nitrospira* may be present in these home aquarium biofilters alongside AOA and AOB.

My final thesis research objective focused on nitrifiers in a municipal wastewater treatment plant. In municipal WWTPs, advances in engineering have led to the development of wastewater treatment processes that promote the growth of nitrifying microorganisms, alongside heterotrophs and denitrifiers to promote increased nitrogen removal, while reducing energy consumption and improving resistance to cold operational temperatures. In Chapter 4, I assessed the impact of installing a membrane aerated biofilm reactor (MABR) system on the microbial community composition and nitrifiers in the current conventional activated sludge system in a municipal WWTP and additionally characterized the microbial community composition and functionality within the MABR biofilm. Both 16S rRNA gene and metagenomic sequencing were used to determine microbial community composition in the plant and assess the functional potential of the microbial communities, with a focus on nitrification and denitrification.

Chapter 2

Comammox *Nitrospira* among dominant ammonia oxidizers within aquarium biofilter microbial communities

2.1 Introduction

Aquariums represent artificial environments for housing aquatic life, typically fish and plants, with residential and commercial applications. Nitrogenous waste in the form of ammonia/ammonium ($\text{NH}_3/\text{NH}_4^+$), excreted by fish or produced from organic matter degradation, can exceed concentrations that are toxic to aquatic life, especially in newly established aquariums. Concentrations of un-ionized ammonia (NH_3) greater than 0.1 mg/L can lead to chronic stress and disease of aquarium fish if not removed through nitrification or water changes (Andrews *et al.*, 1988; Parker, 2002). To maintain a healthy aquarium, biofilters with established microbial communities that can perform nitrification are used to convert ammonia into nitrate, via nitrite, although this intermediate can also lead to toxicity, which reinforces the importance of establishing nitrification rapidly within biofilters to ensure aquatic health.

The first step of nitrification, ammonia oxidation, can be performed by either ammonia-oxidizing bacteria (AOB; e.g., *Nitrosomonas*) or ammonia-oxidizing archaea (AOA; e.g., *Nitrosotenuis*, *Nitrosopumilus*) (Hatzenpichler, 2012). Nitrite oxidation is subsequently performed by nitrite-oxidizing bacteria (NOB; e.g., *Nitrobacter*, *Nitrospira*). More recently, specific *Nitrospira* spp. were found that catalyze complete ammonia oxidation (“comammox”), possessing genes for both ammonia and nitrite oxidation. Comammox *Nitrospira* were first discovered in a deep oil exploration well and a recirculating aquaculture system (RAS) (Daims *et al.*, 2015; van Kessel *et al.*, 2015), and were earlier predicted to be associated with biofilm growth exposed to low ammonia concentrations (Costa *et al.*, 2006). All known comammox bacteria belong to the genus *Nitrospira*, previously only considered to contain canonical NOB (Daims *et al.*, 2016), and are classified into two distinct clades, comammox *Nitrospira* clade A and

clade B (Daims *et al.*, 2015). Since their discovery, comammox *Nitrospira* have been identified in many natural and engineered environments, including wastewater and drinking water treatment plants, soil, lake sediments, and groundwater-fed rapid sand filters (Pinto *et al.*, 2016; Pjevac *et al.*, 2017; Fowler *et al.*, 2018; Orellana *et al.*, 2018; Spasov *et al.*, 2020; S. Xu *et al.*, 2020). Although factors that govern environmental distributions and abundances of comammox *Nitrospira* remain unclear, several studies demonstrate that comammox *Nitrospira* associate with relatively low ammonia concentrations (Pjevac *et al.*, 2017; Spasov *et al.*, 2020). Kinetic studies reveal that the cultivated comammox representative *Nitrospira inopinata* has a high affinity for ammonia ($K_{m(app)} = 63 \pm 10$ nM NH_3), suggesting a competitive advantage within oligotrophic environments (Kits *et al.*, 2017).

Initial aquarium filter studies concluded that AOB were solely responsible for ammonia oxidation in these systems (Hovanec and DeLong, 1996; Burrell *et al.*, 2001). Similarly, NOB were identified as being responsible for completing nitrification in association with AOB, with *Nitrospira* spp. serving as strict nitrite oxidizers (Hovanec *et al.*, 1998). However, the first cultivated AOA representative, *Nitrosopumilus maritimus* SCM1, was isolated from gravel collected from a marine tropical aquarium (Könneke *et al.*, 2005), and several studies in subsequent years revealed a dominance of AOA in marine aquaculture biofilters (Urakawa *et al.*, 2008; Brown *et al.*, 2013). A subsequent survey of ammonia oxidizers within biofilters collected from hobbyist freshwater aquariums, using qPCR analysis of the ammonia monooxygenase A gene (*amoA*), demonstrated that AOA were numerically dominant in freshwater aquariums compared to their AOB counterparts (Sauder *et al.*, 2011). This survey also found that the relative abundance of AOA was negatively correlated with total ammonia concentration. Another study identified a dominance of AOA in freshwater aquarium biofilters, observing long-term stability within the sampled filter material (Bagchi *et al.*, 2014). Given the identification of comammox *Nitrospira* in aquaculture systems (Bartelme *et al.*, 2017), and the favourable high surface area for biofilm growth and low ammonia environment predicted for optimal growth of comammox *Nitrospira*, it was hypothesized that comammox *Nitrospira* would be relatively abundant within aquarium biofilters.

Here the microorganisms involved in aquarium nitrification were revisited, with an emphasis on ammonia oxidizers, and the abundance of AOA, AOB, and comammox *Nitrospira* present in representative freshwater and marine aquarium biofilters was determined using qPCR analysis of the *amoA* gene, along with subsequent sequencing of *amoA* genes from comammox *Nitrospira*. The total microbial community composition using 16S rRNA gene sequencing was also explored and aquarium characteristics that may influence microbial community composition were evaluated. Overall, this study demonstrated that both comammox *Nitrospira* and AOA dominate nitrifier communities within freshwater aquarium biofilters.

2.2 Materials and methods

2.2.1 Sample collection

Aquarium filter and water samples were collected in 2019 from members of the Kitchener Waterloo Aquarium Society (KWAS), community members, and local pet stores; a total of 38 freshwater and 8 saltwater tanks were sampled. Small pieces of sponge, foam, or floss (as appropriate for each biofilter) were collected using sterile scissors and forceps and then stored in Ziplock bags. During transport to the University of Waterloo, samples were kept on ice and subsequently stored at -70°C prior to DNA extraction. Aquarium water samples were collected alongside filter samples and kept on ice during transport. Prior to storage at -20°C, water samples were distributed into two 50 mL Falcon tubes, one of which was filtered through a 0.45 µm syringe filter for chemical analysis. Participants also provided aquarium information including temperature, maintenance history, water source, and the number and species of fish and plants.

2.2.2 DNA extraction

DNA was extracted from aquarium biofilter samples using the DNeasy PowerSoil Kit (Qiagen). Filter samples were thawed and a ~2 cm³ portion from the inside of the sample that had no contact with

the collection bag was cut into small pieces and placed into the 2 mL bead beating tube using sterile scissors and forceps. The extraction was performed following the manufacturer's protocol, with the addition of a 10 min pre-incubation at 70°C prior to bead beating to help maximize cell lysis. Subsequent homogenization was performed using a FastPrep-24 bead beater (MP Biomedical, Santa Ana, CA) for 45 s at 5.5 m s⁻¹. Extracted DNA was visualized on a 1% agarose gel using Gel Red Nucleic Acid Gel Stain (Sigma) and quantified using Qubit dsDNA HS Assay Kit (ThermoFisher Scientific). Extracted DNA samples were stored at -20°C until further use.

2.2.3 Quantitative PCR

Quantitative PCR (qPCR) was performed on biofilter DNA extracts to determine both 16S rRNA and *amoA* gene copies. The 16S rRNA genes associated with bacteria were quantified using the primer set 341F/518R (Muyzer *et al.*, 1993) and *Thermoproteota* 16S rRNA genes were amplified with primers 771F/957R (Ochsenreiter *et al.*, 2003). The *amoA* genes were amplified using comaA-244F(a-f) and comaA-659R(a-f) to target clade A comammox *Nitrospira* (Pjevac *et al.*, 2017), crenamoA-23F/crenamoA-616R to target AOA (Tournu *et al.*, 2008), and amoA1F/amoA2R to target AOB (Rotthauwe *et al.*, 1997).

2.2.3.1 Generation of qPCR gene standards

DNA templates used to generate standard curves for the comammox *Nitrospira amoA*, AOA *amoA*, AOB *amoA*, and AOA 16S rRNA gene qPCR were generated using PCR with the same primers described in Table 2.1. Following PCR amplification of these target sequences, PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). For the 16S rRNA gene qPCR standard, the target gene was amplified from a pUC57-Kan vector containing the target 16S rRNA gene fragment from *Thermus thermophilus* flanked by M13 primers. The M13 primers were used to amplify the 16S rRNA gene target and following PCR amplification the PCR product was also purified as described for the other four DNA template targets. Standards were stored in aliquots at concentrations of

around 10^{10} copies/ μL at -20°C . The DNA concentrations of standard aliquots were measured on the same day the qPCR was performed using a Qubit dsDNA HS Assay Kit (ThermoFisher Scientific).

Measurements were done in duplicate using 4 μL of sample, and the average concentration was used to determine the copy number concentrations for the qPCR standard curves alongside the molecular mass of each standard amplicon sequence as described below.

For the AOA, AOB, bacterial, and archaeal targets, exact sequences of the amplicons were known, and these sequences were used to determine the exact copy numbers added to each qPCR based on the molecular weight of the fragment (Table 2.1, Table 2.2). Since the standard for clade A comammox *Nitrospira* was amplified from a mixture of different comammox *Nitrospira amoA* sequences from aquariums, a molecular weight of an exact sequence could not be used to determine the copy number. Therefore, the average molar mass per bp of 650 g/mol/bp was used to determine the fragment molecular weight used for copy number calculations. Copy number was calculated using the following formula:

$$DNA\ copies = \frac{m \times N_A}{M \times 10^9\ ng/g}$$

Where m is the mass in ng of standard template DNA added to the qPCR reaction, N_A is Avogadro's number, and M is the molecular weight of the dsDNA fragment (Table 2.1).

2.2.3.2 Preparation of qPCR standard curve dilution series and detection limits

The standard curve DNA samples were prepared using a freshly thawed standard aliquot that had a concentration of $\sim 10^{10}$ copies/ μL . Standard dilutions were prepared in a 10-fold dilution series from magnitudes of 10^9 copies/ μL to 10^0 copies/ μL in 100 μL volumes. Each dilution was made with 10 mM Tris-HCl buffer containing 0.05% Tween-20 to minimize adherence of any DNA to the tube.

Detection limits for each qPCR were defined as the lowest magnitude standard that was present in the final standard curve for each gene target. Although standards at a magnitude of 10^0 copies were included, none of these amplified in a successful manner and were omitted from the standard curve range.

Table 2.1 qPCR standard DNA template information for all five gene targets.

Target gene	Primers	Primer reference	Standard source	Amplicon size (bp)	Fragment mass (g/mol)*
16S rRNA bacterial	341F/518R	Muyzer <i>et al.</i> , (1993)	<i>Thermus thermophilus</i>	182	444231
16S rRNA archaeal	771F/957R	Ochsenreiter <i>et al.</i> , (2003)	<i>Ca. Nitrosotenuis aquarius</i>	227	140151
AOB <i>amoA</i>	amoA1F/ amoA2R	Rotthauwe <i>et al.</i> , (1997)	<i>Nitrosomonas europaea</i>	491	303255
AOA <i>amoA</i>	crenamo A23F/616R	Tourna <i>et al.</i> , (2008)	<i>Ca. Nitrosotenuis aquarius</i>	629	388505
CMX <i>amoA</i>	comaA- 244F/A-659R pooled	Pjevac <i>et al.</i> , (2017)	Aquarium samples (four amplicons pooled for template)	415	**269750

* Fragment molecular masses for linear dsDNA correspond to the sequences listed in Table 2.2.

** Calculated based on average molar mass of 650 g/mol/bp as sequence of the DNA target was variable.

Table 2.2 qPCR standard gene sequences with location of target forward and reverse primers indicated in bold. The amplified sequence consists of the region between and inclusive of bolded primer sequences.

Standard source	Sequence
<i>Thermus thermophilus</i> 341F/518R gene standard	GTAAACGACGGCCAGTGAATTCGAGCTCGGTACCTCGCGAATGCATCTAGATATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAACGGGCCCCACT CCTACGGGAGGCAGCAGT TAGGAATCTTCCGCAATGGGCGCAAGCCTGACGGAGCGACGCCGCTTGGAGGAAGAAGCCCTTCGGGGTGTAACCTCCTGAACCCGGGACGAAACCCCGACGAGGGGACTGACGGTACCGGGGTAATA GCGCCGGCCAACTCCGTG CCAGCAGCCGCGGTAAT ACGGAGGGCGCGAGCGTTACCCGGATTCACTGGGCGTAAGGGCGTGTA GCGCGCTGGGGCGTCCCATGTGAAAGACCAGGCTCAACCGTGGGGGAGCGTGGGATACGCTCAGGCTAGACGGTGGGAGAGG GTGGTGGAATTCCTGGAGTAGCGGTGAAATGCGCAGATACCGGGAGGAACGCCGATGGCGAAGGCAGCCACCTGGTCCACCCGT GACGCTGAGGCGCAAGCGTGGGGAGCAAACCGGATTAGATACCCGGGTAGTCCACGCCCTAAACGATGCGCGCTAGGTCTCT GGGTCTCCTGGGGGCCGAAGCTAACGCGTTAAGCGCGCCGCTGGGGAGTACGGCGCAAGGCTGAAACTCAAAGGAATTGACG GGGGCCCGCACAAAGCTTGGCGTAATCATGCTCATAGCTGTTTCCTG
<i>Ca. Nitrosotenuis aquarius</i> 771F/518R gene standard	ACGGTGAAGGATGAAAGCT GGGGGAGCAAACCGGATTAGATACCCGGGTAGTCCCAGCTGTAAACGATGCAGACTCGGTGATGC ATTGGCTTGTGGCCAAATGCAGTGCAGCGGAAGCCGTTAAGTCTGCCGCTGGGAAGTACGTACGCAAGTATGAAACTTAAAG GAATTGGCGGGGAGCACCACAAGGGGTGAAGCCTGCGGTT CAATTGGAGTCAACGCCG
<i>Nitrosomonas europaea</i> amoA1F/amoA 2R gene standard	CCCCCTCTGGAAGCCCTTCTTC ACCGAATGCGGTAACATCATTGCGATGTACGATACGACCTCTTTTACCTTTAACGTAGAAAAA GGCTGTACAGTAACTTTTCCAAGATACCACCATACCGTGAACATCAACATTGATACGAACGCAGAGAAGTGTGCAATAAC TGTGGTATGACCACCAAGGTACGCGTGAACCTTGCTCAATATGACGAACATACTCGGGTGTACCTGTACGAACATACAGATG TCCCATGTAAATCAGCCATCGACAGCAATGTGCCTTCTACAACGATTGGCAAATGGGTTGGTCCAAAAATCGGCCAGTTACCCGG ATAGAACAGCAGACCGAAGAATCCACCTCCAACCCAGAGCGGTACCAGCCAGTTGCGTGTACGATACAGCGTGAAGTCCAGCAT CAGCGCACCCCGGAAGCATAATGCCCGGTGTTACGAAGTTGATGGGGTAGTGT GACCACCAGTAGAATCCCC
<i>Ca. Nitrosotenuis aquarius</i> crenamoA23F/6 16R	ATGGTCTGGCTTAGACGAT GTACGCACTACTTGTTCATAGTAGTCTAGCAGTCAACTCAACCCCTGCTTACAATCAACGCAGGA GACTACATCTTCTACACTGACTGGGCATGGACTTCGTATGTCGTGTTCTCAATATCACAGACATTGATGTTGGTGGTAGGTGCA ACTTACTATCTGACATTTACCGGAGTTCAGGAACCCGAACATACTACGCGCTGATTATGACCGGTGATACATGGATCGCAAAA GGCGCATGGTTTGTCTAGGTTACCCATATGACTTTCATTGTTACACCAAGTTGGTTACCATCAGCAATGCTGATTGATAGCA TACTGGGCTACAAAGAAGAACAAGCACTCACTGATACTATTCCGGTGGTGTGTTGTGTGGAATGTCACTGCCATTGTTCAACATG GTAAATCTAATTACCGTGGCTGATCCATTGGAGACTGCTTTCAAATATCCAAGACCAACATTGCTCCATACATGACTTCCAATA GAACCCCAAGTGGGCAAGTTCTATAACAGTCCAGTTGCATCCGGTGCAGGCGCAGGCGCTGTATTATCAGTAACCTTTGCCGCT CTGGGATGTAAAGCTGAATAC GTGGACGTACAGATGGATGGC
<i>Nitrospira inopinata</i> comaA-244F/A-659R pooled*	TATAACTGGGTCAATTA TGCTCAGCCGTTTGGGGCAACGATAACCATTTTAGCGTTAGGTGCCGGAATAAGGGTGGCGTTTAC ACCTCTTGGTGGTGGTGGTCCAACATACCGCCAAATTTCTGCATGCCGGCCACGTTGCTTCTAGCGCCTTGGTTCTTGATTTC ACCTTGTGTGTAACTAGAACTGGACTTTGACCCGAGTGATCGGGGCGTGGATGACGCGATTTTGTCTATCCGAGCAATTGG CCTATCTTTGCTTACAGCCATACTCCGCTTGTGGTGGATGGACCTTGCTTTTCATGGGCCACTATATGGGCTTTATGTATGTG CGGACCGGAACCTCCTGAATATATCCGTATGATTGAAGTTGGGTGCGTGCAGACGTTCCGTGGG CCACAGCAGCATGATT
P5-P7 16S rRNA standard control **	TGTAACACGACGGCCAGTGAATTCGAGCTCGGTACCTCGCGAATGCATCTAGATCGTATCACCGGTTGTGTGA AATGATACGG CGACCACCGAGAT CAACGAACCTGAACCTGGCAGACTAT CCCGCCGGGAGTGGTGAT ACCGACGAAAACCGCAAGAAAAAGCAGT CTTACTTCCATGATTCTTTAATTATGCCGGGATCCATCGCAGCGTAAT TCGATATGCCGCTCTTCGCTT GAGAGTTTGTATGAT GGTCTAGGGTGAACGCTGGCGCGTGCTTAAGACATGCAAGTCTGTCGGGCGCGGGGTTTACTCGGTGTCAGCGCGGAGCAG GGTGAATAACCGGTGGGTGACCTACCCGGAAGAGGGGGACAACCCGGGGAACCTCGGGCTAATCCCCCATGTGGACCCGCCCT TGGGGTGTGTCCAAGGGCTTTGCCCGCTTCCGGATGGGCGCGCGTCCCATCAGCTAGTTGGTGGGTAATGGCCCAACAGGC GACGACGGGTAGCCCGCTGAGAGGGTGGCGGCCACAGGGGCACCTGAGACACGGGCCCCACTCTACGGGAGGCAGCAGTTAG GAATCTTCCGCAATGGGCGCAAGCCTGACGGAGCGACGCGCTTGGAGGAAGAAGCCCTTCGGGGTGAACCTCCTGAACCCGG GACGAAACCCCGACGAGGGGACTGACGGTACCGGGGTAATAGCGCGCGCCAACCTCCGTGCCAGCAGCCCGGTAAATACGGAGG GCGCGAGCGTTACCCGGATTCACTGGGCGTAAGGGCGGTGTAGGCGGCTGGGGCGTCCCATGTGAAGACCACGGCTCAACCG TGGGGGAGCGTGGGATACGCTCAGGCTAGACGCTGGGAGAGGGTGGTGAATTCCCGGAGTAGCGGTGAATGCGCAGATACCG GGAGGAACGCCGATGGCGAAGGCAGCCACCTGGTCCACCCGTGACGCTGAGGCGCGAAAGCGTGGGGAGCAACCCGATTAGAT ACCCGGGTAGTCCACGCCCTAAACGATGCGCGCTAGGTCTCTGGGTCTCCTGGGGGCCGAAGCTAACGCGTTAAGCGCGCGGCC TGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCGCACAAAGCGGTGGAGCATGTGGTTTAAATTCGAAGC AACGCGAAGAACCTTACCAGGCCTTGACATGCTAGGGAACCCGGGTGAAAGCCTGGGGTGCCCGCGAGGGGAGCCCTAGCACA GGTGCTGCATGGCGTCTGACGCTGCGGTGAGGTGTTGGGTAAAGTCCCGCAACGAGCGCAACCCCGCCCTTAGTTGGCA GCGGGTAGGCGGGGCACTCTAACGGGACTGCCCGCAAGAGCGGGAGGAAGGAGGGGACGACGCTGTGGTCAAGCATGGCCCTTACG GCCTGGGCGACACACGCTGCTACAATGCCCACTACAAGCGAGGCGACCCCGGCAACGGGAGCTAATCGCAAAAAGGTGGGCCCA GTTCGGATTGGGGTCTGCAACCCGACCCCATGAAGCCGAATCGCTAGTAATCGCGGATCAGCCATGCCCGGTGAATACGTTT CCGGGGCTTGTACACACCGCCGCTCAGCCATGGGAGCGGGCTTACCCGAAGTGCAGGGGAGCCTACGGGCGAGCGCGGAGGG TAGGGCCCGTGACTGGGGCGAAGTCTGAACAAGTAATCGGATCCCGGGCCGCTGACTGCAGAGGCGCTGCATGCAAGCTTGGC GTAATCATGGTCATAGCTGTTTCCTG

* Only a representative sequence of the pooled amplicon template used for CMX *amoA* gene qPCR.
 **The P5-P7 16S rRNA standard control was used exclusively during qPCR inhibition testing.

The detection limits for each of the five assays were as follows: for the *amoA* gene targets the detection limits for AOA, AOB, and comammox *Nitrospira* were 50, 70, and 75 copies per reaction, respectively. For the 16S rRNA archaeal gene target, the detection limit was 10 copies. Although the lowest amplified standard for the bacterial 16S rRNA gene target was ~40 copies, this fell within the range of copies detected in the NTC (as background contamination) of 30-60 copies. Therefore, the detection limit was set for this gene target at the next lowest standard of 400 copies, which was still well below the average range of detection at a magnitude of 10^5 copies.

2.2.3.3 qPCR protocols

All qPCR amplifications were run as technical duplicates using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The 10 μ L reaction volumes contained 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 5 μ g of bovine serum albumin, primers, and 1-10 ng of template DNA. The amount of forward and reverse primer used per sample for amplification varied depending on the primer set. For the crenamoA-23F/crenamoA616R and amoA1F/amoA2R primer sets, 4 pmoles of each primer were used, the 341F/518R primer set used 3 pmoles of each primer, 2 pmoles of each primer were used for the 771F/957R primer set, and 5 pmoles were used for the comaA-244F(a-f) and comaA-659R(a-f) primer pair.

The PCR conditions for the 341F/518R and 771F/957R primer sets involved an initial 3 min denaturation at 98°C, then 35 cycles at 98°C for 30 s followed by a combined annealing and extension step at 55°C for 45 s. For clade A comammox *Nitrospira amoA* gene amplification, an initial denaturation for 3 min at 98°C was also used, followed by 35 cycles of 30 s at 98°C, 45 s at 52°C, and 1 min at 72°C. Both the AOA and AOB *amoA* gene amplification began with an initial denaturation step for 3 min at 98°C, followed by 35 cycles of 30 s at 98°C, 30 s at 55°C or 60°C for the AOA and AOB amplification, respectively, and 1 min at 72°C. Following all qPCR amplifications, a melt curve was run from 65-95°C with 0.5°C interval increases, each lasting for 2 s. Gel-purified PCR amplicons generated from *Ca*.

Nitrosotenuis aquarius, *Nitrosomonas europaea*, and pooled aquarium biofilter DNA templates were used as qPCR standards for AOA, AOB, and comammox *Nitrospira amoA* gene targets, respectively, whereas purified PCR amplicons generated from *Thermus thermophilus* and *Ca. N. aquarius* DNA were used as standards for bacterial and *Nitrososphaeria* 16S rRNA gene targets, respectively (Table 2.1, Table 2.2). Starting DNA copy numbers in standards used for qPCR were determined based on the DNA concentrations of the PCR amplicons, with standard curves ranging from 10^0 to 10^7 gene copies. Analysis of qPCR data, including quantification and melt curve analysis, was done through the CFX Manager Software (version 1.5; Bio-Rad). Efficiencies across all qPCR assays ranged from 82.6-99.9%, with R^2 values > 0.99 for all standard curves. Final qPCR products were also verified using 1% agarose gels. Clade B comammox *Nitrospira* primers (Pjevac *et al.*, 2017) were not used for qPCR because no clade B *amoA* gene signal was detected during initial end-point PCR screening using all previously mentioned primer sets (data not shown). Statistical analysis of qPCR gene copy data was done in R (v 4.3.2) to perform non-parametric Wilcoxon rank sum and Kruskal Wallis rank sum tests to test for significant differences between *amoA* gene abundances of all three groups of ammonia oxidizers. Additional post-hoc analysis was done using the Dunn's test function from the FSA (v. 0.9.5) R package. All uncertainty values for qPCR results represent calculated standard deviation (SD) values.

2.2.3.4 Testing for inhibition of qPCR

To test for PCR inhibitors in extracted DNA samples used as template for qPCR, an inhibition assay based on a previously described protocol was conducted with modifications (Nolan *et al.*, 2006). An in-house 16S rRNA gene standard template was used, which contained P5-P7 Illumina adapter sequences that allow amplification of a 164 bp target fragment (Table 2.2). The qPCRs were run in technical duplicates for samples, with each reaction containing 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 5 μ g of bovine serum albumin, primers, 1-10 ng of template DNA, and 5 pmol of both the P5 forward primer and P7 reverse primer to a final volume of 15 μ L. Each sample containing template DNA was also spiked with 10,000 copies of the P5-P7 16S rRNA standard control

sequence target. Additionally, eight replicate reactions were run as “control samples” containing only ~10,000 copies of the standard to provide a baseline for where the C_q value is expected to be for this target copy number. The conditions for qPCR were as follows: initial 3 min denaturation at 98°C, then 40 cycles at 98°C for 10 s followed by a combined annealing and extension step at 60°C for 30 s, with a final melt curve was run from 65-95°C with 0.5°C interval increases, each lasting for 2 s. The qPCR was run using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and analyzed using CFX Maestro Software (version 2.3; Bio-Rad).

Control samples containing 10,000 copies of the standard template had an average C_q value of 22.3 ± 0.2 (min 22.0, max 22.6; n = 16). Standard template spiked with aquarium DNA had an average C_q value of 22.7 ± 0.3 (min 22.1, max 23.5; n = 46). All C_q values for template spiked with aquarium DNA were within 1 cycle of the non-spiked controls, demonstrating minimal PCR inhibition by co-extracted inhibitors.

2.2.4 Water chemistry

Water that was filtered through a 0.45 µm syringe filter prior to storage at -20°C was used for subsequent assays. Total ammonia was quantified using the orthophthaldialdehyde (OPA) fluorometric assay (Holmes *et al.*, 1999; Poulin and Pelletier, 2007) and the Griess reagent was used to determine nitrite/nitrate concentrations (Miranda *et al.*, 2001). The lower limits of detection for these assays were 0.7 µg/L NH₃-N (50 nM) and ~3 µg/L NO₂⁻-N (0.2 µM) for the OPA assay and Griess assay, respectively. The pH of water samples was measured using a LAQUAtwin pH-33 meter (Horiba Advanced Techno Co., Ltd.). Additionally, general water hardness (GH) and carbonate hardness (KH) were measured using the GH and KH Test Kit (Freshwater; API). General hardness, which is a measure of primarily dissolved magnesium and calcium ions, was quantified using degrees of GH (dGH) where 1 dGH = 17.9 ppm of calcium carbonate equivalents. Carbonate hardness was measured using degrees of KH (dKH) where 1 dKH = 17.9 ppm of CaCO₃, which measures specifically concentrations of carbonate in the water. Alkalinity was also measured using the MultiTest Marine pH & Alkalinity kit (Seachem). A measure of

total alkalinity quantifies the ability of water to buffer or neutralize acid and expressed here as meq/L, where 1 meq/L = 50 ppm of CaCO₃.

2.2.5 16S rRNA gene sequencing and analysis

Primers 515F-Y and 926R were used to target the V4-V5 region of 16S rRNA genes (Quince *et al.*, 2011; Parada *et al.*, 2016). For each sample, 25 µL PCR amplifications contained 1X ThermoPol buffer, 15 µg of bovine serum albumin, 200 µM of dNTPs, 0.2 µM of both forward and reverse primers, 0.625 units of Hot Start *Taq* DNA Polymerase (New England Biolabs, MA, USA), and 1-10 ng of template DNA. The PCR cycling conditions consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 1 min, with a final extension time of 7 min at 68°C. Individual samples were amplified in triplicate, with unique barcoded adapters attached to primers to allow sequencing of pooled samples (Bartram *et al.*, 2011). Additional no template and positive controls (1:1 *Aliivibrio fischeri* and *Thermus thermophilus* DNA) were included as samples during PCR amplification and for sequencing.

The resultant triplicate PCR products for each sample were pooled and quantified using a 1% agarose gel stained with GelRed (Biotium, CA, USA). Quantified pooled triplicates for each sample were then combined in equimolar concentrations based on gel quantification to create an amplicon library, which was sequenced using the MiSeq Reagent Kit v2 (2 x 250 cycles, Illumina, Canada) on a MiSeq System (Illumina, CA, USA) at the University of Waterloo following manufacturer's protocols and guidelines. Following demultiplexing of paired-end sequence reads using MiSeq Reporter software version 2.5.0.5 (Illumina), analysis was done using QIIME2 (version 2020.6) implemented through the AXIOME3 pipeline (Min *et al.*, 2021). Quality trimming, primer sequence removal, denoising, paired-end sequence merging, chimera removal, and final generation of an amplicon sequence variant (ASV) table was done using DADA2 (Callahan *et al.*, 2016). Taxonomic classification of ASVs was done through AXIOME3 using the SILVA database release 138, which classifies AOA within the phylum

Crenarchaeota (Quast *et al.*, 2013). A phylogenetic tree of ASV sequences was generated using FastTree (Price *et al.*, 2010).

Additional ASV table analysis was performed using the AXIOME3 pipeline, including rarefaction to the lowest sample count (8417) prior to calculation of beta diversity metrics (weighted UniFrac, Bray-Curtis) and generation of triplot ordinations. Correlations between environmental variables and beta diversity were determined using the `envfit()` function in the R `vegan` package (version 2.6-4), to fit environmental variables onto the ordination space. PERMANOVA testing was performed through QIIME2 (version 2020.6) using ‘beta-group-significance’ available through the ‘diversity’ plugin. All DNA sequences were deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA780914. The variances for relative abundances shown in results represent calculated standard deviation values.

2.2.6 Comammox *Nitrospira amoA* gene sequencing

The *amoA* genes of clade A comammox *Nitrospira* were amplified by PCR for each of the 38 freshwater aquarium biofilter samples using the *comaA*-244F(a-f) and *comaA*-659R(a-f) forward and reverse primers following previously established protocols (Pjevac *et al.*, 2017). The PCR was set up in triplicate using 25 µL reaction volumes, each containing 1X ThermoPol buffer, 5 µg bovine serum albumin, 200 µM of dNTPs, 500 µM of each forward and reverse primer, 0.625 units of *Taq* polymerase, and 1-20 ng of template DNA and amplified using 35 cycles. Alongside the freshwater biofilter samples, *amoA* genes of comammox *Nitrospira* from additional aquarium enrichments containing comammox *Nitrospira* (section 2.2.7) and an additional biofilm sample from a tertiary treatment system of a Guelph, ON municipal WWTP were amplified for sequencing. The WWTP biofilm sample was used as a positive control and contained two known clade A comammox *Nitrospira* *AmoA* sequences. After amplification, the PCR product was confirmed using gel electrophoresis and triplicate samples were pooled.

Final *amoA* gene PCR products were sent to the Centre for Analysis of Genome Evolution and Function (CAGEF) at the University of Toronto (Mississauga, ON). Bead purification was used to clean up PCR products prior to library preparation. The NEB Next Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) was used to generate sequencing libraries, with the addition of barcoded Illumina adapters to end of the amplified *amoA* gene amplicons. Additional size selection was performed prior to sequencing and fragment size was verified on a bioanalyzer. The prepared amplicon library was then sequenced (2 x 250 cycles) on an MiSeq System (Illumina) following the manufacturer's protocol. Raw DNA sequence reads are available through the NCBI Sequence Read Archive (project number PRJNA780914).

Demultiplexed raw sequence reads were processed using QIIME2 (version 2020.6) implemented through the AXIOME3 pipeline (Min *et al.*, 2021). Similar to 16S rRNA gene sequence data processing, quality trimming, primer removal, denoising, paired-end sequence merging, chimera removal, and final generation of an amplicon sequence variant (ASV) table was done using DADA2 (Callahan *et al.*, 2016). From the ASV table, individual ASVs were further sorted using a custom R script to identify and combine reverse complement ASV pairs present in the data. These reverse complement pairs were present because of the non-preferential ligation of barcodes to either end of the PCR products amplified for sequencing. After merging reverse complement sequence pairs, ASV translated nucleotide sequences were analyzed using tblastn available through NCBI Web BLAST against the AmoA amino acid sequence of *Nitrospira inopinata* using default algorithm parameters. Any sequences that did not align with the *N. inopinata* AmoA amino acid sequence were removed from the data, as they were not true comammox *Nitrospira amoA* gene amplicons. Alignments with *amoA* gene sequences from other ammonia oxidizers such as *N. europaea* (AOB) and *N. maritimus* (AOA), which align with some identity to the AmoA sequence of *N. inopinata*, were used as controls to confirm that any sequence that did not align was an off-target product in the amplicon sequence data.

The final *amoA* gene ASV table was used for subsequent data analysis. The ASVs were collapsed based on 100% amino acid identity and resulting unique amino acid sequences were used to generate a phylogenetic tree with MEGA X (version 10.1.8) (Kumar *et al.*, 2018). A heatmap of the collapsed *amoA* amplicon data was generated through R (version 4.1.2) using the *ggdendro* (0.1.23), *ggtree* (3.2.1), and *ggplot2* (3.4.1) packages. The triplot analysis and visualization of the amplicon data was also done with a custom R script implementing the *vegan* (2.6-4), *ggplot2* (3.4.1), and *ecodist* (2.0.9) packages. Beta diversity of *AmoA* sequences across samples was calculated using the *vegdist()* function from *vegan*. The *envfit()* function from *vegan* was used to calculate correlations and fit environmental variables (pH, temperature, dGH, dKH, months since sponge replacement, alkalinity, ammonia, nitrite, and nitrate) onto the ordination space. Additionally, the distribution of individual *amoA* gene sequences across samples was fitted to the ordination using the *wascores()* function from the *vegan* package, which computes the weighted averages for a variable across samples in an ordination.

2.2.7 Enriching comammox *Nitrospira* from an aquarium biofilter

The comammox *Nitrospira* aquarium biofilter enrichment cultures included in the *amoA* gene sequencing (Aq-C1 and Aq-C2) originate from biomass from a freshwater aquarium biofilter. The original parent culture was started by taking 5 mL of water directly from within the biofilter while it was not running and inoculating it into 45 mL of AOM medium (Daims *et al.*, 2015), containing 0.5 mM NH₄Cl in a 100 mL Schott bottle. The enrichment was incubated in the dark at room temperature. After ~5 weeks, the culture had oxidized all the ammonia to both nitrite and nitrate and was re-fed ammonia at the same concentration of 0.5 mM as was originally present in the AOM medium. Two weeks later, DNA extracted from the culture was used to run PCR with the *comaA*-P primers targeting the comammox *Nitrospira* clade A *amoA* gene (Pjevac *et al.*, 2017). The PCR detected the presence of comammox *Nitrospira amoA* genes, indicating their presence in the enrichment culture.

Given the detection of comammox *Nitrospira* and active ammonia oxidation, a sub-culture was created from this original enrichment culture by transferring 2 mL of the parent culture into 48 mL of

fresh AOM medium (0.5 mM NH₄Cl), and an additional 5 mL of water from the original aquarium filtered through a 0.1 µm syringe filter in a new 100 mL Schott bottle. Incubation of the cultures was continued at room temperature in the dark. After 2 weeks, the new sub-culture had oxidized all ammonia into nitrate and was sub-cultured again following the same protocol as just described. This newest sub-culture was the Aq-C1 enrichment culture. The Aq-C1 enrichment culture was continually re-fed 25 µL of 1M NH₄Cl every few weeks once it had oxidized all the ammonia into nitrate and PCR did detect the presence of comammox *Nitrospira*. Around 2 months after incubation, Aq-C1 was sub-cultured again following the same protocol – this newest subculture was Aq-C2. After several weeks, Aq-C2 had detectable comammox *Nitrospira* (via PCR) and was oxidizing all provided ammonia to nitrate, indicative of comammox activity.

Both Aq-C1 and Aq-C2 enrichment cultures were re-fed 25 µL of 1M NH₄Cl every few weeks as they oxidized the ammonia to nitrate. They were continually kept in the dark at room temperature. When enrichment cultures had a nitrate concentration greater than 500 mg/L N-NH₃, most of the AOM medium was then removed from the Schott bottle, leaving behind the solid calcium carbonate, and replaced with fresh AOM medium to decrease the overall nitrate concentration. Approximately 6 months after starting Aq-C2, DNA was extracted from both Aq-C1 and Aq-C2, which were both still actively oxidizing ammonia and had detectable comammox *Nitrospira*. This extracted enrichment culture DNA was used for the comammox *Nitrospira amoA* sequencing described in section 2.2.6. All DNA extractions were performed on 2 mL of enrichment cultures using the DNeasy UltraClean Microbial Kit (Qiagen) following the manufacturer's protocol.

2.2.8 Network analysis

To explore direct interactions between microbial community members, nitrifiers, and metadata within freshwater biofilters network analysis was performed using FlashWeave, which predicts ecological interactions between microbial community members, while removing spurious correlations that result from indirect network edges (Tackmann *et al.*, 2019). To help prevent spurious associations during

network analysis, the freshwater ASV table was filtered to remove any ASVs that were not present in at least three of the samples. Additionally, because FlashWeave is unable to handle missing values, samples that were missing large portions of metadata, FW-22, FW-23, and FW-24, were removed from the ASV table prior to filtration. The filtered table was used to generate a correlation network using the FlashWeave – sensitive mode in FlashWeave (version 1.6), with the `learn_network` function using the parameters, `sensitive=true`, `heterogeneous=false`, `max_k=3`, `alpha=0.05`, and `sparse=false`. The output network was then filtered using Cytoscape to select only nodes corresponding to genera of known nitrifiers (e.g., “*Nitro*” or “*Nitroso*” containing genera) and the first neighbours of those nodes, along with any edges existing between these selected nodes. These selected nodes and edges comprised the final visualized network. Filtered and original network tables were manually checked to ensure that all ASVs of known nitrifier genera were included. All FlashWeave network analyses were run using Julia (version 1.9.2) (Bezanson *et al.*, 2017) and Cytoscape (version 3.10.0) was used for network visualization (Shannon *et al.*, 2003).

2.3 Results

2.3.1 Aquarium samples

Aquarium biofilter and water samples (Table 2.3) were collected from 38 freshwater aquariums (FW-01 to FW-38) and 8 saltwater aquariums (SW-01 to SW-08). Samples were collected from residences and retail pet stores in the Region of Waterloo and the city of Mississauga in Southwestern Ontario, Canada. Approximately half of the sampled aquariums contained live plants, and the average number of fish across all aquariums was ~22, ranging from 0-150. Several aquariums were populated with cichlids, algae eaters (e.g., *Plecostomus*), or guppies, and many contained mixed tropical or marine fish; one aquarium housed a turtle. Aquarium ages averaged 3 years, ranging from 1 month to 13 years, and were subject to diverse maintenance routines (Table 2.3). Water used for most aquariums was sourced from municipal, reverse osmosis, distilled, or bottled water, with water changes occurring at

frequencies ranging from once a week to once every eight weeks. For most aquarium biofilters, the sponge or floss material was rarely or never replaced. However, several owners replaced sponge/floss material as often as once a week to a frequency of every two years of use. At the time of aquarium establishment, five of the freshwater biofilters and one saltwater biofilter were inoculated with aquarium supplements, and within six months prior to sampling, only one freshwater aquarium and two saltwater aquariums had been exposed to antibiotics (Table 2.3). Specifically, FW-11 was treated with Levamisole-HCl and ICH-X (Malachite Green), and both SW-05 and SW-06 were treated with cupramine and erythromycin. Sampled aquariums had a wide range of conditions for size (5-280 gallons), temperature (19.3-28.8°C), alkalinity (0-14 meq/L), pH (6.2-9.3), general hardness (1-52 dGH), and carbonate hardness (1-38 dKH) (Table 2.4). Measured concentrations of total ammonia were relatively low overall, with an average concentration of ~59 µg/L NH₃-N or total ammonia nitrogen. Nitrite was usually undetected, or low in most aquariums; only three aquariums had concentrations >1 mg/L NO₂⁻-N. In contrast, nitrate concentrations were high in most aquariums (>1 mg/L NO₃⁻-N), with an average concentration of 16.7 mg/L NO₃⁻-N, with the lowest and highest measured concentrations at 15.3 µg/L NO₃⁻-N and 106.7 mg/L NO₃⁻-N, respectively.

Table 2.3 Summary of aquarium sample metadata, including aquarium type, maintenance, location, number of fish, and sampling dates.

Sample ID	Sampling date	Location	City	Aquarium type	Filter material	Live plants (y/n)	Added supplements (y/n)	Antibiotic treatment (< 6 mo.)	Water source	Fish type	Aquarium age (years)	Fish	Last sponge replacement (months)	Frequency of water changes (weeks)
FW-01	03/05/2019	Residential 1	Waterloo	Fresh	Sponge	Yes	No	No	Tap water	Discus, angel	7	12	12	1
FW-02	03/05/2019	Residential 2	Kitchener	Fresh	Sponge	Yes	No	No	Tap water	Guppies	0.5	10	Never	1
FW-03	03/05/2019	Residential 3	Guelph	Fresh	Sponge	No	No	No	Tap water	Cichlids, pleco	3	37	12	4
FW-04	03/05/2019	Residential 3	Guelph	Fresh	Sponge	Yes	No	No	Tap water	Mixed tropical	2	67	12	4
FW-05	03/05/2019	Residential 4	Drayton	Fresh	Sponge	No	No	No	Tap water	Cichlids, petricola	9	10	60	1
FW-06	03/05/2019	Residential 4	Drayton	Fresh	Sponge	No	No	No	Tap water	Cichlid	9	5	60	1
FW-07	03/05/2019	Residential 5	Drayton	Fresh	Sponge	No	No	No	Tap water	Guppies, pleco	0.1	8	1	4
FW-08	03/05/2019	Residential 5	Waterloo	Fresh	Sponge	Yes	No	No	Tap water	Guppies, pleco	2	20	Never	2
FW-09	03/05/2019	Residential 5	Waterloo	Fresh	Sponge	Yes	No	No	Tap water	Barbs, pleco	1	13	Never	4
FW-10	03/05/2019	Residential 6	Fergus	Fresh	Floss	Yes	No	No	Tap water	Cichlid, pleco	0.5	2	Never	8
FW-11	03/05/2019	Residential 6	Fergus	Fresh	Floss	Yes	Yes	Yes	Tap water	Mixed tropical	1	25	Never	4
FW-12	03/09/2019	Residential 7	Mississauga	Fresh	Floss	No	No	No	Tap water	Cichlid	0.5	1	1	5
FW-13	03/09/2019	Residential 7	Mississauga	Fresh	Sponge	No	No	No	Tap water	Turtle	9	1	1	4
FW-14	03/18/2019	Residential 8	Waterloo	Fresh	Sponge	No	No	No	Tap water	Cichlid	12	3	8	4
FW-15	04/02/2019	Residential 9	Drayton	Fresh	Floss	Yes	No	No	Tap water	Mixed tropical	3	20	6	4
FW-16	04/02/2019	Residential 5	Drayton	Fresh	Sponge	Yes	No	No	Tap water	Guppies, plecos	0.3	30	4	4
FW-17	04/02/2019	Residential 10	Kitchener	Fresh	Floss	No	No	No	Tap water	Endlers, crayfish	4	20	24	2
FW-18	04/02/2019	Residential 11	Cambridge	Fresh	Floss	Yes	Yes	No	Tap water	Plecos	0.75	10	1	1
FW-19	04/02/2019	Residential 11	Cambridge	Fresh	Floss	No	Yes	No	Tap water	Cichlids	0.3	10	1	1
FW-20	04/02/2019	Residential 12	Kitchener	Fresh	Sponge	Yes	Yes	No	Tap water	Cichlids	6	12	Never	2
FW-21	04/02/2019	Residential 14	Guelph	Fresh	Sponge	Yes	No	No	Tap water	Danios	0.75	4	Never	4
FW-22	04/02/2019	Residential 15	Waterloo	Fresh	Sponge	No	No	No	Tap water	Unknown	2	Unknown	Unknown	Unknown
FW-23	04/02/2019	Residential 15	Waterloo	Fresh	Sponge	No	No	No	Tap water	Unknown	2	Unknown	Unknown	Unknown
FW-24	04/02/2019	Residential 15	Waterloo	Fresh	Sponge	No	No	No	Tap water	Unknown	2	Unknown	Unknown	Unknown
FW-25	04/02/2019	Residential 16	Guelph	Fresh	Sponge	Yes	No	No	Tap water	Mixed tropical	0.5	75	Never	1
FW-26	04/02/2019	Residential 16	Guelph	Fresh	Sponge	Yes	No	No	Tap water	Cichlids	Unknown	5	2	1
FW-27	04/02/2019	Residential 16	Guelph	Fresh	Sponge	No	No	No	Tap water	Cichlids	0.5	30	Never	1
FW-28	04/02/2019	Residential 16	Guelph	Fresh	Sponge	No	No	No	Tap water	Mixed tropical	1	25	Never	1
FW-29	04/02/2019	Residential 3	Guelph	Fresh	Sponge	Yes	No	No	Tap water	Cichlids	1	103	9	4
FW-30	04/02/2019	Residential 3	Guelph	Fresh	Floss	No	No	No	Tap water	Cichlids, plecos	1	40	5	4
FW-31	04/02/2019	Residential 17	Waterloo	Fresh	Sponge	Yes	Yes	No	Ground and RO water	Mixed tropical	3	50	Never	4
FW-32	04/02/2019	Residential 18	Waterloo	Fresh	Sponge	No	No	No	Tap water	Guppies, tetras	2	12	Never	2
FW-33	04/02/2019	Residential 18	Waterloo	Fresh	Sponge	Yes	No	No	Bottled water	<i>Microctenopoma</i>	0.5	2	Never	3
FW-34	04/02/2019	Residential 18	Waterloo	Fresh	Sponge	No	No	No	Bottled water	Mixed tropical	1	30	Never	3
FW-35	04/02/2019	Residential 18	Waterloo	Fresh	Sponge	Yes	No	No	Tap and distilled water	<i>Cynodoricthys</i>	0.5	2	Never	3
FW-36	05/02/2019	Residential 19	Kitchener	Fresh	Sponge	Yes	No	No	RO water	Tetras, rasboras	13	8	6	3
FW-37	05/12/2019	Residential 7	Mississauga	Fresh	Sponge	No	No	No	Tap water	Turtle	9	1	1	4
FW-38	06/05/2019	Residential 22	Unknown	Fresh	Sponge	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
SW-01	04/02/2019	Residential 13	Guelph	Salt	Floss	Yes	No	No	Tap water	Damsels, clownfish	3	8	4	4
SW-02	05/13/2019	Residential 20	Kitchener	Salt	Sponge	Yes	No	No	Tap water	Mixed marine	13	12	Never	8
SW-03	05/17/2019	Retail 1	Kitchener	Salt	Floss	Yes	No	No	RO water	Mixed marine	1.5	10	0.25	1
SW-04	05/17/2019	Retail 1	Kitchener	Salt	Floss	Yes	No	No	Tap water	Mixed marine	1.5	12	0.25	1
SW-05	05/17/2019	Retail 1	Kitchener	Salt	Floss	No	No	Yes	Tap water	Tangs, wrasse	1.5	3	0.25	1
SW-06	05/17/2019	Retail 1	Kitchener	Salt	Floss	No	Yes	Yes	Tap water	Mixed marine	0.5	150	0.25	1
SW-07	05/17/2019	Retail 1	Kitchener	Salt	Floss	Unknown	Unknown	Unknown	Unknown	None	Unknown	Unknown	Never	1
SW-08	05/21/2019	Residential 21	Unknown	Salt	Floss	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown

Table 2.4 Water chemistry, temperature, and aquarium size data for all aquarium biofilter samples collected.

ID	Size (gallons)	Temperature (°C)	pH	Alkalinity (meq/L)	dGH	dKH	Total NH ₃ -N (µg/L)	NO ₂ ⁻ -N (µg/L)	NO ₃ ⁻ -N (µg/L)
FW-01	120	28.0	8.6	14.0	3	20	16.7	21.6	2394.0
FW-02	15	25.0	8.1	14.0	21	10	0.0	0.0	4274.5
FW-03	55	22.0	8.0	4.5	30	7	0.0	19.9	27829.2
FW-04	29	24.0	8.0	2.5	31	7	12.5	45.1	39762.7
FW-05	30	24.4	8.3	3.5	32	9	0.0	0.0	4127.9
FW-06	20	24.4	8.2	3.0	32	7	0.0	14.1	5097.5
FW-07	10	25.6	8.2	4.0	14	11	16.7	2013.6	2262.0
FW-08	20	24.4	8.1	4.5	16	13	3.7	13.2	6225.1
FW-09	30	24.4	8.2	4.0	15	11	20.3	0.0	7403.5
FW-10	29	25.6	8.5	7.5	3	18	0.0	0.0	30337.0
FW-11	75	25.0	8.4	5.0	2	13	0.3	0.0	17516.9
FW-12	90	23.9	6.8	0.0	27	1	1.8	16.3	21473.1
FW-13	5	20.0	8.4	5.0	31	15	232.5	94.7	4425.8
FW-14	110	26.7	8.3	3.0	24	11	0.0	0.0	4313.0
FW-15	50	23.9	8.7	7.5	25	20	25.8	0.0	9535.9
FW-16	50	25.6	8.6	4.5	18	12	0.0	20.3	8203.7
FW-17	5	20.0	8.1	1.5	25	10	12.0	0.0	5149.2
FW-18	65	26.0	8.1	2.5	31	10	0.0	18.0	3364.0
FW-19	90	26.0	8.3	4.0	33	12	0.7	15.7	8223.9
FW-20	110	25.6	8.1	2.5	28	9	22.9	0.0	2756.5
FW-21	5	25.0	9.3	3.5	17	7	0.0	0.0	15.3
FW-22	unknown	27.7	7.5	2.0	52	4	250.8	233.0	106676.6
FW-23	unknown	27.7	8.3	7.5	46	17	109.0	246.2	37921.3
FW-24	unknown	27.7	8.1	4.0	38	8	70.6	46.8	46290.2
FW-25	125	27.0	8.6	12.0	4	28	59.7	15.0	14986.4
FW-26	15	22.0	8.6	10.5	3	23	14.9	0.0	1004.8
FW-27	20	28.8	8.6	16.5	5	38	16.6	0.0	90721.8
FW-28	155	27.3	8.1	4.5	8	13	54.2	0.0	34680.7
FW-29	29	26.7	8.2	4.0	39	12	0.0	0.0	1976.2
FW-30	75	23.9	8.3	4.0	41	9	0.0	17.1	27163.0
FW-31	46	23.9	7.9	1.5	19	3	0.0	0.0	6683.2
FW-32	10	19.3	8.0	4.0	17	10	0.0	0.0	899.5
FW-33	10	19.3	8.2	0.5	2	1	0.0	0.0	62.9
FW-34	10	19.5	6.2	0.5	4	1	650.6	0.0	4599.3
FW-35	10	19.7	6.9	0.5	1	2	0.0	0.0	849.5
FW-36	10	25.0	7.1	0.5	40	3	0.0	0.0	1012.2
FW-37	5	20.0	8.6	7.0	46	18	237.8	40.1	4943.2
FW-38	unknown	unknown	6.5	1.5	36	4	136.9	1029.4	60340.8
SW-01	55	22.6	8.0	4.0	n/a	11	16.2	0.0	24861.7
SW-02	180	25.6	8.0	5.0	n/a	14	0.0	42.4	59595.8
SW-03	240	25.6	8.1	4.5	n/a	12	0.0	42.4	2365.4
SW-04	280	25.6	8.1	8.0	n/a	22	299.8	221.5	9156.8
SW-05	170	25.6	8.3	6.5	n/a	16	8.2	0.0	21177.8
SW-06	200	25.6	8.2	4.5	n/a	13	45.4	14.0	4082.3
SW-07	unknown	25.6	8.1	5.5	n/a	15	12.7	45.2	3412.8
SW-08	unknown	unknown	7.7	12.5	n/a	36	362.4	8231.9	1564.5

2.3.2 Aquarium biofilter microbial communities

A total of 12,241 unique amplicon sequence variants (ASVs) were identified from the 16S rRNA gene sequence data for all biofilter samples, with a total of 969,251 reads and an average of 21,071 reads per sample. Positive sequencing controls contained expected ASVs of both *Aliivibrio fischeri* and *Thermus thermophilus* at relative abundances of ~60% and ~40% in both replicates, respectively. Both *Proteobacteria* and *Bacteroidota* dominated phylum-level ASV affiliations for all fresh and saltwater samples (Figure 2.1). Freshwater biofilter samples had an average relative abundance of $40.1 \pm 9.4\%$ for *Proteobacteria* and $23.7 \pm 9.0\%$ for *Bacteroidota*, whereas saltwater biofilters had average relative abundance of $46.3 \pm 6.3\%$ and $30.4 \pm 9.5\%$ for the two phyla, respectively. Members of the *Planctomycetota* were also detected consistently across all samples, ranging between a relative abundance of 1-13%. Several additional phyla were present in most samples at >1% relative abundance, including *Acidobacteria*, *Chloroflexi*, *Cyanobacteria*, *Myxococcota*, *Nitrospirota*, and *Verrucomicrobiota* (Figure 2.1).

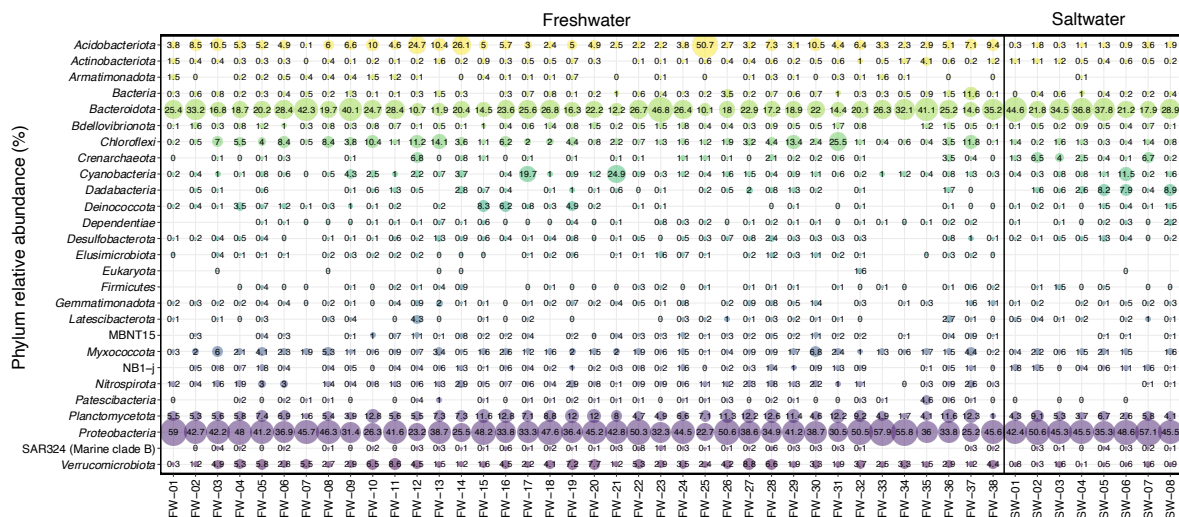


Figure 2.1 The relative abundance plot of 16S rRNA gene profiles of aquarium biofilter samples at the phylum level shows taxonomy assigned using the SILVA database release 138. Size of the bubble and the number represent the relative abundance (RA) of each phylum within a biofilter sample. Only phyla present in at least one biofilter at a relative abundance of >1% are shown.

Microbial community profiles of saltwater and freshwater samples were unique, grouping distinctly from one another in multidimensional space (Figure 2.2A, pseudo-F = 9.6, $p = 0.001$). Although salinity explained the overall composition of sampled aquarium biofilters, other measured parameters also affected microbial community composition. Within freshwater aquarium biofilter samples specifically, correlation analysis revealed that aquarium age, general water hardness, and aquarium temperature all correlated significantly with 16S rRNA gene profiles ($R^2 > 0.3$, $p < 0.05$; Figure 2.2B). For freshwater samples, several taxa grouped in the centre of ordination space, indicating that they were common to most freshwater biofilter samples, including ASVs affiliated with *Comamonadaceae*, *Vicinamibacteraceae*, *Rhodobacter*, and *Terrimonas*. In contrast, *Blastocatellaceae*-associated taxa grouped with samples that had lower general hardness (Figure 2.2B), indicating that these bacteria were at higher relative abundances in soft water. When considering saltwater samples specifically, only aquarium size correlated with overall distribution of microbial community profiles ($R^2 > 0.3$, $p < 0.05$), and this was also true for the combined analysis with salt and freshwater samples. However, the majority of saltwater biofilter samples were collected from the same retail location, where the aquariums were relatively large compared to most at-home aquariums (170-280 gallons; Table 2.4), and the single home saltwater sample had a size of 55 gallons. With a size range of 5-155 gallons, most freshwater aquariums were smaller with 23 of 38 being less than 55 gallons. This difference in size distribution between salt and freshwater aquarium samples collected, along with the observation that they have different overall microbial community compositions, is likely responsible for the correlation between aquarium size and microbial community composition.

At the taxonomic family level, community profiles were more distinct between saltwater and freshwater biofilter samples (Figure 2.3). Members of the families *Saprospiraceae* and *Rhodobacteraceae* were detected in both fresh and saltwater biofilters; *Rhodobacteraceae* had higher relative abundance in saltwater samples (between 7-24%) than in freshwater samples (between 2-9%).

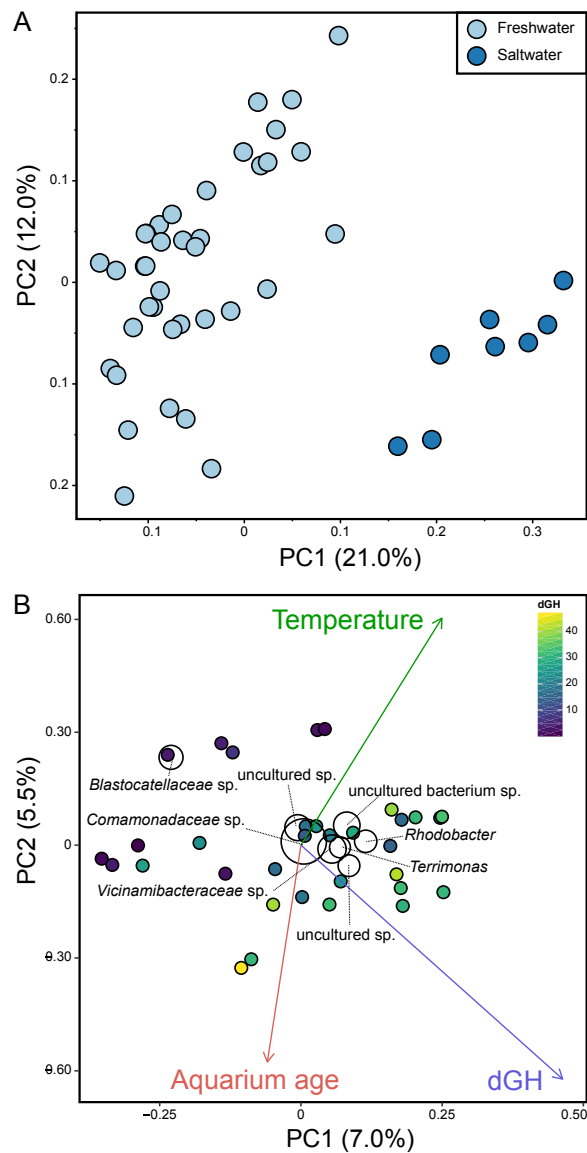


Figure 2.2 Principal coordinates and triplot analysis of 16S rRNA gene profiles of aquarium biofilters (A, B). Grouping between the saltwater and freshwater samples is shown in the PCoA plot (A) which was calculated using the weighted UniFrac metric on samples rarefied to the lowest sample count. An ordination based on the Bray-Curtis metric using only the freshwater samples (B) shows that the freshwater biofilm communities are correlated with temperature, aquarium age, and dGH ($R^2 > 0.3$, $p < 0.05$). Taxa collapsed at the phylum level present at an average abundance of greater than 1% are displayed on the plot by the labelled black circles. Size of the circles illustrates the average relative abundance of the species across samples, while their placement reflects the correlation specific species have with different samples. The axes on both plots display the percent variation within the samples illustrated by each of the two principal components displayed (A, B).

Taxa from families *Cyclobacteraceae* and *Woeseiaceae* taxa appeared only in saltwater biofilter samples at >2% relative abundance thresholds, whereas members of *Chitinophagaceae*, *Microscillaceae*, *Comamonadaceae*, and *Sphingomonadaceae* were present in all freshwater samples either above or below the relative abundance threshold used (Figure 2.3). Overall results from 16S rRNA gene sequencing revealed distinct microbial communities in saltwater and freshwater aquarium biofilters.

2.3.3 Ammonia oxidizers in aquarium biofilters

Ammonia oxidizer abundance was explored using qPCR targeting *amoA* genes of AOA, AOB, and comammox *Nitrospira*. Additionally, 16S rRNA genes of bacteria and archaea were quantified. An initial screening with primers sets targeting *amoA* genes from two different clades of *Nitrospira*, clade A and clade B (Pjevac *et al.*, 2017), revealed that there were no detectable clade B comammox *Nitrospira* present in the aquarium biofilter samples (data not shown). Therefore, only primers associated with clade A comammox *Nitrospira* were used for subsequent qPCR assays.

Clade A comammox *Nitrospira amoA* genes were detected for all 38 freshwater aquarium biofilter samples and were dominant among ammonia oxidizers for 30 of the freshwater biofilters, with *amoA* genes of AOA dominant in the other 7 freshwater biofilters (Figure 2.4). Overall, the average number of comammox *Nitrospira amoA* gene copies detected in freshwater biofilters at $2.2 \times 10^3 \pm 1.5 \times 10^3$ copies/ng of DNA was significantly greater than the average concentration of AOA *amoA* genes at $1.1 \times 10^3 \pm 2.7 \times 10^3$ copies/ng ($p < 0.001$). The AOB *amoA* genes had the smallest concentration in the freshwater biofilters, with an average of $3.2 \times 10^1 \pm 1.1 \times 10^2$ copies/ng of DNA, which was significantly lower than *amoA* concentrations of AOA and CMX ($p < 0.001$). The AOB-specific *amoA* genes occurred at >1% relative abundance (compared to CMX and AOA) in only five other freshwater biofilter samples and dominated in one sample (FW-34), albeit at a concentration of only 6.8×10^1 copies/ng that suggests ammonia oxidizers were not well established in this biofilter (Figure 2.4, Table 2.5). For the eight saltwater biofilters, AOA were the dominant ammonia oxidizers in five biofilters, AOB in two, and one

biofilter had similar proportions of AOA and AOB (Figure 2.4). The average concentration of AOA *amoA* genes in saltwater biofilters was $2.5 \times 10^2 \pm 2.5 \times 10^2$ copies/ng of DNA and was not significantly different than the average *amoA* gene concentration for AOB of $2.0 \times 10^2 \pm 1.5 \times 10^2$ copies/ng.

In addition to *amoA* gene copies, 16S rRNA genes associated with bacteria and archaea were quantified. All aquarium biofilter samples were dominated by bacterial 16S rRNA genes (>94%), whereas 16S rRNA genes associated with archaea were present at lower relative abundances across all samples (Table 2.5, Figure 2.4). The average concentration of bacterial 16S rRNA gene copies detected with qPCR was $3.8 \times 10^5 \pm 2.8 \times 10^5$ copies/ng of DNA and $2.4 \times 10^5 \pm 7.9 \times 10^4$ copies/ng of DNA in freshwater and saltwater biofilters, respectively. For the archaeal associated 16S rRNA genes there were $1.5 \times 10^3 \pm 4.0 \times 10^3$ copies/ng of DNA and $3.4 \times 10^3 \pm 2.9 \times 10^3$ copies/ng of DNA in the freshwater and saltwater biofilters, respectively. The large standard deviation for archaeal 16S rRNA gene copies in freshwater samples highlight the broad range of copies detected amongst the freshwater biofilters (Table 2.5). Highlighting consistency in results between qPCR gene targets, most samples that had a higher relative abundance or concentration of archaeal 16S rRNA genes also had high relative abundances of AOA *amoA* genes (Figure 2.4).



Figure 2.3 Relative abundance plot of 16S rRNA gene profiles of aquarium biofilter samples at the family level (>2% RA), grouped and coloured by phylum association. Taxonomy has been assigned using the SILVA database release 138, with taxonomy shown either at the family level or at the lowest assigned taxonomic level above family. Taxa present in at least one sample at greater than 2% relative abundance are displayed on the plot.

Table 2.5 Gene copies for both *amoA* and 16S rRNA gene targeted qPCRs for each sample. Copies are expressed per ng of extracted DNA.

Sample ID	Type	Comammox <i>Nitrospira amoA</i>		AOB <i>amoA</i>		AOA <i>amoA</i>		Bacterial 16S rRNA gene		<i>Thermoproteota</i> (AOA) 16S rRNA gene	
		Gene copies per ng gDNA	SD	Gene copies per ng gDNA	SD	Gene copies per ng gDNA	SD	Gene copies per ng gDNA	SD	Gene copies per ng gDNA	SD
FW-01	Freshwater	2679.1	104.2	2.3	0.2	48.0	3.4	357650.7	48439.3	337.9	35.8
FW-02	Freshwater	1260.1	113.1	23.5	1.2	4.1	0.2	300566.0	46456.8	1.5	0.6
FW-03	Freshwater	3328.1	130.7	3.2	1.2	73.7	1.8	259100.2	24032.9	652.1	25.4
FW-04	Freshwater	3597.9	244.1	18.4	4.4	202.1	2.8	292563.9	41634.0	250.3	16.5
FW-05	Freshwater	1192.6	13.9	0.8	0.1	78.2	1.8	150118.1	5238.3	356.4	16.9
FW-06	Freshwater	6682.5	794.2	5.5	0.6	1062.7	225.2	277426.1	12601.3	830.7	0.1
FW-07	Freshwater	1396.3	84.2	46.3	8.4	2.6	1.0	582230.8	97091.0	26.5	12.8
FW-08	Freshwater	2351.5	4.1	1.7	0.4	1.6	0.7	378127.9	37235.2	10.6	5.3
FW-09	Freshwater	1730.8	223.3	6.1	2.7	65.8	12.2	375128.7	15513.8	816.5	125.5
FW-10	Freshwater	596.2	28.9	0.4	0.4	28.3	9.2	436530.5	69169.3	30.5	12.0
FW-11	Freshwater	1235.2	159.7	0.9	0.4	0.7	0.1	180345.5	2670.1	42.3	5.6
FW-12	Freshwater	2453.8	98.4	1.4	0.1	13453.9	1552.3	381424.0	81179.0	21162.7	4009.6
FW-13	Freshwater	3761.0	2.4	12.3	3.3	17.1	0.8	238815.4	1040.4	99.7	14.1
FW-14	Freshwater	5010.0	1046.4	1.7	0.2	2368.9	210.1	240915.5	44783.9	1792.9	210.7
FW-15	Freshwater	1455.3	131.4	0.5	0.1	1976.5	279.9	334006.2	267705.7	2488.2	370.2
FW-16	Freshwater	1445.2	216.3	1.6	0.1	15.6	1.6	354042.2	8000.5	146.9	9.5
FW-17	Freshwater	2364.1	447.8	2.2	0.2	256.3	33.6	612831.7	136570.8	405.7	102.7
FW-18	Freshwater	842.5	61.6	0.5	0.1	1.8	0.1	106739.5	19987.6	2.0	0.2
FW-19	Freshwater	645.3	83.0	0.6	0.2	70.4	12.9	238073.3	70781.2	356.5	95.3
FW-20	Freshwater	960.3	58.1	109.0	10.4	BDL	-	100728.4	3753.2	0.5	0.0
FW-21	Freshwater	545.3	21.1	1.7	0.1	358.6	11.3	386154.4	109426.1	524.1	16.6
FW-22	Freshwater	1179.2	130.8	138.1	31.9	8.8	2.3	602839.7	28901.7	474.5	31.6
FW-23	Freshwater	4368.9	77.4	689.0	196.6	116.7	6.4	1210216.3	237183.9	202.6	36.6
FW-24	Freshwater	1765.9	227.4	12.1	2.2	2019.4	223.8	1307997.8	1389547.8	2093.8	172.0
FW-25	Freshwater	461.4	36.7	2.1	0.4	646.3	9.3	101620.5	23826.8	1310.2	178.9
FW-26	Freshwater	2238.6	476.5	8.3	0.2	165.9	32.3	566035.2	153888.7	1056.8	32.0
FW-27	Freshwater	2786.2	430.2	3.5	0.0	178.4	46.7	463383.5	30854.2	437.5	88.3
FW-28	Freshwater	884.1	130.8	2.0	0.3	2359.7	357.4	149997.0	7441.8	1968.6	584.8
FW-29	Freshwater	4973.7	957.6	4.9	0.6	1134.3	217.1	545152.5	51591.8	1805.4	263.4
FW-30	Freshwater	2926.2	398.5	1.4	0.2	462.2	4.9	267397.7	35865.0	512.1	55.5
FW-31	Freshwater	1674.7	118.6	2.5	0.6	2575.8	343.2	121920.8	14029.9	743.9	65.6
FW-32	Freshwater	1570.9	0.5	1.4	0.1	138.5	3.6	311943.9	22899.5	264.6	35.4
FW-33	Freshwater	1138.0	112.3	5.9	0.1	BDL	-	387222.8	149070.7	2.5	0.3
FW-34	Freshwater	34.6	0.0	68.3	10.4	BDL	-	176799.5	7174.0	2.2	0.2
FW-35	Freshwater	1467.0	301.3	4.5	0.4	1.2	0.0	957348.9	799163.3	10.3	4.4
FW-36	Freshwater	4729.0	383.1	3.9	0.9	9922.1	542.0	432699.9	68186.8	14471.5	2056.0
FW-37	Freshwater	3233.5	296.8	15.3	1.4	779.2	62.0	119497.4	14233.0	338.1	4.8
FW-38	Freshwater	1594.3	179.4	0.8	0.5	2.1	0.9	136777.2	23155.9	40.0	4.9
SW-01	Saltwater	BDL	-	218.5	8.4	631.1	81.2	204763.7	45638.6	4830.5	364.3
SW-02	Saltwater	BDL	-	348.4	14.2	354.3	42.3	166481.8	36453.6	6319.1	1725.6
SW-03	Saltwater	BDL	-	2.3	0.9	69.0	16.3	137252.7	29006.7	2367.6	57.8
SW-04	Saltwater	BDL	-	107.1	6.4	194.3	15.2	240120.1	14018.5	3476.7	411.5
SW-05	Saltwater	BDL	-	261.3	12.8	29.9	2.2	393031.5	238249.5	896.2	233.9
SW-06	Saltwater	BDL	-	264.3	29.2	59.2	2.4	286943.4	18283.7	165.9	12.5
SW-07	Saltwater	BDL	-	393.7	96.9	596.8	8.5	240783.8	17034.1	8470.4	516.0
SW-08	Saltwater	BDL	-	18.5	3.3	28.4	5.8	263155.1	4273.9	480.5	69.6

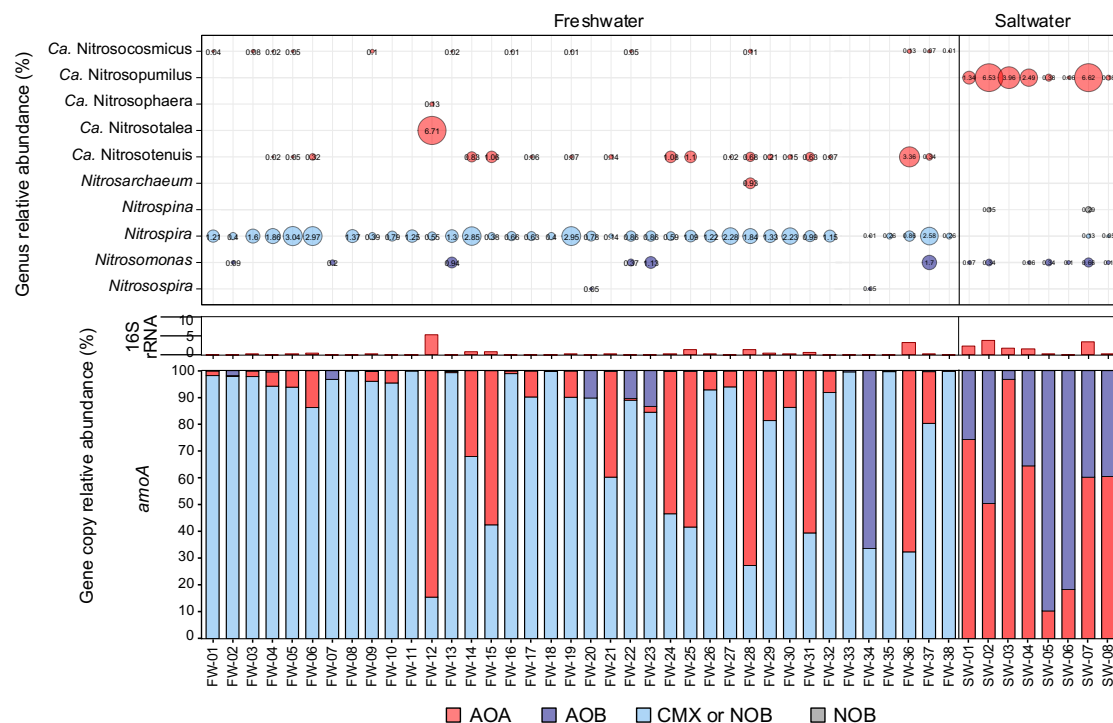


Figure 2.4 Abundance of microorganisms associated with ammonia oxidation were explored using both 16S rRNA gene profiles and gene abundances of the *amoA* gene, a marker for ammonia oxidation. Relative gene abundance of 16S rRNA genes from the archaeal phylum *Thermoproteota*, associated with AOA is shown in the middle. The qPCR results of relative abundance of *amoA* genes from all three groups of ammonia oxidizers (AOA, AOB, and CMX) detected in biofilter samples are shown with the gene relative abundance of each group determined using the gene copy proportion for a single group relative to the total *amoA* gene copy number per ng of DNA from all three groups (bottom). The genera associated with ammonia or nitrite-oxidizing microorganisms present in the aquarium biofilter samples identified from the 16S rRNA gene sequences are illustrated by the bubble plot (top) with the size and number for each bubble representing the relative abundance of each genus in each sample. Colours of bars and bubbles represent the respective groups of ammonia oxidizers, nitrite oxidizers, or comammox *Nitrospira*.

For all saltwater samples, *Nitrosopumilus* was the only AOA genus identified. Detected genera of known AOB included *Nitrosomonas*, which were found in all but one sample. The NOB present in saltwater samples included *Nitrospina* and *Nitrospira* genera, the latter of which were likely strict nitrite oxidizers as no comammox *Nitrospira* were detected in the saltwater samples by qPCR amplification. However, not all saltwater samples contained sequences affiliated with known NOB genera (i.e., SW-01, SW-03, SW-04, and SW-05), which might be explained by the short time that the biofilter material was kept (~ 1 week) for SW-03 – 05. For the others, it remains possible that NOB might be present below the detectable limits of the 16S rRNA gene sequencing or located elsewhere in the aquarium system. In contrast to the saltwater samples, freshwater samples had a higher diversity of AOA, including *Nitrosocosmicus* and *Nitrosotenuis* spp., which were detected in many of the freshwater biofilters. Additionally, AOA genera *Nitrososphaera*, *Nitrosotalea*, and *Nitrosoarchaeum* were detected but each present in only one sample. Two AOB genera, *Nitrosomonas* and *Nitrosospira*, were detected in several freshwater samples. The genus *Nitrospira* was found in all but two freshwater samples and likely represents both comammox *Nitrospira* and canonical NOB *Nitrospira*, which are indistinguishable using 16S rRNA genes. No other NOB-associated genera were detected in the freshwater samples.

Although the overall relative abundances of ammonia oxidizers detected with 16S rRNA gene sequencing matched well to qPCR relative abundances, there were some discrepancies. For example, samples FW-07 and FW-33 did not have any detectable *Nitrospira* via 16S rRNA gene sequencing but did show detectable comammox *Nitrospira* with *amoA* gene qPCR. Additionally, FW-33 did not have any genera associated with known nitrifiers from 16S rRNA gene data. The lack of detected ASVs associated with nitrifiers for some samples might be explained by their low relative abundance within the community, which might have fallen below detection limits during Illumina library preparation, where bias during PCR amplification and sequencing often favors more abundant organisms in the samples. The sequences of 16S rRNA gene primers used for sequencing are meant to target a broad range of prokaryotes within a sample, whereas the qPCR primers for functional gene detection of *amoA* genes

were designed to amplify a more specific sequence, reducing the bias that is seen with the 16S rRNA gene primers.

Because there were several ASVs with taxonomic assignments that could not be resolved below the family level to nitrification-associated taxonomic families, it is possible that these ASVs represent poorly characterized nitrifiers or other related taxa with other encoded functions. For example, the *Nitrosomonadaceae* at the family level comprised a greater relative abundance (Figure 2.3) than what is seen at the genus level for AOB (Figure 2.4). Many of these *Nitrosomonadaceae* ASVs were not assigned to a known AOB genus and it cannot be confirmed based on classifications at the 16S rRNA family level that they are AOB. There was also a discrepancy in the relative abundance of *Nitrosomonadaceae* at the family level and the number of AOB detected via qPCR that may suggest they are not all AOB. Although there were some differences between qPCR and 16S rRNA gene sequencing results for AOA and comammox *Nitrospira* abundances, the discrepancy observed in relative abundance of AOB detected with qPCR and *Nitrosomonadaceae* from the 16S rRNA gene data was consistent for almost all samples.

In addition to identifying dominant ammonia oxidizers present in the biofilters, correlations among ammonia oxidizer abundance and aquarium characteristics were explored. Results indicated that total ammonia concentration was likely linked to ammonia oxidizer relative abundances. For the samples collected, there was only one aquarium that had a relatively high concentration of total ammonia (FW-34; 651 $\mu\text{g/L NH}_3\text{-N}$) and this sample also had the highest relative abundance of AOB based on *amoA* gene quantification (Figure 2.4). Although as previously noted, the concentration of AOB *amoA* genes detected in this sample was quite low (68.0 copies/ng DNA), even though AOB is the dominant ammonia oxidizer in the sample which suggests that this biofilter has an overall low abundance of ammonia oxidizers present. The majority of other freshwater biofilters contained ammonia-oxidizing genes at concentrations 10 to 100 time greater than what is found in FW-34. As for AOB in all other freshwater samples, they fell below 14% relative abundance, with most AOB *amoA* genes making up <1% of the total ammonia oxidizers detected with qPCR. Despite AOB being the dominant ammonia oxidizer in FW-34, this sample

has a low overall abundance of both comammox *Nitrospira* and AOB when examining the overall microbial community based on 16S rRNA gene sequencing, where associated genera of *Nitrospira* and *Nitrosomonas* are both present at a relative abundance of <0.05%, suggesting it has a poorly established nitrifier community.

2.3.4 Clade A comammox *Nitrospira* in freshwater biofilters

To further explore the distribution of comammox *Nitrospira* within freshwater biofilters, amplicon sequencing of *amoA* genes from clade A comammox *Nitrospira* was performed for all freshwater samples. Final processed *amoA* gene sequence data yielded 248 unique ASVs and 76 unique translated AmoA amino acid sequences (Table 2.6). Approximately half (i.e., 43) of these sequences shared 100% identity to the NCBI RefSeq non-redundant protein database, including several previously cultured comammox *Nitrospira* species (Table 2.7). The remaining 33 AmoA sequences were unique to this study. In most freshwater samples, one detected AmoA sequence dominated compared to the other sequences detected (Figure 2.5). More than half of the identified AmoA sequences were grouped into clade A.2, whereas most of the remaining sequences were grouped with sequences previously classified into clade A.1. A single AmoA sequence grouped as a third sub-clade, likely corresponding to clade A.3, and was present only at <1% relative abundance in two of the freshwater biofilters. One of the dominant sequences that grouped within clade A.1, found in most samples, was AmoA-35 (Figure 2.5), whose sequence was previously identified in a wetland environment (Table 2.7). Among sequences from clade A.2, AmoA-49 appeared predominantly in many samples (Figure 2.5). This sequence was previously found in agricultural soil samples (Table 2.7).

Samples containing DNA from aquarium enrichment cultures Aq-C1 and Aq-C2, originally inoculated from the FW-14 aquarium biofilter, were also included for *amoA* sequencing (Figure 2.5). The goal of the enrichment culture was to experimentally demonstrate the nitrification activity of the biofilm material by enriching nitrifiers, including the same comammox *Nitrospira* spp. that were detected in the biofilter of origin. However, the differential contribution of nitrifiers in the aquarium enrichment

(including AOA, NOB, and comammox *Nitrospira*) was not assessed here. Four *AmoA* sequences were detected in both enrichment culture samples, with three belonging to clade A.2 and the other belonging to clade A.1. These *AmoA* sequences were detected within the original inoculum filter sample (FW-14) and in many of the other sampled freshwater biofilters. An additional sample from a Guelph, Ontario wastewater treatment plant rotating biological contactor (RBC) enrichment culture, already known to contain several strains of clade A comammox *Nitrospira*, was also included during sequencing. The comammox *Nitrospira* *AmoA* amino acid sequences identified in the RBC sample matched with previous *amoA* gene sequences from the Guelph RBCs (Spasov *et al.*, 2020) and were detected in several of the freshwater biofilter samples (Figure 2.5).

To explore potential factors that may be correlated with comammox *Nitrospira* spp. distributions across all freshwater biofilter samples, ordination analysis was performed using the comammox *Nitrospira amoA* gene amplicon data (Figure 2.6). Ammonia concentration, carbonate hardness, and alkalinity were all significantly correlated with comammox *Nitrospira* community composition ($R^2 > 0.3$, $p < 0.05$; Figure 2.6), suggesting possible niche preference of certain comammox *Nitrospira* to different water quality parameters, such as ammonia concentration and alkalinity. Several residence-specific patterns were also apparent (e.g., FW-25 to FW-28), but generally samples did not group within ordination space based on location sampled.

Table 2.6 Summary of amplicon read counts before, during, and after final filtration of *amoA* amplicon sequence data. Target sequence for amplification of comammox *Nitrospira amoA* gene is 381 bp in length.

Read counts	Original ASV table	Post R script filtering	ASV table post BLAST filtering (final)	Proportion of original reads (%) post filtering
<i>Average read length (bp)</i>	<i>350.8</i>	<i>349.5</i>	<i>380.5</i>	<i>n/a</i>
Total number of ASVs	1535	976	248	16.2
Total read count	150430	149601	110464	73.4
RBC Positive Control	5813	5813	5813	100.0
PCR Negative Control	8	8	8	100.0
Aq-C1	1367	1362	1360	99.5
Aq-C2	1459	1459	1457	99.9
FW-01	3458	3429	3164	91.5
FW-02	4243	4237	3905	92.0
FW-03	1940	1895	1359	70.1
FW-04	821	808	575	70.0
FW-05	3755	3698	3595	95.7
FW-06	3776	3697	3561	94.3
FW-07	3806	3802	2070	54.4
FW-08	4328	4326	3000	69.3
FW-09	4594	4576	1871	40.7
FW-10	1936	1855	700	36.2
FW-11	2856	2829	2397	83.9
FW-12	5007	5001	4598	91.8
FW-13	4396	4358	3008	68.4
FW-14	4101	4077	3975	96.9
FW-15	3604	3560	3339	92.6
FW-16	5735	5735	2945	51.4
FW-17	3108	3101	2243	72.2
FW-18	6508	6492	5319	81.7
FW-19	3691	3673	2078	56.3
FW-20	5823	5817	5513	94.7
FW-21	4711	4679	3588	76.2
FW-22	5039	5029	3615	71.7
FW-23	3762	3762	1451	38.6
FW-24	3279	3273	1712	52.2
FW-25	968	924	757	78.2
FW-26	3600	3569	2672	74.2
FW-27	6239	6203	4391	70.4
FW-28	3832	3821	2882	75.2
FW-29	4527	4508	3977	87.9
FW-30	2803	2803	2673	95.4
FW-31	3087	3085	2744	88.9
FW-32	2818	2809	2144	76.1
FW-33	4258	4256	2142	50.3
FW-34	1681	1624	54	3.2
FW-35	6694	6674	2470	36.9
FW-36	3260	3247	2512	77.1
FW-37	4292	4280	3421	79.7
FW-38	5268	5268	5227	99.2

Table 2.7 GenBank identifiers and description for AmoA sequences previously identified.

AmoA ID	GenBank accession	Species/metagenome association or description
AmoA-01	QEP54817.1	ammonia monooxygenase subunit A, partial [uncultured bacterium] pasture soil
AmoA-02	VWF18674.1	Guelph RBC Group D <i>Nitrospira</i> sp. MAG
AmoA-03	VWF18671.1	Guelph RBC Group A <i>Nitrospira</i> sp. MAG
AmoA-04	NJI18594.1	Guelph RBC Group C <i>Nitrospira</i> sp. MAG
AmoA-05	QIH54442.1	ammonia monooxygenase, partial [<i>Nitrospira</i> sp. enrichment culture] fast sand filter
AmoA-12	ASO97026.1	ammonia monooxygenase, partial [uncultured bacterium] drinking water treatment plant
AmoA-15	QXU65026.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-17	QXU65031.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-18	QXU65075.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-19	QXU65075.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-20	QXU65075.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-26	QXU65099.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-29	QXU65161.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-30	WP_090742150.1	<i>Ca. Nitrospira nitrosa</i>
AmoA-31	LVWU01000285.1	<i>Nitrospira</i> sp. HN-bin3 MAG
AmoA-32	MBS0156953.1	methane/ammonia monooxygenase subunit A [<i>Nitrospira</i> sp.] activated sludge metagenome
AmoA-33	QXU65083.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-34	QXU65170.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-35	QXU65063.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-36	QXU65144.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-37	QXU65138.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-38	QXE97099.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] agricultural soil
AmoA-41	QXE97092.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] agricultural soil
AmoA-42	QPD98999.1	ammonia monooxygenase subunit A, partial [uncultured bacterium] forest soil
AmoA-43	QXE97180.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] agricultural soil
AmoA-45	ASO97339.1	ammonia monooxygenase, partial [uncultured bacterium] rice paddy soil
AmoA-46	QEP54822.1	ammonia monooxygenase subunit A, partial [uncultured bacterium] vegetable soil
AmoA-49	QXE96988.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] agricultural soil
AmoA-52	OQW57195.1	<i>Nitrospira</i> sp. ST-bin4 MAG
AmoA-54	QEP54815.1	ammonia monooxygenase subunit A, partial [uncultured bacterium] pasture soil
AmoA-55	BBI25072.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] nitrifying granule
AmoA-57	QOD39640.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-59	QXU65066.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-63	QXU65054.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-64	ASO96973.1	ammonia monooxygenase, partial [uncultured bacterium] groundwater well
AmoA-65	THJ16210.1	<i>Nitrospira</i> sp. CG24B MAG
AmoA-67	ASO97348.1	ammonia monooxygenase, partial [uncultured bacterium] recirculating aquarium filter
AmoA-68	CUS38627.1	<i>Ca. Nitrospira nitrificans</i>
AmoA-69	VWF18676.1	Guelph RBC Group G <i>Nitrospira</i> sp. MAG
AmoA-70	QXU65036.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-71	QXU65169.1	ammonia monooxygenase subunit A, partial [uncultured <i>Nitrospira</i> sp.] tidal flat wetland estuary
AmoA-73	UBY00756.1	ammonia monooxygenase, partial [uncultured <i>Nitrospira</i> sp.] tributary sediment

Note: Bolded AmoA sequences indicate amino acid sequences from comammox *Nitrospira* from enriched cultures or representative MAGs.

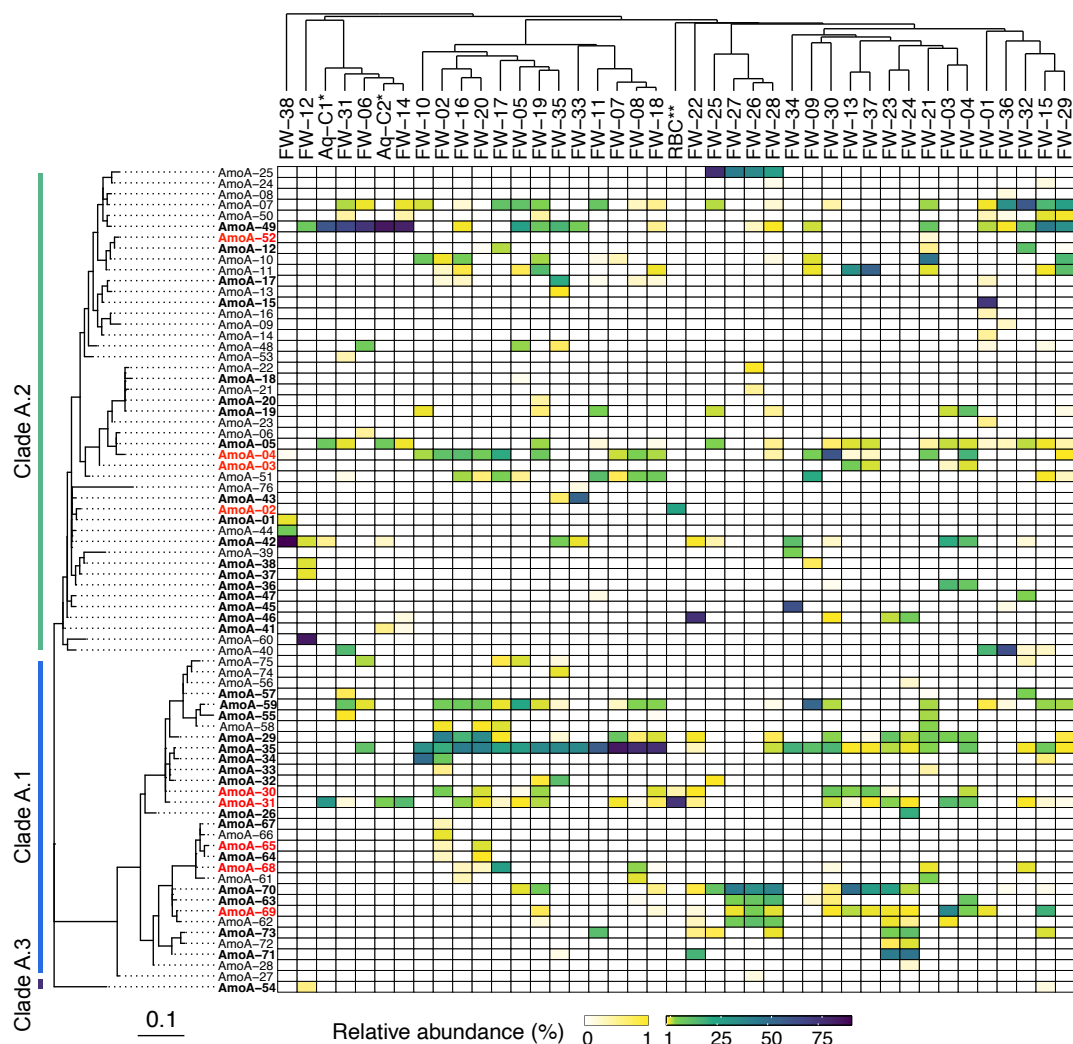


Figure 2.5 Heatmap shows the relative abundance of different clade A comammox *Nitrospira* detected across freshwater aquarium biofilter samples based on AmoA (ammonia monooxygenase A) amino acid sequences (A). Biofilter samples were sorted using hierarchical clustering based on sample profile similarity. A maximum likelihood tree displays the relation of AmoA sequences detected within biofilter samples with the Le Gascuel evolutionary model and discrete Gamma distribution to model evolutionary rate differences among sites (Le and Gascuel, 2008). Additional positive control sample (RBC**) was included and originates from a wastewater treatment plant, and samples from enrichment cultures inoculated from biofilter FW-14 (Aq-C1* and Aq-C2*) are also shown on the heatmap. Bolded AmoA sequences indicate amino acid sequences already present in the NCBI NR database. AmoA sequences highlighted in red are those that are identical to clade A comammox *Nitrospira* spp. either associated with cultured members or those represented by metagenome bins.

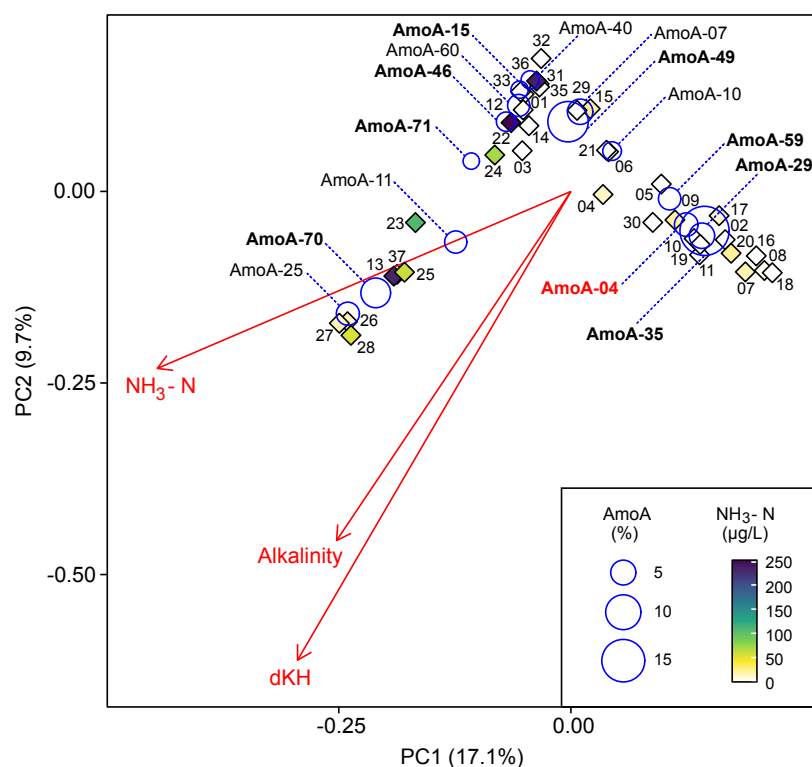


Figure 2.6 A triplot ordination of clade A *amoA* gene sequences detected in each freshwater biofilter (labelled with their associated FW sample number) based on the Bray-Curtis metric shows significant correlations of detected *amoA* gene sequences with biofilter alkalinity, carbonate hardness, and total ammonia concentration ($R^2 > 0.3$, $p < 0.05$). Vector arrow size represents the magnitude of the coefficient of determination. AmoA amino acid sequences detected at above 2% as a weighted average abundance across all samples are displayed on the plot by the labelled blue circles. Circle size represents the weighted average relative abundance of the sequence across all samples, while their placement reflects the correlation specific species have with different samples. Ordination axes display the percent variation within the samples explained by each principal component (PC1 and PC2). Bolded AmoA sequences indicate amino acid sequences already present in the NCBI NR database. AmoA sequences highlighted in red are those that are identical to clade A comammox *Nitrospira* spp. either associated with cultured members or those represented by metagenome bins.

2.3.5 Analysis of microbial community networks involving nitrifying microorganisms

To better understand ammonia oxidizer distributions, correlations among nitrifiers and other taxa within the 16S rRNA gene sequence data were explored using FlashWeave, which infers interactions among microbial populations while incorporating environmental metadata to remove indirect network interactions (Tackmann *et al.*, 2019). The interaction network was filtered to show microbial taxa (ASVs) that had significant correlations with known nitrifiers (e.g., AOB, AOA, CMX, and NOB taxa) in the freshwater biofilters ($p < 0.05$, Figure 2.7, Figure 2.8). The filtered network contained a total of 62 nodes and 59 edges. All edges were positive except for one, which represented mutual exclusions of the connected *Nitrospira* and *Denitratisoma* ASVs. The magnitude of significant edge weights ($p < 0.05$) ranged from 0.33-0.99, with an average of 0.49 ± 0.21 (SD). Of the 15 nitrifier nodes in the network, 3 were associated with AOA genera (e.g., *Nitrosocosmicus*, *Nitrosotenuis* spp.) and 12 nodes were associated with the *Nitrospira* genus (Figure 2.7). There were no ASVs belonging to AOB genera present in the network. As the FlashWeave analysis also considers metadata, three nodes in the network represented the City of Guelph sampling location, water source (Distilled Water), and nitrate (NO_3^- -N). Each of these metadata categories had positive edge connections to distinct *Nitrospira* nodes. The *Nitrospira* and AOA ASVs had comparable levels of connectivity within the network with 4.08 ± 1.93 (SD) edge connections and 3.00 ± 0.00 (SD), respectively. *Nitrospira* ASVs showed higher connectivity to one another, with four edges between neighboring *Nitrospira* nodes. In contrast, only one edge connected an AOA node belonging to a *Nitrosocosmicus* sp. and a *Nitrospira* node. This suggests that coexistence of several *Nitrospira* spp. may be an important characteristic of their ecology in freshwater aquarium biofilters, but not for AOA and AOB, the latter of which were not present at all within the network.

Only six of the total edges within the network connected between heterotroph ASVs. Most non-nitrifier ASVs in the network likely represent aerobic heterotrophic bacteria, with several possibly associated with denitrification (e.g., *Denitratisoma*, *Planctomycetota*) and/or nitrogen fixation (e.g.,

Rhizobiales, *Commamonadaceae*), suggesting further nitrogen cycling beyond nitrification within some of these freshwater biofilters. Despite an inability to distinguish between canonical and comammox *Nitrospira* in the network analysis, the number of positive connecting edges between *Nitrospira* ASV nodes and other putative heterotrophs suggests that their coexistence with *Nitrospira* may be important for nitrifier establishment within biofilter communities (Figure 2.7).

Figure 2.7 Network analysis displays FlashWeave edge weight values between nitrifier ASVs and other ASVs across freshwater biofilter microbial communities. Each node represents an individual ASV based on 16S rRNA gene sequencing and network edges represent the FlashWeave edge weight value. Only significant edges associated with nitrifier genera ($p < 0.05$) with a correlation magnitude illustrated by edge width are shown in the network. Colour and node shape show the different phyla and associated nitrifier group, respectively, and node size displays the average relative abundance of the ASV across all 38 freshwater biofilter samples. The lowest taxonomic assignment of each ASV is shown on the node, followed by the individual ASV number ID.

2.4 Discussion

This study provides a new perspective on aquarium ammonia-oxidizers, revealing that most freshwater home aquarium biofilters are dominated by comammox *Nitrospira*. Although previous work showed that AOA were dominant over AOB, prior to the ability to detect comammox *Nitrospira* counterparts (Sauder *et al.*, 2011), here a more complete picture is provided including all three known ammonia oxidizing groups. The identification of comammox *Nitrospira*, alongside AOA as dominant ammonia oxidizers in freshwater biofilters, further overturns a decades old dogma that AOB are responsible for autotrophic ammonia oxidation and *Nitrospira* responsible for only nitrite oxidation in home aquarium biofilters (Hovanec and DeLong, 1996; Hovanec *et al.*, 1998). This could have important implications for the future development of improved aquarium supplement products that contain active cultures of nitrifying microorganisms and are designed to support the establishment of aquarium nitrification, which until now have focused only on the incorporation of AOB and NOB in their nitrifying consortia (Dhanasiri *et al.*, 2011; Sauder *et al.*, 2011). In addition, these results have provided further insight into the ecology of nitrification in aquarium biofilters, which as their own enclosed ecosystems can make excellent analogs for the study of nitrifying communities in natural environments.

2.4.1 Dominant ammonia oxidizers in aquarium biofilters

Our reassessment of ammonia-oxidizing microorganisms in aquarium biofilters revealed an abundance of comammox *Nitrospira*, revising previous descriptions of known aquarium ammonia oxidizers (Sauder *et al.*, 2011). The ubiquitous presence and observed co-occurrence of multiple comammox *Nitrospira* strains within freshwater aquarium biofilters is consistent with that of similar environments, such as recirculating aquaculture system biofilters, groundwater-fed biofilters, and aquaponics systems, along with WWTP rotating biological contactors (Bartelme *et al.*, 2017; Fowler *et al.*, 2018; Spasov *et al.*, 2020; Heise *et al.*, 2021). Within biofilters, the relatively low ammonia concentrations and high surface area for biofilm growth are ideal predicted conditions for microorganisms with slow growth rates and high yields, such as comammox *Nitrospira*, making their presence in

freshwater biofilters unsurprising (Costa *et al.*, 2006). The detection of solely clade A comammox *Nitrospira* in freshwater biofilters reflects current studies, where most comammox *Nitrospira* associated with wastewater treatment systems (e.g., activated sludge and rotating biological contactors) and freshwater environments also belong to clade A *Nitrospira* (Xia *et al.*, 2018; Beach and Noguera, 2019; Roots *et al.*, 2019; Cotto *et al.*, 2020; Spasov *et al.*, 2020). In contrast, clade B comammox *Nitrospira* are identified frequently as the dominant comammox bacteria in forest soils, paddy soils, plateau soils, and river sediments, with experimental confirmation of their active contributions to nitrification in forest soils (Xia *et al.*, 2018; Wang *et al.*, 2019), suggesting some niche preference for between clades for either aquatic or soil environments. However, co-occurrence of clade A and B comammox *Nitrospira* has been observed in metagenomic surveys of terrestrial subsurface samples (Poghosyan *et al.*, 2019), and clade B were dominant in a groundwater-fed rapid sand filter, existing alongside clade A comammox *Nitrospira* (Fowler *et al.*, 2018). Overall, the underlying factors affecting the distributions of these clades are not entirely understood; however, results show clade A comammox *Nitrospira* dominate in freshwater aquarium biofilters.

The comammox *Nitrospira* clade A.1 and A.2 detected in the freshwater biofilters are similarly found in aquatic environments or wastewater treatment systems, and soil or waste/drinking water treatment systems, respectively (Li *et al.*, 2021). As for clade A.3, these *Nitrospira* are associated more commonly both natural and agricultural soil environments (Li *et al.*, 2021), making their rarity in the freshwater biofilters expected based on previously observed niche preferences. Although about half of the detected AmoA sequences are previously known, including cultivated representatives *Ca. N. nitrosa* (AmoA-30) and *Ca. N. nitrificans* (AmoA-68), the remainder represent unknown strains of comammox *Nitrospira*. This suggests much unexplored diversity in this group, requiring further research to determine if any unique metabolic features exist within these newly detected comammox *Nitrospira* strains.

Despite the ubiquitous and dominant presence of comammox *Nitrospira* within freshwater aquarium biofilters, there were still seven freshwater aquariums whose ammonia oxidizers were

dominated by AOA. There are no clear factors indicating why AOA were more abundant than comammox *Nitrospira* within certain aquariums. It is possible that the differential dominance of these groups in biofilters was the result of stochastic community assembly processes, although there may be other deterministic factors at play, including alternative metabolic substrates, which were not examined here.

In saltwater aquarium biofilters, the absence of comammox *Nitrospira* reflects current research that has not identified them in marine environments, including a recent study that observed comammox *Nitrospira* abundance was negatively correlated with salinity (Liu *et al.*, 2022). Although, comammox *Nitrospira* have been detected in brackish, salt marsh, and estuarine environments (Sun *et al.*, 2020; D. Q. Wang *et al.*, 2021), indicating niche partitioning driven by a salinity tolerance below concentrations of marine environments. Collecting saltwater aquarium biofilters for this study was challenging as fewer saltwater aquarists exist compared to freshwater aquarists who could provide biofilter samples. However, the acquisition of future datasets with more balanced sampling of salt and freshwater biofilters would be ideal to support conclusions that the role of autotrophic ammonia oxidation is occupied by AOA and AOB in fully marine environments.

2.4.2 Niche differentiation of ammonia oxidizers in aquarium biofilters

Previously, pH and ammonia concentration were identified as key factors governing ammonia-oxidizing microorganism abundance in the environment (Lehtovirta-Morley, 2018). Here, results support observations from earlier studies that comammox *Nitrospira* and AOA are dominant in relatively low ammonia conditions, with several cultivated representatives having high affinities for ammonia including *Nitrospira inopinata*, *Ca. N. kreffii*, *Nitrosopumilus maritimus*, and *Ca. Nitrosotenuis aquarius* (Martens-Habbena *et al.*, 2009; Sauder *et al.*, 2017; Sakoula *et al.*, 2021). At this time, there are few cultivated representatives of comammox *Nitrospira* where ammonia affinities have been measured experimentally requiring further research to confirm a generalized high ammonia affinity across all *Nitrospira* spp. In contrast, kinetic studies across many genera of AOA have revealed a wider range of ammonia affinities ($K_{m(app)}$), with lineage III AOA such as *Nitrosocosmicus* having lower ammonia affinities comparable to

those of AOB spp. (Jung *et al.*, 2022). A higher relative abundance of *Nitrosotenuis* spp. was observed compared to *Nitrosocosmicus* spp. in the freshwater samples, which may reflect the lower ammonia niche occupied by *Nitrosotenuis* spp. in the biofilters. Experimental data so far highlights the importance of ammonia concentrations on ammonia oxidizer abundance in biofilters, which should be evaluated under controlled experimental conditions with varied ammonia concentrations, as most aquariums naturally have low levels of ammonia with well-established nitrifying communities in their biofilms.

Differentiation in microbial community composition at a functional level between saltwater and freshwater was evident amongst nitrifiers, not only with the presence and absence of comammox *Nitrospira* in fresh and saltwater aquariums, respectively, but in the AOA, AOB, and NOB species present in the biofilter communities. *Nitrosopumilus* was the only AOA genus detected in saltwater biofilters and was originally discovered in marine aquarium gravel (Könneke *et al.*, 2005). As for freshwater aquariums, AOA from the genera *Nitrosotenuis* and *Nitrosocosmicus* were common, including both *Ca. Nitrosotenuis* aquarius and *Ca. Nitrosocosmicus* hydrocola, which were each discovered in a freshwater aquarium biofilter and a tertiary wastewater treatment system, respectively (Sauder *et al.*, 2017, 2018). This similar niche distinction of nitrifiers in saltwater and freshwater was previously observed in aquarium surveys (Sauder *et al.*, 2011) and noted in the structure of nitrifying communities in moving bed biofilm reactors (Gonzalez-Silva *et al.*, 2016). Regarding nitrite oxidation, only a few saltwater biofilter samples had detectable NOB genera belonging to either *Nitrospira* or *Nitrospina* spp. at a low relative abundance (<0.5%) within the community and detectable nitrite in the water (Table 2.4), suggesting nitrite oxidizers were not well established within the sampled biofilter material.

The observed correlation between ammonia concentration and comammox *Nitrospira amoA* gene distribution suggests ammonia niche preferences among the clade A comammox *Nitrospira* inhabiting the freshwater biofilters. Similarly in a study of groundwater fed rapid sand filters, ammonia concentration explained some of the distribution of comammox *Nitrospira* (based on *amoA* gene sequences) (Fowler *et al.*, 2018). Additionally, the existence of high functional diversity of comammox *Nitrospira* within a

system, which has been observed in rotating biological contactors biofilm of a WWTP (Spasov *et al.*, 2020), might be another factor influencing the distribution and coexistence of comammox *Nitrospira* spp. that could be explored with follow-up metagenomic analyses.

The presence of AOA and comammox *Nitrospira* identified via genes for ammonia oxidation suggests they are performing ammonia oxidation in these systems; however, both have the potential for alternative metabolisms making it important to confirm the ammonia oxidation activity of AOA and comammox *Nitrospira* with activity-based experiments. Some AOA have known potential for mixotrophy, whereas most comammox *Nitrospira* can use different forms of nitrogen such as urea, cyanate, and guanidine (Palatinszky *et al.*, 2015; Sauder *et al.*, 2017; Spasov *et al.*, 2020; Zhao *et al.*, 2021). Additional factors that were not explored in this study that may contribute to niche differentiation and differential abundance between AOA and comammox *Nitrospira* include dissolved oxygen (DO) concentrations, which have been noted in other studies where comammox *Nitrospira* are found at high abundance in environments with low DO concentrations (Beach and Noguera, 2019; Roots *et al.*, 2019), and inhibition of ammonia oxidizers by organic compounds and metals (Cao *et al.*, 2011; Lehtovirta-Morley *et al.*, 2014; Gwak *et al.*, 2020), which should be evaluated in future work.

2.4.3 Factors influencing aquarium biofilter microbial community composition

In this study, the correlations of freshwater biofilter microbial community composition with temperature, aquarium size, and general water hardness is consistent with previous studies that demonstrated the impact of environmental, biological, and physical factors, such as temperature, filter support material, and fish species in the differentiation of biofilter microbial communities of RAS, water treatment, and aquaponic systems (Sugita *et al.*, 2005; Wakelin *et al.*, 2010; Schmutz *et al.*, 2017). The presence of phyla *Proteobacteria*, *Bacteroidota*, and *Planctomycetota* found across all biofilters in this survey in similar proportions was also observed in previous research of freshwater aquaria (Smith *et al.*, 2012), and freshwater and saltwater RAS biofilters (Sugita *et al.*, 2005; Schreier *et al.*, 2010; Hüpeden *et*

al., 2020). This might suggest a common general microbial community composition found within biofilter systems.

Alongside nitrification, other biofilter microbial community members play significant roles in waste removal from the water to maintain a healthy environment for an aquarium's animal residents. These roles can include the degradation of organic wastes (e.g., dissolved organic matter) by heterotrophic bacteria and microbially mediated processes like denitrification, anaerobic ammonium oxidation (anammox), methanogenesis, and dissimilatory nitrate reduction to ammonia (DNRA) (Schreier *et al.*, 2010). Denitrification was not measured in this study, but microorganisms capable of denitrification may have been present in the biofilters. Within three freshwater samples (FW-05, FW-37, FW-47) very low relative abundances (<0.2%) of *Ca. Anammoximicrobium* were detected, suggesting the potential for low levels of anammox activity within the freshwater biofilters (Khramenkov *et al.*, 2013). Although, considering the high level of aeration designed to occur in many aquarium biofilters, it is unlikely that such anaerobic metabolisms are commonplace. Overall, the occurrence of these microbiological processes is dependent on the conditions within the biofilter (e.g., dissolved oxygen, pH) and the microbial community composition, which highlights the importance of an increased understanding of the ecology of aquarium biofilters and factors affecting community assemblage to optimize the functionality of biofilter microbial communities, both in home aquariums and commercial systems.

2.4.4 Interactions between nitrifiers and heterotrophic biofilter community members

Beyond their primary metabolic functions within the biofilters, relationships between microbial community members could be important to establish and support nitrification activity. Network analysis revealed that many positive connections exist between nitrifiers and other putative heterotrophs within the freshwater aquarium biofilters (Figure 2.7), suggesting that the coexistence of these microorganisms may be mutually beneficial. Several *Nitrospira* ASVs have connections with members from the phylum *Myxococcota* (e.g., *Haliangium* spp.), which have been identified as predatory bacteria including in environments like activated sludge systems and freshwater lakes (Osaka *et al.*, 2008; Ezzedine *et al.*,

2020; Kamada *et al.*, 2023). It was hypothesized that micropredators could benefit nitrifiers, as the predation within the biofilter might lead to reduced competitive pressure for resources (Terborgh, 2015). Additionally, many heterotrophic species across freshwater biofilters had strong positive connections with various *Nitrospira* and AOA ASVs, leading to the hypothesis that there may be either commensal or mutualistic relationships between these heterotrophs and nitrifiers. Heterotrophs exhibiting growth promoting effects on AOA and NOB *Nitrospira*, and conversely growth promoting effects of nitrifiers on heterotrophs have been identified in previous studies (Keluskar *et al.*, 2013; Sedlacek *et al.*, 2016; Bayer *et al.*, 2019), supporting the possibility of symbiotic relationships between microbial community members. Mutualistic relationships have also been observed in anammox granules, bacteria associated with the phylum *Chlorobi* appeared to be active protein degraders, while also recycling nitrate to nitrite, which are likely beneficial for the activity of their anammox bacteria neighbours (Lawson *et al.*, 2017).

A major challenge in the study of nitrifiers and especially for comammox *Nitrospira*, is obtaining pure cultures, as nitrifiers frequently grow in co-cultures alongside other heterotrophic microorganisms, which further supports the hypothesis for cooperative community interactions between nitrifiers and heterotrophs (van Kessel *et al.*, 2015; Sakoula *et al.*, 2021). Network analyses suggest the existence of many interactions between biofilter microbial community members; however, these results alone are not sufficient to make strong conclusions regarding microbial interactions, but instead serve as a hypothesis generation tool to identify potential species interactions that can be tested in future experiments. Overall, more research is needed to elucidate nitrifier-heterotroph interactions between community members, and whether their coexistence in biofilms plays a significant role in the abundances or activities of ammonia or nitrite oxidizers within aquatic biofilter environments.

2.4.5 Conclusions and future work

This study has further clarified the presence of ammonia oxidation in home aquarium biofilters, revealing that comammox *Nitrospira* are ubiquitous and with high strain level diversity in freshwater biofilters, where the dominant ammonia oxidizers are either comammox *Nitrospira* or AOA. Future

research should further address factors involved in niche differentiation of nitrifiers, elucidating interactions with other microbial community members and exploring group-specific contributions to ammonia oxidation rates in the biofilters. Additionally, an evaluation of how ammonia oxidizer distribution may vary between different biofilter substrates and surfaces is still needed, as this study focused on either sponge or filter floss material. An improved understanding of this microbially mediated process in aquariums is important to help improve and optimize current water treatment in both the home aquarium and aquaculture industries and could lead to future development of aquarium biofilter supplements containing comammox *Nitrospira*, improving upon those currently available that contain mainly AOB and NOB, and rarely AOA (Sauder *et al.*, 2011; Urakawa and Sipos, 2020).

Chapter 3

Microbial community succession of home aquarium biofilters associated with early establishment of comammox *Nitrospira*

3.1 Introduction

Nitrification is an aerobic respiratory process that converts relatively toxic ammonia ($\text{NH}_3/\text{NH}_4^+$) waste to less toxic nitrate (NO_3^-) via nitrite (NO_2^-) as an intermediate. This process is critical for closed aquatic systems, such as home aquariums and recirculating aquaculture systems (RAS), because ammonia released as nitrogenous waste from fish or through organic matter decomposition can increase to harmful levels (e.g., $> 0.1 \text{ mg/L NH}_3\text{-N}$), negatively impacting resident aquatic life. When establishing a new aquarium, it is essential to establish a microbial community within the associated biofilter to promote nitrification.

Nitrification can occur in two discrete biological steps, with ammonia-oxidizing bacteria (AOB; e.g., *Nitrosomonas* spp.) or archaea (AOA; e.g., *Nitrosotenuis* spp.) performing the initial oxidation of ammonia to nitrite, followed by further oxidization of nitrite to nitrate by nitrite-oxidizing bacteria (NOB; e.g., *Nitrospira* spp.). In contrast, complete ammonia-oxidizing bacteria or “comammox” (CMX) bacteria, are capable of oxidizing ammonia through to nitrate. Complete ammonia oxidation may be favourable in aquarium environments because it circumvents the release of nitrite into the environment, which can also be toxic to aquatic organisms (Eddy and Williams, 1987). Prior to the discovery of AOA and comammox *Nitrospira* in 2005 and 2015 (Könneke *et al.*, 2005; Daims *et al.*, 2015; van Kessel *et al.*, 2015), respectively, AOB were considered by the aquarium industry as dominant ammonia-oxidizers, with AOB supplements sold for use by aquarists to expedite nitrogen cycling in new aquariums (Hovanec and DeLong, 1996; Burrell *et al.*, 2001). However, recent studies reassessing the presence of all three groups of ammonia oxidizers have revealed that comammox *Nitrospira* and AOA are among the dominant ammonia oxidizers in established freshwater aquarium biofilters, with AOB and AOA dominant in

saltwater biofilters in the absence of comammox *Nitrospira* (Sauder *et al.*, 2011; McKnight and Neufeld, 2024). With a revised understanding of the ammonia oxidizers involved in aquarium nitrification, there is still a need to explore the dynamics of microbial community members during biofilter establishment, including all three potential groups of ammonia oxidizers that often coexist in aquarium biofilm communities (Bartelme *et al.*, 2017; Pjevac *et al.*, 2017; Fowler *et al.*, 2018).

Aquarium biofilters are a type of fixed-film bioreactor, often on carrier materials like ceramic beads and polyurethane sponges that support biofilm growth, with flow rates to help control biofilm thickness and ensure sufficient oxygen and nutrient transfer into the biofilm (Malone and Pfeiffer, 2006). In a newly established aquarium, biofilter nitrifier communities accumulate in response to the availability of ammonia and nitrite, with the initial growth of ammonia oxidizers in the biofilm, followed by an increase in NOB in response to the nitrite production of strict ammonia oxidizers. The result of a well-established nitrifying community is to maintain a low-level of total ammonia nitrogen (TAN; $\text{NH}_3/\text{NH}_4^+$), with undetectable levels of nitrite, and accumulating levels of nitrate that can be lowered via water changes or uptake by live plants.

Although the establishment of nitrifiers in aquarium biofilters can be accelerated by seeding with nitrifiers (Carmignani and Bennett, 1977), nitrifying communities also develop in biofilters without supplementation, suggesting that microbial nitrifiers are introduced to the aquarium from external origins, such as drinking water systems, fish, plants, and other environmental sources (Wang *et al.*, 2017; Palomo *et al.*, 2022). However, no research has explored whether it is common to have all three groups of ammonia oxidizers naturally introduced into a home aquarium, and only a few studies have explored temporal dynamics of ammonia oxidizers within these systems (Bagchi *et al.*, 2014).

Dynamics within biofilter microbial communities are complex and interactions occur not only between autotrophic nitrifiers, but also with the many heterotrophic community members who play an important role in the degradation organic wastes that accumulate in aquarium systems (Blancheton *et al.*, 2013). There is competition for both resources (e.g., oxygen, C and N sources) and space within the

biofilm among different autotrophic ammonia oxidizers and heterotrophic bacteria, because the diversity of the microbial community increases during biofilm maturation (Jiang *et al.*, 2019). The groups of ammonia oxidizers have evolved different strategies for niche differentiation depending on the environmental conditions. With higher growth rates, AOB are better able to dominate in an environment early but can be displaced by both AOA and comammox *Nitrospira*, which tend to possess higher ammonia affinities and are thus better able to compete for substrate in oligotrophic environments (Stahl and De La Torre, 2012; Kits *et al.*, 2017). This pattern of ammonia oxidizer succession was observed in an experimental freshwater aquarium biofilter, where the AOB that had initially established were outcompeted by AOA after a 6-month stabilization period (Bagchi *et al.*, 2014). Currently, it is unclear how comammox *Nitrospira* establish with aquarium biofilters alongside AOA and AOB representatives.

This study monitored biofilters of three independently established freshwater aquariums, from initial start-up to maturation, evaluating the succession of the biofilm microbial populations, with a focus on nitrifiers. It was hypothesized that ammonia oxidizers capable of faster growth (e.g., AOB), with a preference for higher ammonia concentrations, would establish early in time series, and nitrifiers better adapted to lower ammonia niches and with slower growth rates (i.e., AOA, CMX) would establish later in the biofilter sampling period.

3.2 Materials and methods

3.2.1 Home aquarium set-up and sample collection

A home aquarium experiment was conducted to assess ammonia oxidizer abundances in biofilters of newly established freshwater aquariums that were subjected to unregulated feeding and fish stocking schedules. Three individuals participated in this experiment by establishing a new aquarium in their homes from which they collected water and biofilter samples over the first three months (12-weeks) of operation. Participants were provided with a research package that included set-up guidelines, sampling

instructions, documentation sheets, and sampling materials. Beyond this, participants were free to establish an aquarium to their liking to represent realistic and unique home aquarium conditions.

Each of the three participants received a 20-gallon aquarium, an LED light lid, an AquaClear 30 aquarium filter, and a plastic thermometer (Hagen Industries). Each aquarium was established with independent selections of fish, fish food, aquarium treatment products, accessories, and operational conditions (e.g., temperature, water source) (Table 3.1). Aquarium 3 was the only aquarium in this experiment to which Cycle (Hagen Industries), reported previously to contain AOB and NOB (Sauder *et al.*, 2011), was added during set-up; the other two aquariums had no supplements added.

Participants regularly sampled ceramic beads, sponge, and water from their tanks as instructed. Samples were first collected 24 hours prior to the addition of fish, 48 hours after adding fish, then weekly thereafter for 12 weeks. During each sampling event, three biofilter beads were collected using flame-sterilized tweezers and sponge samples of approximately $2.5 \times 1 \times 1 \text{ cm}^3$ in size were collected with flame-sterilized scissors. Both bead and sponge samples were collected and stored in sterile 1.5 mL tubes. Water samples were collected in 50 mL tubes. All samples were stored after collection at -20°C until processed.

3.2.2 Water chemistry

Assays were performed to determine the concentrations of total ammonia ($\text{NH}_3/\text{NH}_4^+$), nitrite, and nitrate in collected aquarium water samples. The adapted ortho-phalaldehyde (OPA) assay was used to determine total ammonia concentration (Holmes *et al.*, 1999; Poulin and Pelletier, 2007). The working reagent consisted of OPA and sodium sulfite solutions mixed into an alkaline borate buffer that reacts with ammonia to generate a fluorescent signal (Poulin and Pelletier, 2007). Fluorescence was measured using an excitation wavelength of 360 nm and an emission wavelength of 465 nm. A standard curve from $12.5 \mu\text{g/L-N}$ – $1000 \mu\text{g/L-N}$ was prepared from stock ammonium chloride solution for the standard curve.

To measure nitrite and nitrate concentrations, colorimetric assays using the Griess reagent for nitrite quantification and both Griess reagent and vanadium chloride solution for nitrate quantification (Miranda *et al.*, 2001). Samples with concentrations exceeding the linear range for quantification were diluted with distilled water prior to quantification. As for quantification of total ammonia, standard curves ranging from 12.5 µg/L-N – 1000 µg/L-N of nitrite and nitrate were generated for both assays. Plates from all assays were read with the FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices, LLC) with the SoftMax Pro 6.1 program (Molecular Devices, LLC). Measurements for all assays were done with three technical replicates for each sample.

3.2.3 DNA extraction

Microbial DNA was extracted from biofilter sponge and ceramic beads using the DNeasy PowerSoil Pro Kit (Qiagen). A 1 x 1 x 2 cm³ piece of each sponge sample was cut into smaller pieces using flame sterilized scissors and tweezers and placed into PowerBead Pro Tubes (Qiagen). After adding lysis solution, tubes were incubated at 65°C for 30 min, followed by bead beating at 5.5 m/s for 45 s using a FastPrep-24 bead beater (MP Biomedical, Santa Ana, CA). After sample lysis, the remainder of the DNA extraction procedure was completed as described by the manufacturer's protocol. For DNA extraction from the ceramic beads, beating beads from the PowerBead Pro Tubes were emptied into sterile 5 mL centrifuge tubes (Globe Scientific Inc.). One ceramic bead was placed into the 5 mL tube using flame sterilized tweezers, along with 800 µL lysis solution (Solution C1, Qiagen). The tubes were then incubated at 65°C for 10 min prior to bead beating, which was performed by attaching tubes to an Analog Vortex Mixer (ThermoFisher Scientific) run at maximum speed (3200 rpm) for 10 min. Tubes were centrifuged for 1 min at 7100 RCF before continuing the extraction following the manufacturer's protocol. Extracted DNA was eluted to a final volume of 100 µL using the kit elution buffer (Solution C6, Qiagen). Subsequently, to assess the quality of extracted DNA, samples were run on a 1% agarose gel stained with EtBr. The Qubit dsDNA HS Assay Kit (ThermoFisher Scientific) and the NanoDrop 2000

(ThermoFisher Scientific) were used to determine sample purity and DNA concentration. Extracted DNA samples were stored at -20°C until further use.

3.2.4 Quantitative PCR

To quantify templates from ammonia-oxidizing microorganisms present in biofilter bead and sponge samples, quantitative PCR (qPCR) was performed on all extracted DNA samples. Primers targeting the ammonia monooxygenase subunit A gene (*amoA*) were used to quantify the abundances of AOB, AOA, and comammox *Nitrospira* within the biofilter samples following previously established protocols (McKnight and Neufeld, 2024). Additionally, qPCR was performed with the 515F-Y/806R primer set targeting the V4 region of bacterial and archaeal 16S rRNA genes (Apprill *et al.*, 2015; Parada *et al.*, 2016). Each qPCR contained 5 µL of 2X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 3 pmoles of both forward and reverse primer, 5 µg of bovine serum albumin, and 1-10 ng of template DNA topped up to a final reaction volume of 10-µL with PCR water. All samples were run as technical duplicates, including standards and no template control samples. Amplification conditions for the 515F-Y/806R primer set included an initial denaturation at 98°C for 3 min, followed by 40 cycles of 98°C for 30 s, 50°C for 15 s, and 72°C for 30 s. A melt curve was run immediately following amplification from 65-95°C, increasing at intervals of 0.5°C for 2 s each. Standard curves were generated for each qPCR run using purified template DNA containing the full-length 16S rRNA gene of *Thermus thermophilus*, with copy numbers ranging from 10¹-10⁸ copies/µL. All qPCR amplifications were run using the CFX Opus 96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) and analyzed with Bio-Rad CFX Maestro Software (version 2.3), including quantification and melt curve analysis. All standard curves generated for qPCR had R^2 values >0.99 and amplification efficiencies ranging from 87-97%. Final qPCR products were confirmed on 1% agarose gels.

3.2.5 16S rRNA gene sequencing

Sequencing of the V4-V5 region of the 16S rRNA gene for all bead and sponge samples was carried out using previously established protocols on a MiSeq System (Illumina) (McKnight and Neufeld, 2024). The sequencing run had a cluster density of 755 thousand/mm², with 86.1% of clusters passing the quality filter, and a Q30 (quality score) of 86.3%. Demultiplexing of the paired-end sequence reads was carried out using MiSeq Reporter software version 2.5.0.5 (Illumina). The AXIOME3 (Automation, eXtension, and Integration Of Microbial Ecology) pipeline, implementing QIIME2 version 2020.6 and DADA2, was used for primer sequence removal, quality trimming, denoising, paired-end sequence merging, and subsequent generation of an amplicon sequence variant (ASV) table (Callahan *et al.*, 2016; Min *et al.*, 2021). Taxonomic classification of ASVs was performed during the AXIOME3 pipeline using the SILVA database release 138 (Quast *et al.*, 2013), along with generation of a phylogenetic tree of ASV sequences using FastTree (Price *et al.*, 2010). Beta diversity metrics were calculated using the q2-diversity plugin also via the AXIOME3 pipeline (Min *et al.*, 2021). Additional statistical analysis of beta diversity was performed in R using the *adonis2* function from the *vegan* package (v. 2.6-4) to test for differences in beta diversity between sample groups (i.e., aquarium and filter material type). The *pairwise.adonis* function from the *pairwiseAdonis* R package (v. 0.4.1) was subsequently used to test for specific differences in beta diversity between individual groups, using Bonferroni correction of *p* values to account for multiple comparisons. Calculation of alpha diversity indices, including observed species (richness), Pielou's index (evenness), and Shannon index (richness and evenness), was done using the *vegan* package (v. 2.6-4) implemented in R (v. 4.3.2).

3.2.6 Metagenomic sequencing and analysis

To evaluate the potential metabolic functionality of the microbial communities established in the biofilter bead and sponge material after three months, metagenomic sequencing was performed on the final bead and sponge samples collected during the home aquarium experiment (week 12 samples). Extracted DNA was sent to The Centre for Applied Genomics (TCAG) in Toronto, ON, CA. For each

aquarium sponge sample, 700 ng of DNA was supplied for sequencing, whereas a minimum of 140 ng of DNA was sent for bead samples because they had a lower yield of extracted DNA. Sequencing libraries were prepared using the TruSeq DNA PCR-Free HT Library Prep Kit (Illumina) following the manufacturer's protocol. Prepared libraries were then sequenced on an NovaSeq 6000 System using a single SP flow-cell lane (2×150 cycles) (Illumina). Demultiplexed sequence data received from TCAG were processed using the quality control portion of the Metagenome ALTAS pipeline to remove duplicates, perform quality trimming, and contaminant removal (i.e., adapter sequences) (Kieser *et al.*, 2020). Subsequently, FragGeneScanRs (v 1.1.0) was used to predict open reading frames (ORFs) within the quality controlled, unassembled, and unmerged forward and reverse metagenomic reads (Van der Jeugt *et al.*, 2022). Hidden Markov model (HMM) profiles for associated functional genes of interest involved in nitrification (AOA_amoA, AOB_amoA, comammox_amoA, nxrB, Nitrobacter_nxrB, and Nitrospira_nxrB) were retrieved, along with the rpoB HMM for taxonomic classification from the FunGene database (Fish *et al.*, 2013). These HMM profiles were used with the MetAnnotate (metannotate-wrapper; v. 0.9.2) tool to quantify the relative abundances and taxonomic affiliations of sequence hits to functional marker genes from the unassembled metagenomic reads (Petrenko *et al.*, 2015). The NCBI RefSeq release 220 (October 2023) was used for taxonomic assignment within MetAnnotate using the default E-value (1×10^{-6}) for usearch-based taxonomic classification. MetAnnotate results were further analyzed and visualized using the MetAnnoviz R package (v. 1.1.0), which normalized functional gene hits for each HMM based on HMM length and the number of length-normalized hits to the rpoB HMM. An E-value of 1×10^{-10} was used as the threshold above which hits to HMMs would not be plotted. Forward and reverse reads from each sample were considered replicates and displayed as the average value of the duplicates for each sample. All of the raw sequence reads associated with both the metagenomic and 16S rRNA gene sequencing were deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA1162135.

3.3 Results

3.3.1 Home aquarium sampling

Home aquariums were established using the provided materials and a common municipal tap water for all three residences. Although participants were provided the same aquariums and biofilters, choices regarding fish stock, decoration, feeding, and maintenance were left up to personal preference (Table 3.1). Once stocked, Aquarium 1 contained 7 fish and live plants, kept at a temperature $\sim 27^{\circ}\text{C}$. Aquarium 2 also contained live plants, but was stocked with 16 fish and was maintained at a water temperature of $\sim 23^{\circ}\text{C}$. Aquarium 3 contained artificial plants, along with 7 fish, and a water temperature of $\sim 24^{\circ}\text{C}$. Each participant used different water conditioner products to treat the municipal tap water and different fish food (Table 3.1).

A total of 14 bead and 14 sponge samples were collected from each aquarium (Table 3.2). To assess the impact of fish addition on the biofilter microbial communities, bead and sponge samples were collected 24 hours before adding fish to the aquarium (pre-fish) and 48 hours after adding fish (post-fish). The following week, bead and sponge samples were collected for “week 1” and subsequently for the following 11 weeks after, for a total of 12 weeks of sampling, in addition to the pre- and post-fish sample collections.

Table 3.1 Contents of participating aquariums in home aquarium experiment.

Aquarium	Fish	Food	Water source/ temperature (°C)	Water treatment	Accessories
1	3 Burmese spotted danios; 4 rainbowfish	TetraMin Tropical Flakes (Tetra)	Municipal water/ 27	AquaSafe (Tetra)	Live plants: Java fern, <i>Anubias</i> ; natural gravel
2	1 <i>Betta splendens</i> ; 3 Rasboras (week 2); 6 <i>Corydoras</i> (week 3); 6 fish (other)	Colour Flakes (Cobalt Aquatics)	Municipal water/ 23	Aquarium Conditioner (Marineland)	Gravel; driftwood; rock; live plants
3	3 Rummy-nose tetra; 3 Red-eyed tetra; 1 Chocolate Oranda goldfish	TetraPro Tropical Color Crisps (Tetra); Omega One Freeze Dried Tubifex Worms (Omega One)	Municipal water/ 24	Aqua Plus (Nutrafin); Cycle (Hagen Industries)	Gravel; toy plane; fake pottery; artificial plants

Table 3.2 Quantity of DNA extracted from bead and sponge samples and water chemistry concentrations. Date column indicates the specific day of sampling. Samples noting <5 ng of DNA were below the detection limit for the Qubit instrument used for DNA quantification.

	Sample	Date	Bead DNA (ng)	Sponge DNA (ng)
Aquarium 1	Pre-fish	09/02	255	241
	Post-fish	09/03	28	44
	Week 1	09/12	165	132
	Week 2	09/19	155	161
	Week 3	09/28	242	652
	Week 4	10/03	425	945
	Week 5	10/10	933	5900
	Week 6	10/20	1050	3140
	Week 7	10/25	640	1640
	Week 8	10/31	288	3900
	Week 9	11/07	326	1560
	Week 10	11/14	307	2470
	Week 11	11/21	356	2250
	Week 12	12/01	344	1780
Aquarium 2	Pre-fish	09/03	512	1130
	Post-fish	09/05	179	647
	Week 1	09/12	213	120
	Week 2	09/19	120	144
	Week 3	09/26	241	210
	Week 4	10/03	263	385
	Week 5	10/10	1210	16810
	Week 6	10/17	604	17770
	Week 7	10/24	1460	9780
	Week 8	10/31	1990	15390
	Week 9	11/07	1280	13950
	Week 10	11/14	2240	15730
	Week 11	11/21	2220	13800
	Week 12	12/05	5920	23120
Aquarium 3	Pre-fish	09/07	<5 ng	<5 ng
	Post-fish	09/09	1070	915
	Week 1	09/17	456	228
	Week 2	09/24	578	970
	Week 3	10/01	224	174
	Week 4	10/08	277	395
	Week 5	10/15	303	3850
	Week 6	10/22	681	2480
	Week 7	10/29	1580	4420
	Week 8	11/05	1610	4900
	Week 9	11/12	1550	8920
	Week 10	11/19	414	8100
	Week 11	11/26	687	10140
	Week 12	12/03	480	9920

3.3.2 Aquarium nitrification

Samples of aquarium water collected during weekly biofilter sponge and bead collections were used to measure total ammonia, nitrite, and nitrate. Before the addition of fish to the aquariums, measurements showed relatively low total ammonia concentrations for both Aquarium 1 and 2 samples (Figure 3.1), with a higher concentration for Aquarium 3 ($\sim 250 \mu\text{g/L NH}_3\text{-N}$). All aquariums had no detectable nitrite and concentrations of nitrate around $2 \text{ mg/L NO}_3^-\text{-N}$ (Figure 3.1). The initial elevated concentration of nitrate likely originates from the municipal tap water used to fill all three aquariums, which is known to have concentrations $\sim 500 \mu\text{g/L NO}_3^-\text{-N}$ in the municipality where participants resided (Water and Wastewater Services, 2022).

After adding the fish, aquarium ammonia concentrations increased, associated with food added and fish waste produced. For Aquariums 1 and 2, a relatively small increase in ammonia was observed after fish addition, but ammonia was undetectable in these two aquariums from week 2 onwards (Figure 3.1). The maintenance of ammonia concentrations below detection limits and increasing nitrite concentrations reflects active ammonia oxidation activity established during week 1. The concentration of nitrite in Aquariums 1 and 2 reached a maximum during week 2 at concentrations of 499 and $687 \mu\text{g/L NO}_2^-\text{-N}$, respectively, and then decreased below detection limits the following week (Figure 3.1). In parallel with nitrite oxidation, nitrate concentrations increased for Aquariums 1 and 2 over the experimental time frame.

In contrast to active nitrification observed in both Aquarium 1 and 2, which both maintained undetectable levels of ammonia from week 2 onwards, Aquarium 3 was not associated with nitrification activity until the second month. Although there was an initial decrease in ammonia concentration for Aquarium 3 between pre-fish and week 1 samples, ammonia increased to $>750 \mu\text{g/L NH}_3\text{-N}$ between week 1 and week 2 and stayed at this level until week 7 (Figure 3.1). At week 7, nitrite concentrations rose to $\sim 400 \mu\text{g/L NO}_2^-\text{-N}$ from previously undetectable levels, indicating that ammonia oxidation activity

was occurring in the biofilter (Figure 3.1). The following week, both ammonia and nitrite were no longer detectable, and nitrate concentrations increased, reflecting that nitrification was occurring in Aquarium 3 (Figure 3.1).

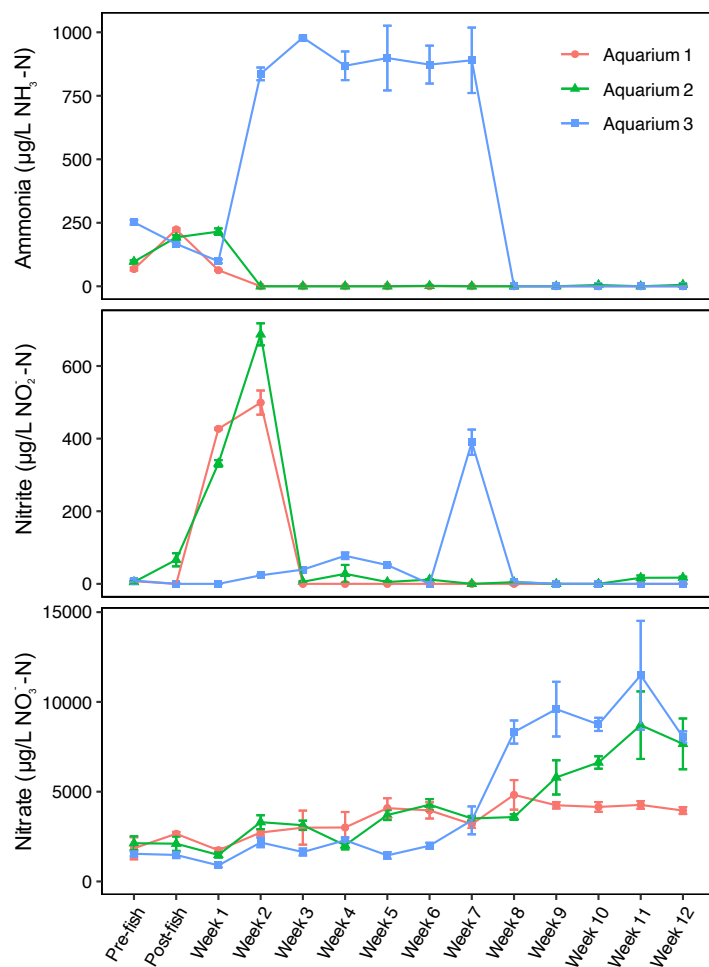


Figure 3.1 Water chemistry measurements quantifying levels of total ammonia, nitrite, and nitrate as micrograms of nitrogen per liter in aquarium water for all three home aquariums during the first 12-weeks following aquarium start-up. Data points represent the average of technical triplicate measurements for each sample, with error bars representing the standard deviation of replicates. The ammonia measurement represents the total ammonia in the sample, including both ionized and un-ionized forms (total ammonia).

3.3.3 Quantification of biofilter ammonia oxidizers with qPCR

To quantify nitrifier community abundances within aquarium biofilters during the first 12 weeks, qPCR was performed to quantify *amoA* genes from AOA, AOB, and clade A comammox *Nitrospira* with primer sets previously used to quantify ammonia oxidizers in freshwater biofilter samples. Additionally, the abundance of prokaryotic templates based on 16S rRNA gene copies was quantified using primers targeting the V4 region of the 16S rRNA gene for bacteria and archaea (Apprill *et al.*, 2015; Parada *et al.*, 2016). During the 12-week sampling period, including pre- and post-fish sample collections, the concentration of 16S rRNA gene copies detected in both the biofilter bead and sponge samples ranged between 10^6 - 10^9 copies (Figure 3.2). The only exception was Aquarium 3 pre-fish samples, where quantities of 16S rRNA genes were only 6,449 and 35,489 copies in the bead and sponge samples, respectively. However, for post-fish samples, these 16S rRNA gene abundances were in the same range as Aquariums 1 and 2 (Figure 3.2). These first samples collected from Aquarium 3 also had <5 ng of total DNA extracted from both bead and sponge samples (Table 3.2).

The succession of ammonia-oxidizing groups (AOA, AOB, and comammox *Nitrospira*) was distinct for each aquarium. Aquarium 1 had detectable clade A comammox *Nitrospira amoA* gene copies as early as week 1 for both bead and sponge sample (Figure 3.2). Comammox *Nitrospira amoA* genes remained elevated at magnitudes of 10^5 - 10^6 copies per bead and at higher levels in sponge samples at 10^6 - 10^7 copies through to week 12. In contrast, AOB *amoA* genes were below the detection limit for both bead and sponge throughout weeks 1-12, including pre- and post- fish sampling (Figure 3.2). Although at a lower abundance than comammox *Nitrospira amoA* genes, AOA *amoA* genes were detectable in both Aquarium 1 bead and sponge from week 3-12, with copy numbers 10-100 times greater per bead than per sponge sample, suggesting a preference for growth in biofilter beads (Figure 3.2). First detectable in week 3, AOA *amoA* genes increased to magnitudes of 10^5 copies per bead from weeks 6 through 12 (Figure 3.2). By the final sampling at week 12, AOA represented more than a quarter of all *amoA* genes detected in biofilter beads.

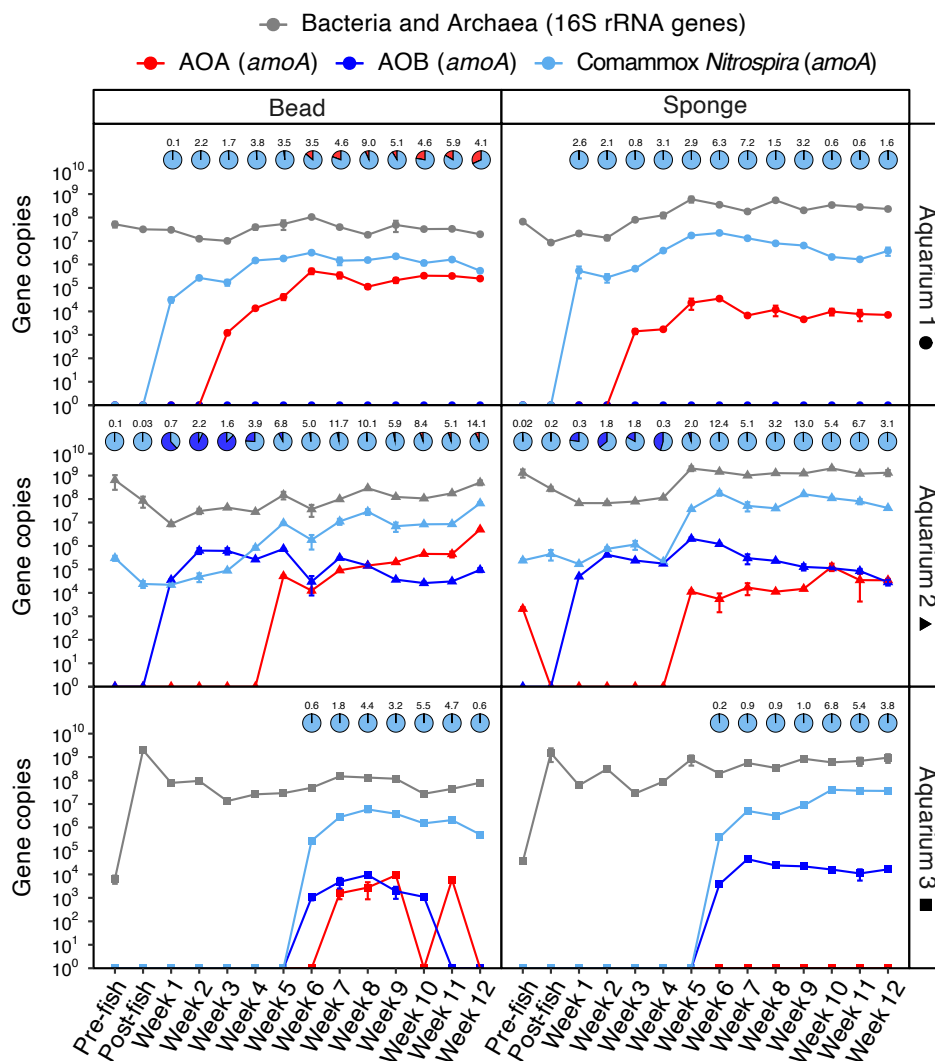


Figure 3.2 Abundances of 16S rRNA and *amoA* gene copies for ammonia oxidizers within bead and sponge samples quantified using qPCR. Data points show the detected copies per nanogram of extracted DNA from the bead and sponge biofilter samples for all four quantified genes: AOA *amoA* (red), AOB *amoA* (blue), comammox *Nitrospira* *amoA* (light blue), and the V4 region of the 16S rRNA gene (grey). The qPCR for each sample was run as technical duplicates with average values shown and error bars representing the standard deviation of duplicates. Data point shapes correspond to each aquarium, with circle, triangle, and square representing Aquarium 1, 2, and 3, respectively. Pie charts represent the relative proportions of ammonia oxidizer groups detected within the sample, and in samples where no *amoA* genes were detected there is no chart displayed. Numbers above pie charts represent proportion (%) relative abundance) of the total community (i.e., 16S rRNA gene copy number) that is represented by ammonia oxidizers (*amoA* gene copy number) for each sample.

For the Aquarium 2 biofilter, comammox *Nitrospira amoA* genes were detected in pre-fish samples at a magnitude of 10^5 copies, suggesting that they were introduced during establishment of the aquarium prior to fish addition (Figure 3.2). By week 5, comammox *Nitrospira amoA* genes increased to concentrations of 10^6 copies and 10^7 copies per biofilter bead and sponge sample, respectively. During weeks 7, 8, and 12, comammox *Nitrospira amoA* genes reached their highest at concentrations above 10^7 copies per biofilter bead, while during weeks 6, 9, and 10, magnitudes of 10^9 copies per sponge samples were detected (Figure 3.2). The AOA *amoA* genes were not present in both bead and sponge until week 5, despite an initial detection of $2.1 \times 10^3 \pm 3.6 \times 10^2$ copies in the sponge sample, suggesting inoculation of AOA into the aquarium during set up (Figure 3.2). Similar to Aquarium 1, AOA *amoA* genes in the Aquarium 2 biofilter beads were detected at magnitudes of 10^5 copies per bead from weeks 7-11, increasing to 10^6 copies per bead in week 12. Although AOA again showed a preference for bead over sponge biofilter material, the discrepancy in magnitude between sample types was not as great as was observed in Aquarium 1 (Figure 3.2). Here, AOA *amoA* genes were detected at magnitudes of 10^4 copies per sponge sample from weeks 7-12, apart from the 10^5 copies detected in week 10. In contrast to Aquarium 1, Aquarium 2 had AOB *amoA* genes detected in both biofilter bead and sponge from week 1 onwards. The AOB *amoA* genes reached maximum copies for bead and sponge samples during week 5, representing >80% of the total ammonia oxidizers in the biofilter beads during weeks 2-3 (Figure 3.2). In the following weeks, AOB *amoA* gene concentrations decreased to 10^3 copies per sponge sample by week 12.

Ammonia oxidizer establishment was delayed for the Aquarium 3 biofilter. Comammox *Nitrospira* were not detected until week 6 sampling, reaching 10^6 copies per bead in the following weeks, and up to 10^7 copies for sponge samples (Figure 3.2). The AOA *amoA* genes were not detectable in Aquarium 3 sponge samples throughout all sample time points (Figure 3.2). In contrast, AOB *amoA* genes were detected in biofilter beads during weeks 6-10 at magnitudes of 10^3 copies per bead, while in

sponge they were detected at 10^3 copies per sponge sample during week 6 and increased to 10^4 copies for the rest of the sampling period (Figure 3.2).

The proportion of the total prokaryotic community represented by ammonia oxidizers changed throughout the 12-weeks of sampling, making up 1.6%, 3.1%, and 3.8% of prokaryotes in biofilter sponge by week 12 in Aquariums 1, 2, and 3, respectively (Figure 3.2). This proportion was even higher in week 12 for the biofilter beads of Aquarium 1 (4.1%) and Aquarium 2 (14.1%), revealing that ammonia oxidizers comprised a higher proportion of the prokaryotic community in biofilter beads than in sponge. However, the relative abundance of ammonia oxidizers was low for Aquarium 3 biofilter beads (0.6%), despite having a similar total DNA yield to that of the Aquarium 1 bead sample (Table 3.2), suggesting that there was not a well-established group of ammonia oxidizers in the beads or that heterotrophic bacteria were more dominant. Ammonia oxidizers were relatively abundant in the sponge of Aquarium 3, making up 3.8% of the total prokaryotic community with a DNA yield from the sponge sample greater than that of Aquarium 1 and less than that of Aquarium 2 (Figure 3.2, Table 3.2).

Despite variations in ammonia oxidizer proportions among aquariums, beads and sponge samples of all three biofilters were dominated by comammox *Nitrospira* from weeks 6-12 (Figure 3.2). Within the biofilter bead communities, both comammox *Nitrospira* and AOA were prevalent ammonia oxidizers, which was not the case for the sponge that was dominated solely by comammox *Nitrospira* at the end of the sampling period. These results suggest that AOA associate primarily with bead surfaces for colonization. The results also indicate that ammonia oxidation activity coincided with the detection of ammonia oxidizers in each aquarium biofilter. Nitrite concentrations of Aquariums 1 and 2 increased between post-fish and week 1 samples, coinciding with the detection of comammox *Nitrospira* in Aquarium 1 and both comammox *Nitrospira* and AOB in Aquarium 2 in sampled biofilter bead and sponge. The delayed start to ammonia oxidation for Aquarium 3, which was evident from weeks 6 and 7, aligned with the first detection of comammox *Nitrospira* in bead and sponge samples at week 6. The

complementarity of nitrite increase, alongside detection of ammonia oxidizers, provides evidence linking biofilter nitrification activity with the nitrifiers detected in biofilter samples.

3.3.4 Microbial community establishment in the aquarium biofilters

To analyze the total microbial community composition of aquarium biofilter beads and sponge over the 12-week sampling period, 16S rRNA gene sequencing was performed for each aquarium. A total of 10,645 ASVs were identified in the dataset, with an average of 24,277 reads per sample (SD = 11,822 reads). Microbial community establishment was assessed using alpha diversity metrics to evaluate how the richness (i.e., number of ASVs) and combined richness and evenness of the microbial community (i.e., Shannon index) changed temporally. During the first few weeks, both the number of detected ASVs (richness), evenness, and Shannon index values for the biofilter bead and sponge samples increased (Figure 3.3). Alpha diversity measures were consistent for week 7-12 samples from each aquarium. By week 12, there were similar numbers of ASVs in Aquarium 1 and Aquarium 2 for the bead and sponge samples, respectively, with higher diversity values for bead samples overall (Figure 3.3). Aquarium 3 had lower diversity values for all time points.

When assessing biofilter community composition at the phylum level, during pre-fish, post-fish, and week 1 sampling both *Proteobacteria* and *Firmicutes* were detected in bead and sponge samples for all aquariums (Figure 3.4). From weeks 2 to 12, genera predominantly from the *Proteobacteria* phylum were detected, along with those associated with *Bacteroidota*, *Planctomycetota*, and *Firmicutes*. At the genus level, many similar genera were detected across all three aquariums in bead and sponge biofilters, including *Bacillus*, *Comamonadaceae* spp., *Hyphomicrobium*, *Caldilineaceae* spp., and *Mycobacterium* (Figure 3.4). *Nitrospira* spp. were also detected for all aquarium bead and sponge samples, corresponding similarly to the time points at which comammox *Nitrospira* were detected with qPCR. There are a few differences between individual aquarium biofilter communities, including a higher relative abundance of *Bacillus* observed in Aquarium 2 when compared with Aquariums 1 and 3, and a higher relative abundance of *Mycobacterium* in Aquarium 3 compared to the other two (Figure 3.4).

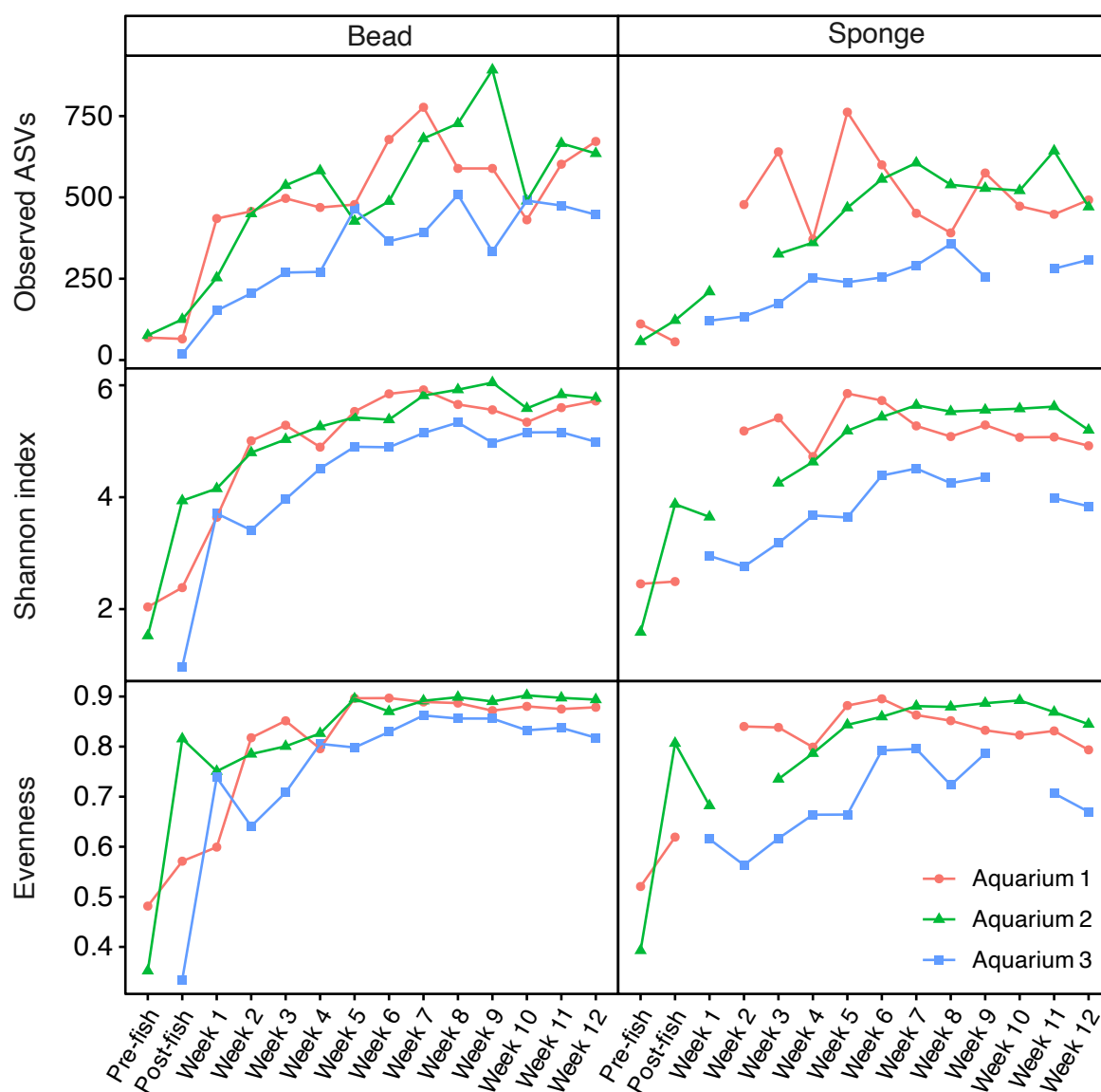


Figure 3.3 Alpha diversity of microbial communities from all aquarium samples across all 14 sampling time points. Alpha diversity values were determined using observed ASVs for richness, the Shannon index for evenness and richness and Pielou's evenness index. Six samples had read counts below the rarefaction depth of 9384 and as a result were removed from the analysis. Omitted samples do not have corresponding evenness, Shannon index, or observed ASV values displayed above.

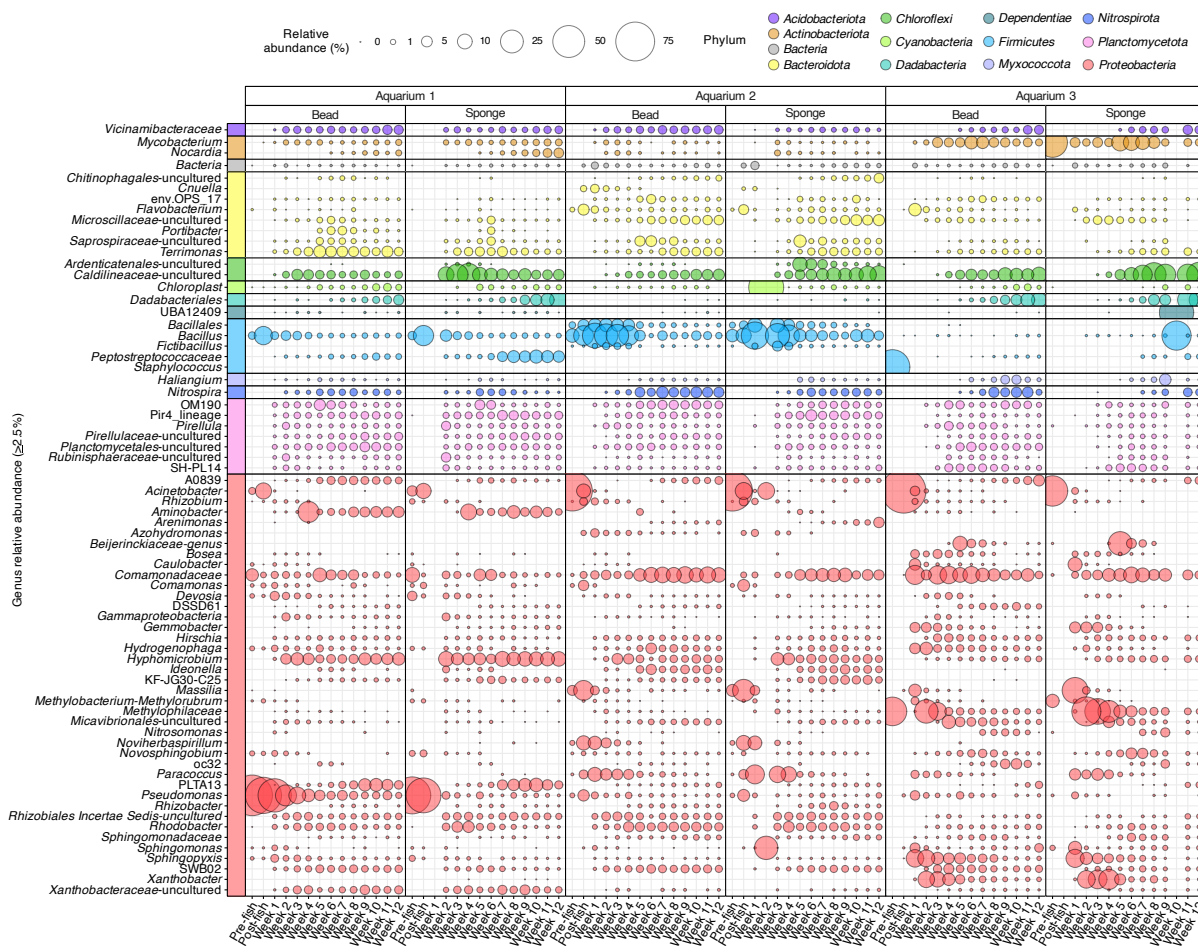


Figure 3.4 Relative abundance of genera across bead and sponge biofilter samples. Bubble size and numbers indicate the relative abundance of each genus within a sample, with colours representing phylum associations. All genera shown on the plot are present in at least one sample at a relative abundance of 2.5% or greater.

The ASV data reveal details of biofilter community establishment occurring from pre-fish sampling to week 12. From week 3 onwards, all biofilter bead and sponge samples were distinct for each aquarium, showing clear differences among the microbial community profiles for each aquarium biofilter (Figure 3.5). Although the unique composition of biofilter communities for Aquariums 1, 2, and 3 were strongest for weeks 9-12 samples, overall beta diversity among aquariums was significantly different across the entire sampling period (PERMANOVA; $R^2 = 0.27$, $p = 0.001$). There were small differences in microbial community composition between bead and sponge samples across the sampling time period for each aquarium (PERMANOVA; $R^2 = 0.03$, $p = 0.002$; Figure 3.5), with no significant interaction between

the biofilter material type (e.g. bead and sponge) and aquarium (two-way PERMANOVA; $R^2 = 0.03$, $p > 0.05$). There was a significant correlation of ammonia concentration to community composition ($R^2 = 0.5$, $p = 0.001$), with the vector pointing in the direction of samples from Aquarium 3 that had the highest ammonia concentrations (Figure 3.5). Beta diversity was also significantly correlated with nitrate concentration, where higher nitrate concentrations were associated with later sampling time points ($R^2 = 0.2$, $p = 0.001$; Figure 3.5). This was expected because nitrate concentrations increased during the 12-weeks in relation to the nitrification activity that occurred for all three aquariums from week 7 onwards. Triplot analysis also allowed us to identify specific taxa that were correlated with the microbial community composition, including *Bacillus* spp. with Aquarium 1 (ASV 10) and Aquarium 2 (ASV 6), and *Caldilineaceae* (ASV 4), *Xanthobacter* (ASV 8), *Methylophilaceae* (ASV 3), and *Sphingopyxis* (ASV 11) with Aquarium 3 (Figure 3.5). Despite the distinct microbial community compositions of all three aquariums, they each showed similar patterns from week 7 onwards, with samples show less dissimilarity to one another for each aquarium, corresponding to the alpha diversity metrics reaching an asymptote in that same time frame (Figure 3.3, Figure 3.5).

Along with a qPCR assessment of ammonia oxidizer template abundances, the relative abundances of nitrifier ASVs was also explored. Overall, 16S rRNA gene profiles were consistent with the detection of ammonia-oxidizing microorganisms quantified by qPCR analysis. In biofilter beads from Aquariums 1 and 2, AOA ASVs of *Ca. Nitrosotenuis* were detected at relative abundances up to ~1% starting from week 5 in Aquarium 1 and week 6 for Aquarium 2 (Figure 3.6). Additionally, two unique *Ca. Nitrosotenuis* ASVs were detected in Aquarium 1, with only one AOA-associated ASV detected in Aquarium 2. There were many different ASVs associated with *Nitrospira* genera detected across all aquarium biofilter bead and sponge samples, which appeared at the same time points that they were detected by qPCR (Figure 3.2). Multiple *Nitrospira* ASVs were present at most sampling time points and several *Nitrospira* were detected for all three aquariums, including ASVs 35, 135, 259, and 580 (Figure 3.6). Although several of these *Nitrospira* ASVs represent comammox *Nitrospira*, it is likely that others

represent canonical NOB *Nitrospira*; however, with these 16S rRNA gene sequences alone it cannot be determined whether ASVs correspond to comammox *Nitrospira* or canonical NOB (Pjevac *et al.*, 2017). Aside from *Nitrospira*, no other NOB-associated genera were detected in any of the biofilter samples. Several AOB ASVs were detected, including five *Nitrosomonas* ASVs and one *Nitrosospira* ASV (Figure 3.6). There were also many ASVs associated at the family level with *Nitrosomonadaceae*. However, without further metagenomic data, their functional role as AOB cannot be confirmed. Additionally, many of these *Nitrosomonadaceae*-associated ASVs appeared when no or very few AOB were detected by qPCR analysis.

Several *Nitrosospira* ASVs identified by 16S rRNA gene sequencing were exclusively associated with Aquarium 2 bead samples from weeks 1-5 (0.1-0.5% RA), likely corresponding to AOB *amoA* genes that were detected with qPCR for the same samples (Figure 3.6). For Aquarium 1, *Nitrosomonas* ASV 1007 was detected for bead and sponge samples for weeks 1, 5, and 9 at less than 0.3% RA, reflecting AOB rarity based on qPCR analysis (Figure 3.2). In contrast, there was some discrepancy in the detection of AOB for Aquarium 3 using 16S rRNA gene sequencing and qPCR. Although AOB *amoA* genes were detected in Aquarium 3 with qPCR from weeks 6-12, the *Nitrosomonas* ASV 128 was detected in Aquarium 3 from weeks 7-9 at relative abundances from 0.8-3.5%. This was higher than the relative abundances of total *Nitrospira* ASVs detected in the same samples at 0.4-1.6%, despite comammox *Nitrospira* dominating over AOB based on qPCR results (Figure 3.2, Figure 3.6). It is possible that the primers used to detect AOB *amoA* genes were not efficiently targeting the *amoA* sequence associated with this *Nitrosomonas* ASV, which was only found in Aquarium 3.

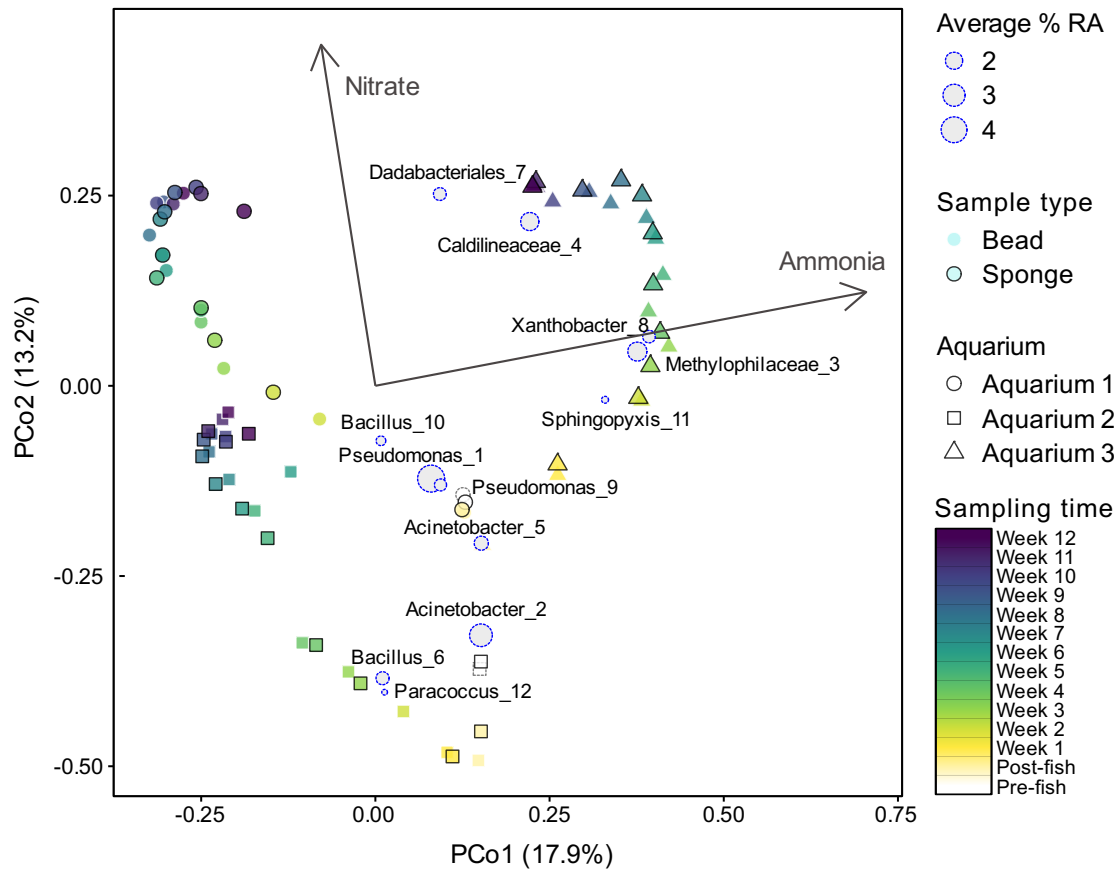


Figure 3.5 Triplot analysis of the 16S rRNA gene sequence data displays the principal coordinate values of each bead and sponge sample, calculated based on the Bray Curtis dissimilarity metric for samples rarefied to 9384 reads (samples below this were removed). Variation explained by principal components 1 and 2 are displayed on the x and y axes, respectively. Blue circles represent average ASV relative abundances (RA) greater than 1% and the location with ordination space reflects relative abundance correlations with microbial community profiles. Grey arrows represent the direction of significant correlations of environmental variables with beta diversity values above a threshold for inclusion ($R^2 > 0.2, p < 0.01$).

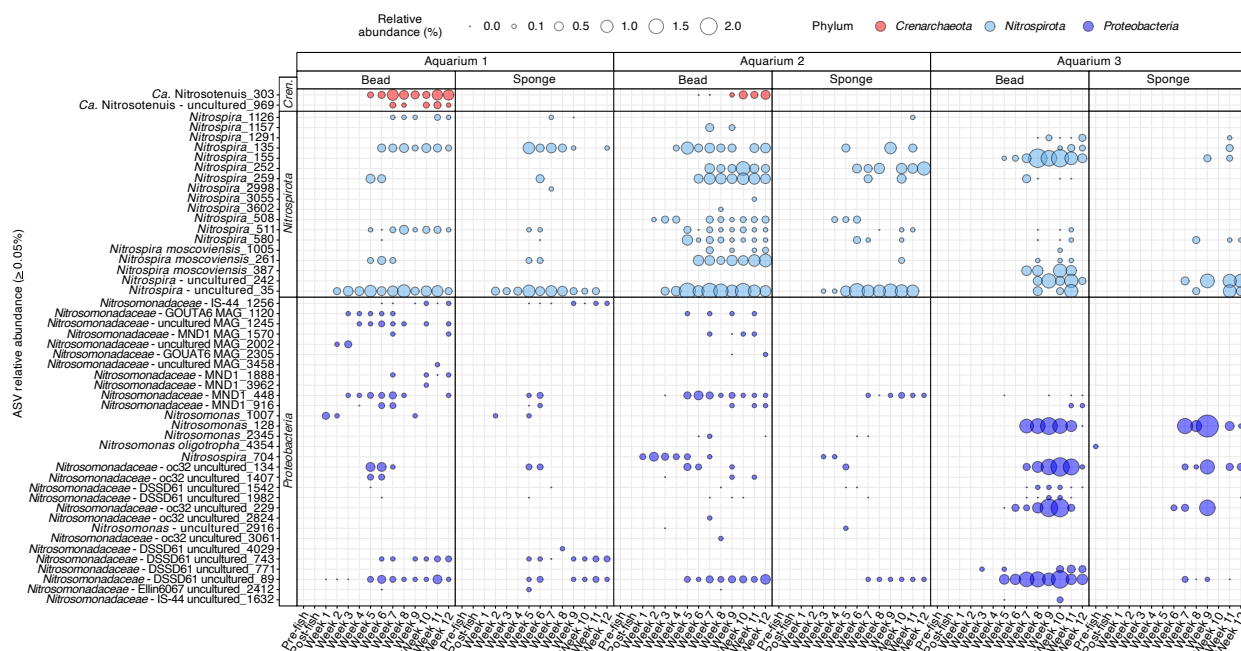


Figure 3.6 Relative abundances of nitrifier-associated ASVs in bead and sponge microbial communities. Bubbles on the plot display the relative abundance (%) of each ASV associated with nitrification in the microbial community of each biofilter bead and sponge sample. The ASVs were filtered based on having the presence of “Nitro” at the beginning of taxonomic rank names. AOA bubbles are red, AOB are dark blue, and comammox *Nitrospira* and NOB *Nitrospira* are light blue. Relative abundances labelled “0” have a percentage value greater than 0 but equal or less than 0.05%. The absence of a bubble indicates 0% relative abundance of the ASV.

3.3.5 Metagenomic analysis of established biofilter microbial communities

In addition to qPCR and 16S rRNA gene sequencing, metagenomic sequencing was performed on week 12 samples from each aquarium to further evaluate the presence of nitrification genes. Specific HMM profiles for different groups of nitrifiers were used to search for corresponding reads, alongside a *rpoB* HMM profile to assign overall taxonomy to the unassembled metagenomic reads. Taxonomic profiling of week 12 samples using *rpoB* hits yielded similar results to the community profiles obtained for the same time point with 16S rRNA gene sequencing, yet with increased taxonomic resolution for several ASVs. Genera that were prevalent for all samples from week 12, including *Nitrospira* and *Hyphomicrobium*, were detected in both 16S rRNA gene sequencing and metagenome-based *rpoB* profiling (Figure 3.4, Figure 3.7). Additionally, *Vicinamibacteraceae* spp. that were unresolved at the

genus level using 16S rRNA sequences were present in samples as the genus *Litorilinea* with *rpoB* profiling (Figure 3.7). Resolved taxonomy at the genus level for *Caldilineaceae* ASVs was also found with *rpoB* taxonomic profiling, revealing the presence of *Litorilinea* spp. in week 12 samples.

The *amoA* HMM profiles reflected those from 16S rRNA gene sequence profiles, detecting AOA in the beads of both Aquarium 1 and 2 (Figure 3.6, Figure 3.8A). Taxonomic assignment for the majority of AOA *amoA* HMM hits were associated with *Ca. N. aquarius*, consistent with 16S rRNA gene sequencing. The other two taxa detected as hits to both the comammox *Nitrospira* and AOB *amoA* HMM corresponded to both comammox *Nitrospira* and AOB, likely due to sequence homology for the AMO proteins of both groups (Figure 3.8). Results revealed that most bacterial ammonia oxidizers in the final bead and sponge samples were associated with comammox *Nitrospira* (~2-14% hits normalized to *rpoB*), with the majority of reads classified specifically to *N. moscoviensis* SBR1015, which corresponds to a comammox *Nitrospira* MAG (Figure 3.8A). For week 12 samples that had detectable AOB using qPCR, HMM hits classified as AOB (including *Nitrosomonas* spp.) were also found for Aquarium 2 bead and sponge samples, and Aquarium 3 sponge at <1% when normalized to *rpoB* genes (Figure 3.8A).

The *nxrB* HMM profiles were dominated by *Nitrospira* spp. (Figure 3.8B). There were also additional hits to the *nxrB* HMM profiles by nitrate reductase genes that can also be detected by these HMM profiles due to homology between these two enzymes (Spasov *et al.*, 2020). Read profiles for the *nxrB* HMM showed that most NOB taxa were representative of the canonical NOB *Nitrospira lenta*, with hits for comammox *Nitrospira* showing similar proportions of the same species across samples for *nxrB* HMM hits as were observed for the *amoA* HMMs (Figure 3.8A). These results suggest that, alongside comammox *Nitrospira*, there was a substantial proportion of nitrifiers in the biofilters by week 12 that are represented by canonical NOB, which likely also contribute to nitrite oxidation within these systems.

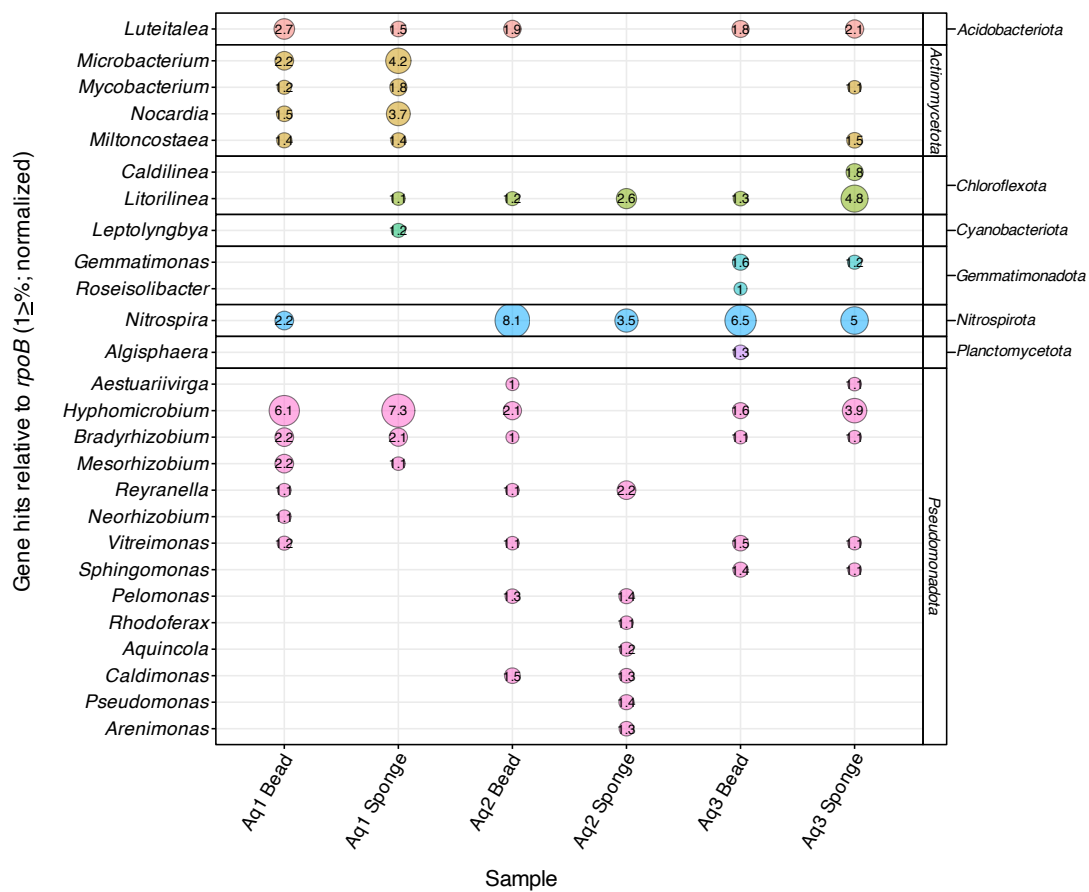


Figure 3.7 Genera of microorganisms detected using the taxonomic marker gene *rpoB* HMM in week 12 bead and sponge samples from metagenomic sequencing. Only genera that were greater than or equal to 1% relative abundance within a sample are shown in the bubble plot, where bubbles and number on plot correspond to the relative abundance of each genera.

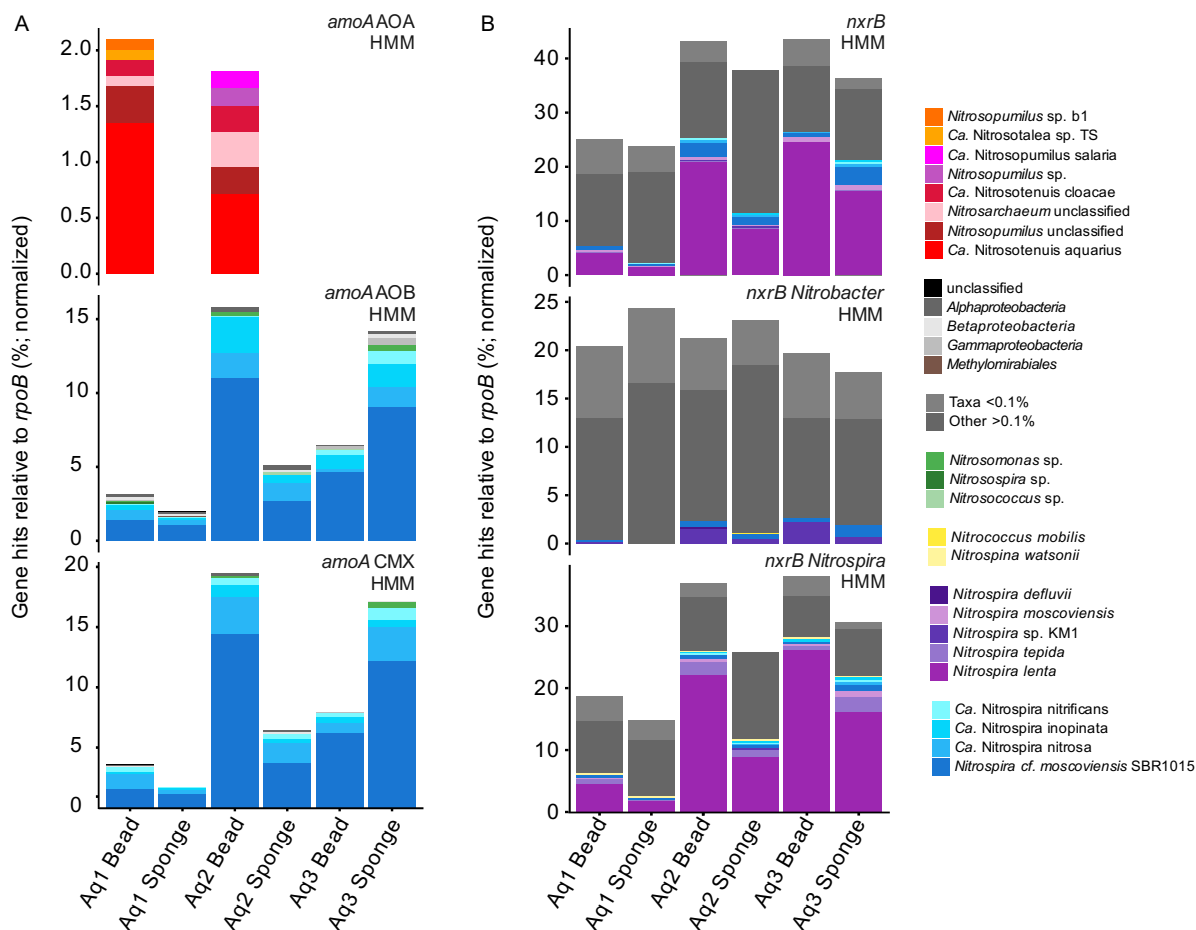


Figure 3.8 Functional gene profiling of nitrifiers for biofilter bead and sponge samples from week 12 using hidden Markov models (HMMs). Stacked bars represent the relative abundance of marker genes for nitrification; (A) *amoA* for ammonia oxidation and (B) *nxrB* for nitrite oxidation, with taxonomy for HMM hits assigned to the closest species. All abundances are length-normalized to *rpoB* hits from unassembled metagenomic reads. The specific HMM model used for reference is noted on the right-hand side of each bar plot. Hits associated with AOA are highlighted in red to orange colours, AOB in green, and comammox *Nitrospira* in blue (A). For nitrite oxidation, comammox *Nitrospira* spp. are highlighted in blue, NOB *Nitrospira* in purple, and other nitrite-oxidizing genera in yellow. All hits for taxa not associated with known nitrifiers are highlighted in grey. The *amoA* gene hits are shown with no percent threshold cut-off (A) and *nxrB* gene hits are shown greater or equal to 0.1% (B), normalized to *rpoB*.

3.4 Discussion

This study shows establishment of all three known groups of autotrophic ammonia oxidizers in freshwater home aquariums and reinforces the dominance of comammox *Nitrospira* in the associated biofilter systems. The results also highlight a need for further research into the factors involved in determining the differences in community composition observed and assessing what methods might improve nitrifier establishment in biofilters to improve fish health (e.g., microbial inoculation from plant sources, bio-supplements), with further research potentially leading to improved aquarium products that will benefit the aquarium industry. Additionally, the use of aquarium systems to test and evaluate the roles of different aquarium parameters will help to improve the understanding of the microbial ecology in these systems.

Prior aquarium microbiome surveys have focused primarily on fully established biofilters, which show a dominance of AOA (Sauder *et al.*, 2011), or comammox *Nitrospira* (McKnight and Neufeld, 2024). The goal of monitoring microbial communities in freshwater home aquariums during their first 12-weeks was to evaluate how nitrifying microbial communities establish initially within aquarium biofilters. After monitoring three independent freshwater home aquariums, despite differences in patterns of community establishment, all biofilters contained similar nitrifiers by the last time point. Reflecting the results of established home aquarium biofilters (McKnight and Neufeld, 2024), comammox *Nitrospira* were found to be dominant in the microbial communities of both the bead and sponge filter components. The dominance of clade A comammox *Nitrospira* after community establishment within these biofilters is expected based on the current understanding of their niche preferences for slower growth, biofilm, and low ammonia concentrations (Costa *et al.*, 2006; Kits *et al.*, 2017).

When sampling biofilm carrier material from aquarium biofilters, differences were observed in microbial community composition between bead and sponge samples, particularly among detected ammonia oxidizers. The results indicate that AOA preferred beads based on qPCR gene abundances (Figure 3.2). This type of carrier preference has been noted in a previous study where AOA showed a

preference for finer sponge material than coarser sponge substrate (Bagchi *et al.*, 2014). Using different biofilter substrates may provide distinct niches for growth within biofilters, which may encourage diverse microbial communities to establish.

When comparing the differences between ammonia oxidizers, faster growth rates of AOB allow them to increase in abundance early on in response to the higher ammonia concentrations present in the aquarium. However, as the ammonia concentrations become stable at concentrations below detectable limits, both comammox *Nitrospira* and AOA can outcompete AOB for acquisition of ammonia due to their often-higher ammonia affinities (Sakoula *et al.*, 2021; Jung *et al.*, 2022). As a result of this competition, AOB will decrease in abundance as the slower-growing comammox *Nitrospira* and AOA succeed them, becoming the dominant ammonia oxidizers in the biofilter. This succession has been previously observed in similar environments (Bagchi *et al.*, 2014), and is reflected in the dominance of both comammox *Nitrospira* and certain AOA species in environments with low ammonia concentrations (Sauder *et al.*, 2012; Fowler *et al.*, 2018; Spasov *et al.*, 2020; McKnight and Neufeld, 2024). With Aquarium 3, there was some succession observed in the biofilter beads, as the AOB *amoA* genes present in the beads decreased below detectable limits in week 11. However, there was less time to further observe these dynamics due to the later establishment of nitrifiers within the biofilters.

Despite a lack of direct NOB quantification using marker genes such as *nxrB* in this study, their diversity was observed in the HMM profiling data from week 12 samples and through the increase in *Nitrospira* ASV diversity in later weeks (Figure 3.8, Figure 3.6). However, future quantification of NOB during biofilter development would be beneficial to further understand the succession of nitrifiers in aquarium biofilters. This diversity of *Nitrospira* spp. within biofilters has been observed in other studies (Hovanec *et al.*, 1998), although there was a lack of knowledge at the time regarding the comammox identity of many of these *Nitrospira*. The NOB detected in these study aquariums were exclusively of the genus *Nitrospira*, which is a highly diverse genus of NOB found ubiquitously in nature, including freshwater and biofilm environments (Daims *et al.*, 2016). Other groups of NOB, including *Nitromarina*,

Nitrospina, and *Nitrococcus* are more frequently found in marine environments (Watson and Waterbury, 1971; Spieck *et al.*, 2014), whereas *Nitrotoga* NOB have been found at higher abundance in colder temperature wastewater systems (Alawi *et al.*, 2009). Overall, the results here are consistent with the niche specialization of different NOB genera.

As observed in similar aquatic biofilm environments, the final communities at 12 weeks contain more than one type of nitrifying species (Bartelme *et al.*, 2017; McKnight and Neufeld, 2024). This functional redundancy within the biofilter is beneficial if one of these groups is lost. Additionally, the nitrifiers have metabolic versatility, adding flexibility to the substrates they can consume in the event where further competition arises between cohabiting groups. In particular, comammox *Nitrospira* exhibit versatility in substrate utilization, as they also possess enzymes to use urea, cyanate, and guanidine (Koch *et al.*, 2019; Palatinszky *et al.*, 2024). The metabolic versatility of comammox *Nitrospira* has been observed in full-scale wastewater treatment systems, where comammox *Nitrospira* actively transcribed urease genes in the system (Yang *et al.*, 2020). Much like comammox *Nitrospira*, AOA are also capable of using organic nitrogen substrates, although these capabilities are not present in all AOA species (Wright and Lehtovirta-Morley, 2023). There is also the potential for mixotrophic activity among AOA, increasing their potential metabolic roles in microbial communities (Mußmann *et al.*, 2011; Qin *et al.*, 2014; Sauder *et al.*, 2017). Despite not directly measuring the activity of individual ammonia oxidizers in this study, the correlation between nitrification activity alongside the presence and/or absence of ammonia and nitrite oxidizers was supportive of their activity within these biofilters. Further experiments would be valuable to elucidate the specific roles of each nitrifier group in the aquarium biofilters and determine the direct contributions of each group to ammonia and nitrite oxidation.

In the context of the microbial community within the aquarium biofilters, nitrifying microbial communities did not develop independently of the other microorganisms that also inhabit the biofilters. Microbial community composition in the biofilters increased in diversity over the 12 weeks and likely played a role in competition for resources including ammonia. As noted in previous studies, heterotrophic

bacteria can play an important role in nitrifier establishment, assisting with initial biofilm formation in the biofilter (Tsuneda *et al.*, 2001; Bassin *et al.*, 2012), which is important in particular for the growth of comammox *Nitrospira* (Zhao *et al.*, 2022). The phyla observed in the three aquarium biofilters during establishment are similar to those of other studies, including the dominance of *Proteobacteria*, along with the presence of microorganisms from phyla including *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*, which have been observed in other freshwater and also marine recirculating aquaculture systems (Moschos *et al.*, 2022). Additionally, a study that observed community succession in a cold freshwater recirculating aquaculture system identified several biomarker taxa in the biofilter microbial community, including those affiliated with the *Nitrospirales*, *Rhizobiales*, and *Sphingomonadales* (Jiang *et al.*, 2019). The presence of genera from these orders was also observed in the freshwater aquarium biofilters, and many of these were present at the end of the 12-week sampling period, as detected with taxonomic profiling from metagenomic sequencing data (Figure 3.7), suggesting commonality in the taxa observed during biofilter community establishment.

In this study, all three independently run aquariums had varied physicochemical parameters. Due to the small sample size and likely spurious correlations that would result from connecting water chemistry parameters to biofilter microbial community differences between aquariums, it is difficult to draw conclusions on the factors that might have direct influence on the microbial community composition. Ideally, conducting further controlled experiments with a larger sample size could help to distinguish among these effects. Previous literature demonstrates that nitrifiers vary in their environmental preferences (Gonzalez-Silva *et al.*, 2016; Al-Ajeel *et al.*, 2022). As solely freshwater aquarium biofilters were examined in this study, the detected nitrifiers had a niche preference for freshwater environments. Although some comammox *Nitrospira* spp. can adapt to higher salinity concentrations (Liu *et al.*, 2020), they have yet to be detected in marine environments, including saltwater aquarium biofilters (McKnight and Neufeld, 2024). This made the inclusion of saltwater aquarium samples less relevant when assessing the presence of AOA, AOB, and comammox *Nitrospira* during

microbial community establishment in aquarium biofilters, as the current understanding is that comammox *Nitrospira* are absent from marine aquariums.

As mentioned, with only three aquariums it was challenging to elucidate factors responsible for the different microbial community compositions observed in the biofilters. However, the differences in nitrifiers amongst the three aquariums raise several questions: Why did AOB establish in Aquarium 2 but not in Aquarium 1? Why was nitrification activity delayed in Aquarium 3? Based on small, measured increases in detectable nitrite and nitrate in Aquarium 3 from week 1 to week 6, there was likely low levels of nitrification occurring; however, consistent nitrification activity was not observed until week 8. Despite some aquarium-specific variation, it is clear from the water chemistry data that nitrification kept ammonia levels below detectable limits by the end of the 12-week sampling period in all three aquariums. There was one notable difference between the aquariums with and without early nitrification, which was the inclusion of live plants in both Aquariums 1 and 2, but not in Aquarium 3. It was hypothesized that the live plants served as a source of microorganisms, introducing nitrifiers that may have decreased the time required to establish an effective nitrifying community. Many rhizosphere and phyllosphere microorganisms would be transferred into the new aquarium upon addition of the plants. In aquaponics systems, nitrifying bacteria are often present in the system and sometimes associated with plant roots, including comammox *Nitrospira* and AOA (Heise *et al.*, 2021; Schmutz *et al.*, 2022). Elevated 16S rRNA gene copies detected in the pre-fish sampling for Aquariums 1 and 2 support the hypothesis that they had a higher initial inoculation of microorganisms prior to the addition of fish, which might have aided in microbial community establishment. In addition to a potential microbial inoculation source, the live plants in Aquariums 1 and 2 provided some nitrate removal from the water via assimilatory processes, which might explain lower nitrate concentrations for these aquariums compared to Aquarium 3 during weeks 9-12.

Results from this study have quantified the abundances of different ammonia oxidizers within the biofilters, coinciding with observed nitrification activity through ammonia, nitrite, and nitrate

concentration measurements. Although these concentrations were measured during the 12 weeks of aquarium sampling, the amount of nitrogen added to the aquarium system (e.g., via fish food) was not quantified, nor is it known the specific amount of nitrogen assimilated by the plants in Aquariums 1 and 2. As a result, it was not possible to determine a specific rate of nitrification for these aquariums. However, by comparing changes in ammonia, nitrite, and nitrate concentrations over the 12 weeks, it could be determined when nitrification activity began. With a better sense of ammonia oxidizer presence in the biofilters during the first 12 weeks of microbial community establishment, future studies quantifying nitrification rates and nitrification activity of specific ammonia and nitrite oxidizers within the biofilters would further clarify their contributions to nitrification in these aquariums.

Aquarium hobbyists seek to establish nitrifying communities within new home aquarium biofilters, to maintain healthy water conditions for their aquatic residents (i.e., fish). Biological supplements commercially available in pet supply stores typically contain AOB and NOB (Dhanasiri *et al.*, 2011; Sauder *et al.*, 2011), which are meant to accelerate the establishment of nitrifiers in the biofilter and help keep ammonia levels low. In this study the sole aquarium that added supplements during set up (i.e., Aquarium 3) was not associated with nitrite or nitrate accumulation until week 6-8 and known nitrifiers were also not detected in biofilter DNA until that time. As discussed, other factors may have delayed nitrification activity, which are not apparent given the present data, however with a sample size of one aquarium, it is not possible to conclude that these supplements would be ineffective when used as instructed in another aquarium. It is known that the use of supplementation in the form of either adding in filter material from a previously established biofilter or other forms of inoculation have been effective ways to promote nitrifier growth in biofilters (Carmignani and Bennett, 1977; Grommen *et al.*, 2002). However, a recent study revealed that only one of five tested quick-start nitrification products sold with the purpose of promoting rapid aquarium nitrification significantly reduced aquarium ammonia concentrations (Scagnelli *et al.*, 2023). The quick start nitrification product that was effective claims to contain *Nitrosomonas*, along with *Nitrospira* – although it is assumed this refers to canonical NOB. The

results from this home aquarium establishment study draw further attention to the high abundance of comammox *Nitrospira* in freshwater aquarium biofilters, which occurs in tandem with detectable nitrification activity in the aquariums. These results suggest that aquarium bio-supplements would benefit from including comammox *Nitrospira* to promote nitrification activity in home aquarium biofilters and a sustainable nitrifying community.

Establishment of nitrifiers in Aquariums 1 and 2 without supplementation demonstrates that nitrifiers can be seeded in aquarium biofilters without the use of bio-supplements. Shared *Nitrospira* and AOA ASVs in these biofilters suggests that these nitrifiers could originate from the municipal drinking water system. Comammox *Nitrospira* have been detected within drinking water systems previously (Pinto *et al.*, 2016; Wang *et al.*, 2017), in addition to both AOA and AOB also residing in drinking water distribution systems (Van Der Wielen *et al.*, 2009; Cruz *et al.*, 2020; Roy *et al.*, 2020). Because participants were provided with gift cards to obtain fish, live plants, and supplies from the same local pet store, the nitrifiers may have originated from this common source and transferred by the fish, plants, and water associated with their purchase.

In the future, long-term monitoring of aquarium biofilter systems past twelve weeks would confirm comammox *Nitrospira* stability in the biofilter community, which has been reported before for AOA in aquarium biofilters (Bagchi *et al.*, 2014). Overall, results from this study have highlighted the previously overlooked presence of comammox *Nitrospira* during the establishment of nitrifying microbial communities in freshwater aquarium biofilters. This suggests a need for future studies to investigate the individual contributions of ammonia and nitrite oxidizers to nitrification in these biofilters during their development.

Chapter 4

Microbiology of a membrane aerated bioreactor upgrade for a municipal wastewater treatment facility

4.1 Introduction

Treatment of wastewater is crucial to prevent the release of excess nutrients and pollutants into receiving waters, which can lead to eutrophication in natural aquatic systems. During wastewater treatment, various physical, chemical, and biological processes are implemented to remove suspended solids, pollutants, along with dissolved organic matter (DOM) and nutrients, reducing the biological oxygen demand (BOD) and toxicity of treated water. The use of conventional activated sludge (CAS) processes is widely implemented in municipal wastewater treatment facilities for the removal of organic carbon, phosphorus, and nitrogen (Jenkins and Wanner, 2014). Nutrients are consumed within a CAS system by a consortium of biologically active microorganisms suspended in flocs within aeration tanks, known as activated sludge, which together with the wastewater is termed mixed liquor (Metcalf and Eddy Inc. *et al.*, 2014).

The CAS systems play an important role in reducing concentrations of ammonia or total ammonia nitrogen (TAN) from wastewater, which can be toxic to both humans and aquatic life (Randall and Tsui, 2002; Dasarathy *et al.*, 2016). Removal of TAN occurs via conventional biological nitrogen removal (BNR) as nitrification, whereby chemolithotrophic microorganisms oxidize ammonia to nitrite for energy (ammonia-oxidizing bacteria, AOB; ammonia-oxidizing archaea, AOA), and nitrite-oxidizing bacteria (NOB) then use the nitrite for energy, further oxidizing it to nitrate (Al-Ajeel *et al.*, 2022). These nitrifiers are commonly found in CAS activated sludge, including species affiliated with the *Nitrosomonas*, *Nitrobacter*, and *Nitrospira* genera (Kim *et al.*, 2011). Additionally, complete ammonia oxidizers (comammox) associated with the *Nitrospira* genus are capable of oxidizing ammonia to nitrate, via nitrite, acquiring energy from both oxidation steps (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Comammox

Nitrospira are dominant ammonia oxidizers within activated sludge of numerous full-scale WWTPs, including municipal systems (He *et al.*, 2022; Zheng *et al.*, 2023). In contrast to many AOB species, comammox nitrifiers favour longer SRTs, attached growth phases, and lower ammonia and DO concentrations, as a result of their slower specific growth rates, ammonia affinities, and increased sensitivity to oxygen (Koch *et al.*, 2019; Roots *et al.*, 2019; Cotto *et al.*, 2020; Zheng *et al.*, 2022).

Dependent on the system configuration, BNR can also include removal of nitrate from the system, often facilitated by anoxic compartments where the microorganisms within the activated sludge will perform denitrification in the absence of oxygen, using nitrate as their terminal electron acceptor (Lu *et al.*, 2014). Total removal of nitrogen from the wastewater via dinitrogen gas is ideal, because although nitrate is less toxic than ammonia or nitrite, high nitrate concentrations can also contribute to eutrophication and have negative impacts on aquatic animal and human health (Camargo *et al.*, 2005; Ansari *et al.*, 2010; Bryan and van Grinsven, 2013).

Although CAS systems have been successfully implemented for over a century in municipal wastewater treatment facilities, several problems are associated with their use. To facilitate nitrification and organic matter degradation via microbial activity, oxygen is delivered to the CAS aeration tanks by continuous aeration. However, the aeration tanks have inefficient oxygen transfer efficiency (OTE) of only 25-35%, and high amounts of energy are consumed running blowers to aerate the system (Metcalf and Eddy Inc. *et al.*, 2014; IESO, 2018). Additionally, nitrous oxide (N₂O), which is 300× more potent than carbon dioxide as a greenhouse gas, is produced as a side-product by AOB during nitrification, which is released from the mixed liquor due to the intense aeration (Law *et al.*, 2012). Nitrous oxide released from wastewater treatment plants accounts for 3% of anthropogenic N₂O emissions globally (IPCC, 2013), with studies indicating that emissions are mainly from aerated sections (Ahn *et al.*, 2010). As for CAS systems in areas with cold winters, they experience decreased nitrification efficiencies during colder seasons that negatively impact nitrogen removal rates (Painter and Loveless, 1983). To address the

inefficiencies of conventional wastewater treatment methods, novel technologies have emerged as alternatives.

The membrane aerated biofilm reactor (MABR) technology was designed to address many of the issues currently encountered with CAS processes (Lu *et al.*, 2021). An MABR cassette, which is placed inside an anoxic tank, contains thousands of vertically attached hollow cords composed of gas-permeable membranes that provide an attached surface for biofilm growth (Figure 4.1). A counter gradient of oxygen and nutrients is established across the biofilm as air is passed through the center of the cords, allowing oxygen to diffuse out into the biofilm, while nutrients diffuse in the opposite direction from the liquid phase into the biofilm surface (Nerenberg, 2016). This redox gradient established across the biofilm is designed to promote simultaneous nitrification and denitrification (SND), and organic carbon removal through the stratification of microorganisms that occurs across the biofilm (Martin and Nerenberg, 2012). Ammonia-oxidizing bacteria establish at the membrane's surface, where they have access to higher oxygen concentrations to oxidize ammonia from the mixed liquor to nitrite, which can be used either by NOB for energy or as electron acceptors by heterotrophic denitrifiers that reside in the anoxic outer biofilm layers (LaPara *et al.*, 2006; Matsumoto *et al.*, 2007; Downing and Nerenberg, 2008).

Previous research has used bench scale units and laboratory conditions to demonstrate feasibility of treating municipal wastewater with MABR systems (Terada *et al.*, 2006; Bunse *et al.*, 2020; Ukaigwe *et al.*, 2021), and pilot studies treating municipal wastewater have demonstrated simultaneous carbon and nitrogen removal (Peeters *et al.*, 2017; Long *et al.*, 2020; Uri-Carreño *et al.*, 2021). Additionally, studies on larger scale pilot systems containing 1-2 MABR cassettes have explored how factors such as ammonia loading (Houweling *et al.*, 2017), temperature (Long *et al.*, 2020), C/N ratios (Ravishankar *et al.*, 2022), and oxidation-reduction potential in the bulk phase (Uri-Carreño *et al.*, 2023) impact MABR performance. Nitrification rates in MABR systems are less affected by colder temperatures (Uri-Carreño *et al.*, 2021), and current implementations have shown increased OTE and reduced energy consumption for aeration (Côté *et al.*, 2015; Peeters *et al.*, 2017). To date, large scale implementations and the long-

term performance of a full-scale MABR system in a municipal WWTP under variable operational conditions have only been recently assessed.

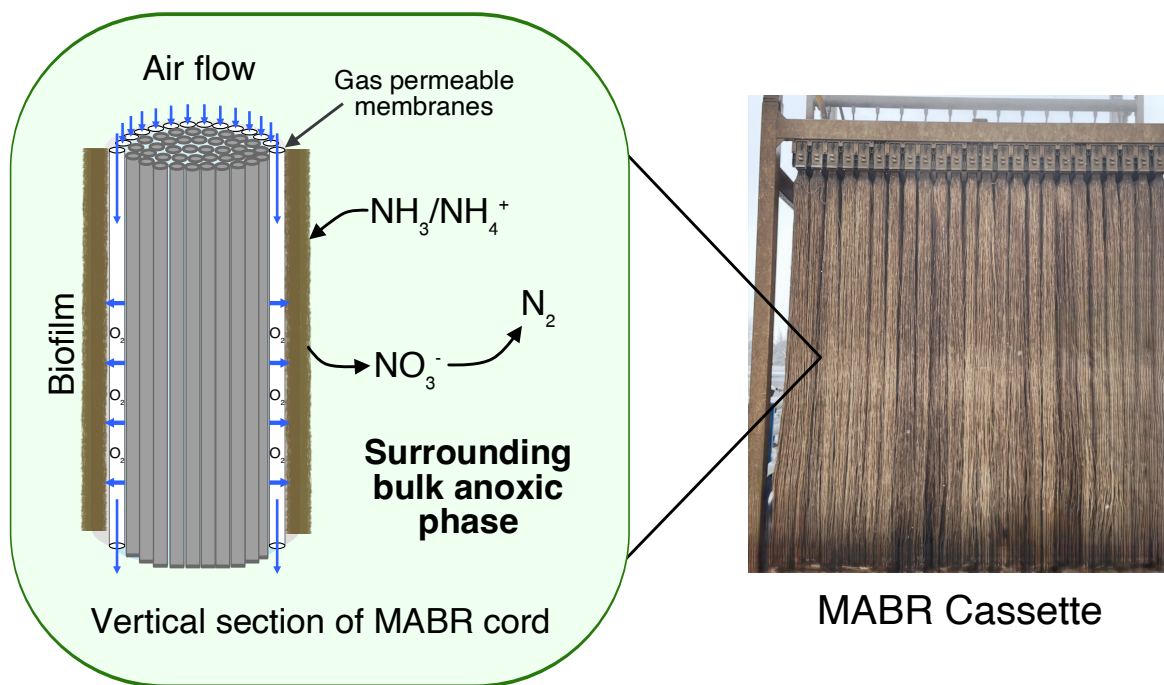


Figure 4.1 Diagram illustrating the composition of an individual MABR cord fiber. Air flows down through several gas permeable membranes, which surround the solid centre of the cord, and diffuses out into the biofilm attached to the cord surface. Ammonia diffuses into the biofilm where nitrifying microorganisms use the ammonia and oxygen that diffuses out from the cord, producing nitrate that serves as an electron acceptor for denitrification by microorganisms on the outer surface of the biofilm and in the surrounding anoxic bulk phase.

The municipal WWTP study site in Southern Ontario was upgraded with an MABR system in 2022 that was installed upstream of the already-existing CAS system. Here, the microbial community of the CAS system was characterized before and after the MABR upgrade to evaluate the impact of the upstream MABR installation on the functional potential of the downstream aeration basin. This study was done collaboratively, with additional research evaluating long-term plant performance with various operational parameters, along with exploring biofilm thickness and microbial community composition in the MABR across different operational conditions (Lakshminarasimman, 2024; Lakshminarasimman and

Parker, 2025). Additionally, the MABR biofilm microbial communities were evaluated during the chosen optimal plant operations conditions to characterize the microorganisms responsible for the observed nitrogen removal from the system. This current study evaluates the impact of an MABR upgrade on the CAS and MABR microbial communities at a municipal scale, with this MABR system being the largest in North America based on media surface area at the time of its installation.

4.2 Materials and methods

4.2.1 Study site and plant operating conditions

Prior to the MABR upgrade, the municipal wastewater treatment site was configured with a conventional activated sludge (CAS) system, which had a rated capacity of 9,320 m³/d. The description of the site has been previously outlined (Lakshminarasimman and Parker, 2025). Wastewater treatment involved initial primary treatment with a 2 mm fine screen to remove larger particulate matter, with subsequent secondary treatment in a CAS system consisting of two aeration tanks with fine bubble diffusers, representing a total volume of 2,404 m³ (Figure 4.2). Following treatment in the aeration tanks, the mixed liquor undergoes chemical phosphorus removal treatment before solid separation in the secondary clarifiers. The remaining secondary effluent is then disinfected using chlorination prior to effluent release.

The MABR upgrade to the site was operational at the end of August 2022, where the new MABR system was installed upstream of the CAS system and received a return activated sludge stream, resulting in a “hybrid” system configuration supporting both attached and suspended growth for secondary treatment (Lakshminarasimman and Parker, 2025). The hybrid MABR system consisted of four parallel anoxic trains that were connected to receive return activated sludge from the secondary clarifiers, with a total volume of 637 m³. Each train contained nine individual ZeeLung cassettes (Veolia Water Solutions and Technologies) for a total membrane surface area across nine cassettes of 69,120 m². The cassettes each contained thousands of membrane cords through which air passed down from the top of the

membrane cord to the bottom. As air passes down the cord, it diffuses out and into the biofilm that forms on the surface of the membrane cords. Since the cassettes are placed in anoxic tanks, a counter diffusion gradient is established across the biofilm with dissolved oxygen and substrates (DOC and N). The membrane exhaust from cassettes is also recycled to a diffuser at the base of the cassettes to provide shearing force to control biofilm thickness on the membrane cords.

To optimize plant function following the MABR upgrade, five different operational conditions were tested to assess the best parameters for MABR air flow and determine the impacts of ammonia and wastewater loading on MABR and plant performance (Lakshminarasimman and Parker, 2025) (Table 4.1). The operational phase with the best nitrogen removal performance was selected for biofilm sampling to assess the microbial community's functional potential for contribution to nitrification, denitrification, and phosphorus removal.

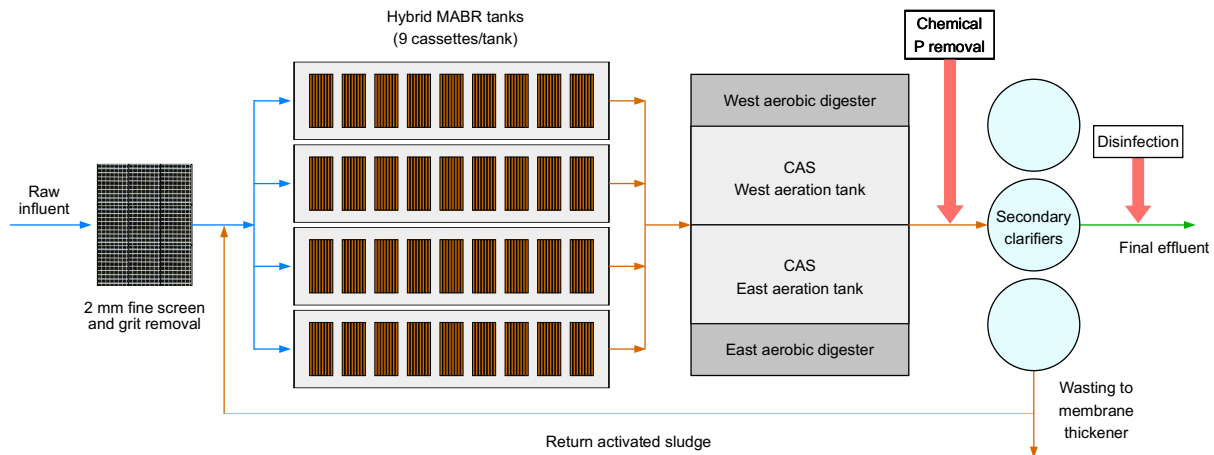


Figure 4.2 Layout of municipal wastewater treatment plant study site. Layout is not shown to scale. Thin arrows indicate direction of flow through the system. Hybrid MABR tank upgrade was operational at the site starting in August 2022. This figure has been modified from Lakshminarasimman (2024).

Table 4.1 Tested operational phases for the upgraded MABR system.

Phase	Operation time (days)	Temperature (°C)	MABR air flow (NL m ⁻² hr ⁻¹)	Tanks	NH ₄ -N loading (g N m ⁻² d ⁻¹)
Phase 1	71	17.0 ± 1.0	6.1 ± 0.1	4	2.5 ± 0.3
Phase 2	61	14.0 ± 0.3	5.4 ± 0.2	3	3.9 ± 0.7
Phase 3	78	12.0 ± 0.7	4.5 ± 0.2	4	3.3 ± 0.4
Phase 4	224	19.0 ± 1.5	5.9 ± 0.2	4	2.9 ± 0.3
Phase 5	76	13.0 ± 0.4	6.3 ± 0.2	4	3.5 ± 0.3

4.2.2 Sampling and water chemistry

Samples were collected monthly from October 2020 to October 2023 from the CAS system with grab samples of mixed liquor from both the east and west aeration tanks to characterize the microbial community in the activated sludge. After transporting samples back to the lab, a 2 mL aliquot was placed in a sterile 2 mL collection tube and stored at -70°C until DNA extraction.

Alongside sampling for microbial community analysis, many water chemistry parameters from the plant were also measured. Using plant influent, effluent, and mixed liquor from within aeration and MABR tanks, total ammonia (NH₃/NH₄⁺), nitrite (NO₂⁻), and nitrate (NO₃⁻) were quantified, along with TKN and BOD₅, using previously described methods (Lakshminarasimman and Parker, 2025). Temperature, airflow, and dissolved oxygen (DO) measurements were collected from online sensors placed throughout the plant (Lakshminarasimman and Parker, 2025). Additionally, several plant performance metrics were calculated throughout the sampling period, as described previously (Lakshminarasimman and Parker, 2025). These performance metrics included: BOD₅ removal, TN removal, OTE in the MABR, nitrification rate in both the aeration tank and MABR, and denitrification rate in the MABR. Electricity consumption was also assessed in the plant before and after the MABR upgrade to evaluate its impact on the reducing electricity consumption needed to operate the CAS at required DO levels.

During sampling of the MABR in October 2023, individual cassettes were lifted from their tanks using a crane and placed on the ground, where they were left to drain excess water. The cassettes were then sprayed using secondary effluent from the system to remove loose materials. Sampled membrane cords were selected from the front side of the cassette, chosen to represent the average biofilm consistency of cords present. Approximately 1.50 m of the 1.95 m long membrane cord was cut using scissors at the top and bottom, leaving sufficient cord behind that was then tied off to prevent leakage of air upon return of the cassette into the tank. The sampled cord was subsequently cut into five 30 cm sections, with each section representing the bottom (1), bottom-middle (2), middle (3), top-middle (4), and top (5) of the cord. Each section was further cut in two, with one of the 15 cm pieces placed into a sterile 15 mL sterile tube for DNA extraction. After transport back to the lab, cord samples were stored at -70°C until further processing. All tools and surfaces used to collect cord samples were sterilized using 70% EtOH and wiped down thoroughly to remove any biofilm material between samplings.

4.2.3 DNA extraction

The DNA was extracted from both the mixed liquor suspended sludge (MLSS) and the MABR membrane cords collected using the DNeasy PowerSoil Pro Kit (Qiagen). As input for DNA extraction from activated sludge samples, 2 mL of MLSS was centrifuged at 10 000 RCF for 5 min to pellet sludge and cellular material. Following supernatant removal, the pellet was resuspended using 800 µL of solution CD1 (Qiagen) and transferred into the PowerBead Pro tube. For the membrane cord samples, a 1 cm section was cut using a sterile scalpel blade and placed into a PowerBead Pro tube using flame-sterilized tweezers, along with 800 µL of solution CD1 (Qiagen). PowerBead Pro tubes containing the sample material were then incubated at 65°C for 10 min and subsequently homogenized using a FastPrep-24 bead beater (MP Biomedical, Santa Ana, CA) for 45 s at a speed of 5.5 m/s. The remainder of the DNA extraction was then completed following the manufacturer's protocol. Extracted DNA was quantified using the Qubit dsDNA HS Assay (ThermoFisher Scientific) and sample purity was assessed

using both gel electrophoresis and the NanoDrop 2000 (ThermoFisher Scientific) to determine 260/280 ratios. Extracted DNA was stored at -20°C until further analysis.

4.2.4 Amplicon and metagenomic sequencing

The DNA extracted from all collected samples was used to perform 16S rRNA gene sequencing to determine the microbial community composition in both the CAS and MABR systems. The V4-V5 region of the 16S rRNA gene was amplified using PCR and universal primers 515F-Y and 926R targeting both bacteria and archaea, following previously described protocols (McKnight and Neufeld, 2024). Demultiplexing of the paired-end sequence reads was carried out using MiSeq Reporter software version 2.5.0.5 (Illumina). The AXIOME3 (Automation, eXtension, and Integration Of Microbial Ecology) pipeline, implementing QIIME2 version 2020.6 and DADA2 was used for primer sequence removal, quality trimming, denoising, paired-end sequence merging, and subsequent generation of an amplicon sequence variant (ASV) table (Callahan *et al.*, 2016; Min *et al.*, 2021). Taxonomic classification of ASVs was performed during the AXIOME3 pipeline using the SILVA database release 138 (Quast *et al.*, 2013), along with generation of a phylogenetic tree of ASV sequences using FastTree (Price *et al.*, 2010). Beta diversity metrics were calculated using the q2-diversity plugin also via the AXIOME3 pipeline (Min *et al.*, 2021).

Metagenomic sequencing was performed on a subset of collected samples. Initial monthly samplings from the CAS basins were sent for metagenomic sequencing, inclusive from October 2020 to March 2021 from both the east and west aeration basins. Samples from this 6-month period comprised a mixture of DNA extracted from samples collected across three consecutive days. A total of 1 µg of DNA for each sample (12 samples total) was sent to The Centre for Applied Genomics (TCAG, Toronto, ON) for metagenomic sequencing. Sequencing was performed using a NovaSeq 6000 System (Illumina), with an SP flow cell (2×150 cycles). Sequencing libraries were prepared using the TruSeq DNA PCR-Free HT Library Prep Kit (Illumina) following the manufacturer's protocol.

Following the MABR upgrade, additional samples were processed for metagenomic sequencing. These samples were collected during October 2023 when the MABR showed high levels of TN removal. Extracted DNA from MABR cord biofilm collected from the middle section of membrane cord samples from cassettes 1, 5, and 9 of tanks 3 and 4 were sent for metagenomic sequencing (6 samples). Additionally, cord biofilm DNA samples from the side and back of cassette 5 from tank 3, and the side of cassette 9 in tank 4 were included (additional 4 samples). Mixed liquor samples collected from both the east and west aeration basins were also sequenced for comparison with the initial metagenomic sequencing of the CAS system done in 2020-2021. A total of 1 µg of DNA was sent for each sample to TCAG (Toronto, ON) for library preparation and metagenomic sequencing. With the objective of obtaining a similar sequencing depth as done for the initial metagenomic sequencing of samples from the CAS system, representing 20-25 million reads per sample, samples were sequenced using 20% of a single lane on an S4 flow cell (2×150 cycles) on a NovaSeq 6000 System (Illumina).

All demultiplexed sequence data were processed using the quality control portion of the Metagenome ATLAS pipeline to remove duplicates, perform quality trimming, and contaminant removal (i.e., adapter sequences) (Kieser *et al.*, 2020). Subsequently, FragGeneScanRs (v 1.1.0) was used to predict open reading frames (ORFs) within the quality controlled, unassembled, and unmerged forward and reverse metagenomic reads (Van der Jeugt *et al.*, 2022). Hidden Markov model (HMM) profiles for associated functional genes of interest involved in nitrification (AOB_ *amoA* and *nxrB*) and denitrification (*narG*, *napA*, *nirS*, *nirK*, *norB*, *nosZ*) were retrieved, along with the *rpoB* HMM for taxonomic classification from the FunGene database (Fish *et al.*, 2013). These HMM profiles were used with the MetAnnotate (metannotate-wrapper; v. 0.9.2) tool to quantify the relative abundances and taxonomic affiliations of sequence hits to these functional marker genes from the unassembled metagenomic reads (Petrenko *et al.*, 2015). The NCBI RefSeq release 220 (October 2023) was used for taxonomic assignment within MetAnnotate using the default E-value (1×10^{-6}) for usearch-based taxonomic classification. MetAnnotate results were further analyzed and visualized using the MetAnnoviz R package (v. 1.1.0),

which normalized functional gene hits for each HMM based on HMM length and the number of length-normalized hits to the *rpoB* HMM. An E-value of 1×10^{-10} was used as the threshold, above which HMM hits would not be plotted. Forward and reverse reads from each sample were considered replicates and displayed as the average value of the duplicates for each sample. All raw sequences for these samples are available on the NCBI Sequence Read Archive under BioProject accession number PRJNA1104605.

4.3 Results and discussion

4.3.1 Effect of MABR upgrade on plant performance

Over three years of sampling, the CAS aeration tanks displayed a consistent seasonal temperature pattern that reflected local regional temperatures throughout the year. Average temperatures in the two aeration tanks ranged from ~ 11 - 22°C , with a minimum temperature of 10.4°C recorded in February 2023, and a maximum temperature 21.4°C in September 2021 (Figure 4.3A). The average raw influent concentration of TKN coming into the plant over the sampling period was 49.2 mg/L ($\text{SD} = 3.8 \text{ mg/L}$), and the raw influent BOD_5 had a mean concentration of 258.1 mg/L ($\text{SD} = 98.5 \text{ mg/L}$). Both the influent BOD_5 and TKN loading concentrations before and after the plant upgrade did not differ, with the operation of the aeration tanks remaining similar before and after the MABR installation (Lakshminarasimman and Parker, 2025). This led to the conclusion that any observed changes in plant efficiency could be attributed to the addition of the MABR system.

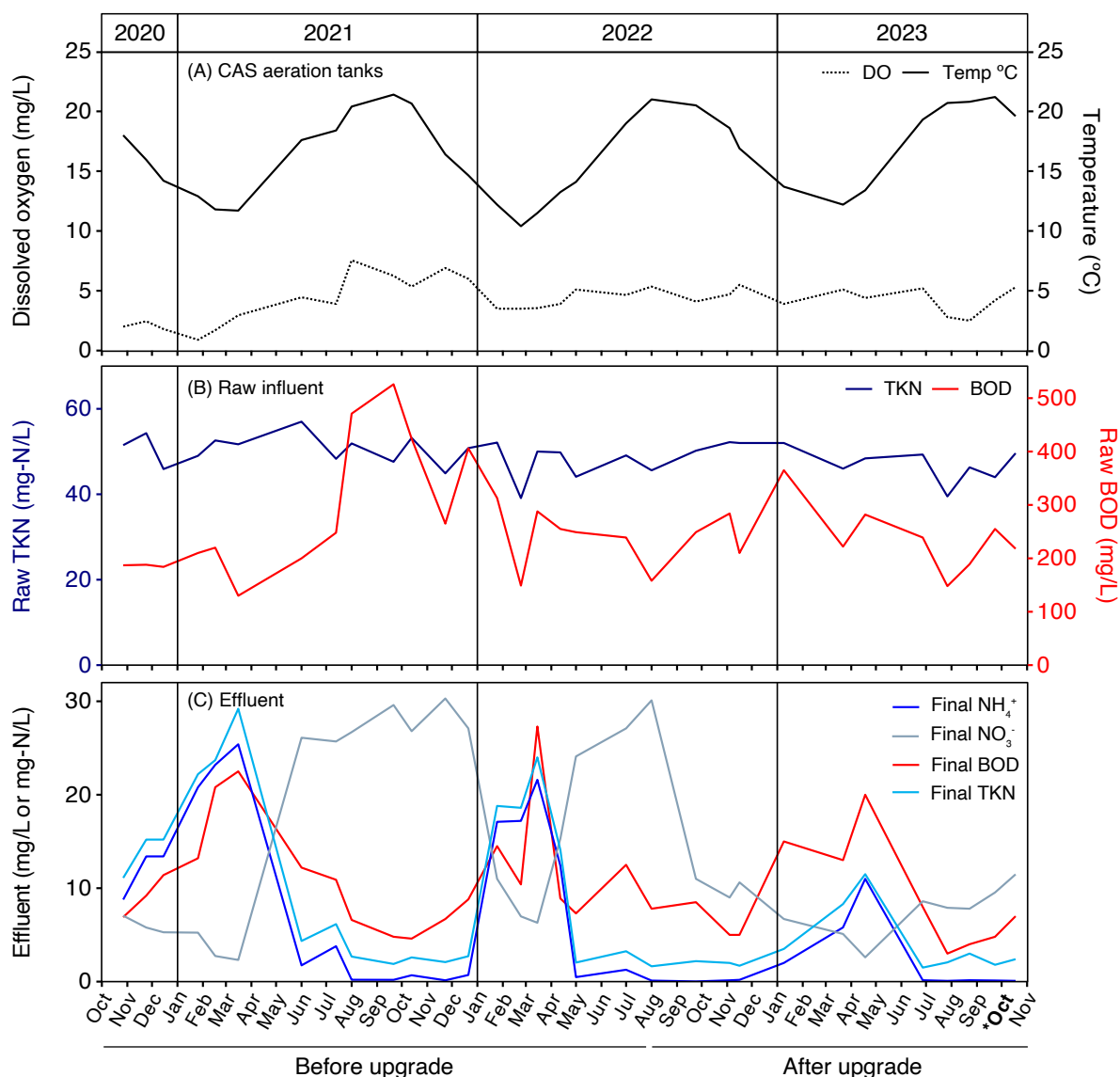


Figure 4.3 Plant water chemistry parameters measured alongside the collection of samples for microbial community analysis, including dissolved oxygen (DO) and temperature of the CAS aeration tanks (A), total Kjeldahl nitrogen (TKN) and biological oxygen demand (BOD) of the raw influent (B), and effluent concentrations (C). Effluent characterization included measurements of final total ammonia and ammonium, final nitrate, final TKN, and final BOD concentrations, represented by different colour lines (C). Measurements from before upgrade correspond to the plant configuration with only the CAS system in operation, whereas measurements from after the upgrade corresponded to the plant with a configuration of both the MABR and CAS systems in operation. The bolded October month represents the corresponding sampling date of the MABR system for metagenomic sequencing.

Before the upgrade, effluent $\text{NH}_4^+\text{-N}$ concentrations were highest during the colder sampling months of Feb-Mar, with corresponding low concentrations of $\text{NO}_3^-\text{-N}$ in the effluent, due to decreased nitrification activity in the aeration tanks (Figure 4.3C). When aeration tanks recorded their highest temperatures from Aug-Oct, $\text{NH}_4^+\text{-N}$ effluent concentrations were below 1 mg/L-N, associated with elevated levels of effluent nitrate (25-30 mg/L-N), indicating high levels of nitrification activity during these warmer months. Both TKN and BOD_5 effluent concentrations followed the same seasonal trend as total ammonia before the upgrade, with elevated concentrations during colder months (Figure 4.3). This seasonal variation in nitrogen removal is commonplace with CAS systems in colder climates and the loss of nitrification activity is often attributed at an operational level to nitrifier “washout”, with an SRT of five days (Houweling *et al.*, 2018). Additionally, temperature is a well know factor affecting nitrification activity, in addition to microbial growth (Shammas, 1986). As mentioned, one of the advantages to implementing an MABR system is to help reduce the loss of plant nitrification activity normally associated with colder months by retaining a large inventory of fixed biomass in the system to extend the SRT, reducing the loss of nitrifiers due to SRT below the washout (Houweling *et al.*, 2018).

Following the plant upgrade, effluent concentrations exhibited a less prominent seasonal pattern. After installation, different operational parameters were tested across several phases with changes to operational conditions in the MABR, including ammonia loading, process airflow, and the number of operational tanks (Table 4.1). The long-term performance of this full-scale hybrid MABR system across the operational conditions was assessed previously (Lakshminarasimman and Parker, 2025). Results showed that MABR nitrification rates were most strongly connected to process airflow, because biofilm thickness was controlled through scouring forces that increased with increasing process airflow, leading to optimal biofilm thickness to support the growth and activity of nitrifiers (Lakshminarasimman and Parker, 2025). The increased process airflow implemented in Phase 4 from Jul-Oct 2023 resulted in effluent ammonia concentrations of <0.2 mg/L-N (Figure 4.3C).

Denitrification activity from the MABR was evident, as the nitrate concentrations decreased following the upgrade, ranging from 5-10 mg/L-N in the summer months to <5 mg/L-N during the winter, in conjunction with continued low levels of total ammonia in the effluent (Figure 4.3C). Together, these results showed nitrification activity, with the nitrate produced subsequently removed via denitrification. The rate of denitrification observed in the MABR was limited by MABR nitrification rates, as nitrification is responsible for supplying the nitrate required for denitrification, with some of the highest denitrification rates also observed during Phase 4 (Lakshminarasimman and Parker, 2025). The assessment of different operational parameters revealed Phase 4 conditions yielded the highest rates of nitrification and denitrification activity in the MABR. As such, the focus of microbial community characterization of the MABR biofilm throughout the rest of this chapter is focused on samples collected during October 2023 at the end of Phase 4.

Following the hybrid MABR system upgrade, the plant was able to sustain nitrification activity even in winter months, in addition to improved total nitrogen removal from the increased denitrification activity in the MABR (Lakshminarasimman and Parker, 2025). Both the nitrification and denitrification activity in the MABR also decreased carbon and nitrogen loading on downstream aeration tanks, given that nitrogen loading decreased from SND activity in addition to carbon, which was removed by denitrification activity (Lakshminarasimman and Parker, 2025). The decreased load on the CAS system and improved OTE of the MABR translated to a plant-wide reduction in volumetric electricity consumption of ~50% because less aeration was required to maintain the required aeration tank DO concentrations (Lakshminarasimman and Parker, 2025). In the winter following Phase 4, despite aeration tanks running at a SRT typically associated with nitrifier washout at cold temperatures (13°C), nitrification activity was sustained and attributed to nitrifier seeding from MABR biomass (Lakshminarasimman and Parker, 2025). With changes in loading to the aeration tank, resulting from carbon and nitrogen removal activity in the MABR, microbial communities present in the aeration tanks

before the upgrade were analyzed, in addition to the subsequent impact of the MABR upgrade on the CAS microbial community composition.

4.3.2 Factors influencing CAS aeration tank microbial community

Before and after the WWTP upgrade, samples were collected from the CAS aeration tanks almost every month, including a MLSS sample collected from either solely the east or west aeration tanks, or both. Activated sludge collected on the same day from both the east and west tanks was highly similar in terms of overall microbial community composition, with samples grouping closely in the ordination space (Figure 4.4B). Triplot analysis using principal coordinates generated with the weighted UniFrac distance metric, which accounts for both the phylogenetic relation of ASVs and their abundance across samples, showed a strong influence of temperature on microbial community composition (Figure 4.4). Although microbial profiles were also associated with final effluent total ammonia and BOD₅ concentrations, these correlations were in opposition to temperature, presumably because higher ammonia and BOD₅ concentrations were present in samples collected during colder months when microbial activity was lower (Figure 4.4A). Across the three-year period, aeration tank samples grouped together by collection year, suggesting a temporal change in microbial community composition. Sample distribution was also strongly associated with temperature, with several ASVs more strongly associated with certain sampling times, such as *Comamonadaceae* ASVs with colder 2022 samples, and *Nitrosomonas* ASVs during warmer periods (Figure 4.4). Even though aeration tank samples from after the MABR upgrade grouped together, especially during warmer months, they did not group separately from before-upgrade samples (Figure 4.4). This suggests that, despite some influence from the installation of the MABR system upstream of the aeration tanks, there was a conserved microbial community composition that was maintained in the aeration tanks and primarily influenced by temperature.

When comparing alpha diversity from before and after the upgrade, microbial community changes were evident. A subset of aeration tank samples collected one year apart, before and after the

upgrade from September-November, January, March, April, July, and August for 2021-2022 and 2022-2023, were used to test for changes in alpha diversity. Generally, Shannon index values were higher in the aeration tanks after the upgrade (Figure 4.4B), with a significant increase in Shannon index value of on average 0.45 between monthly time-points tested (paired t-test: $t = 3.2$, $p < 0.05$). The Shannon index values ranged from 4.17-5.31 before and 4.98-5.58 after the upgrade for compared samples. A significant mean increase of 0.03 in Pielou's evenness was also observed (paired t-test: $t = 4.1$, $p < 0.05$), with values across samples ranging from 0.77-0.83 before to 0.80-0.86 after the upgrade. However, a significant increase in observed ASVs (richness) was not seen following the plant upgrade (paired t-test: $t = 1.9$, $p > 0.05$). Increases in combined evenness and diversity (Shannon index) of the aeration tank microbial communities following the MABR upgrade does support the hypothesis that the MABR biofilm was seeding downstream aeration tanks. However, the additional anoxic tank containing the MABR cassettes could also contribute to an increase in the microbial inventory of the suspended sludge because it also supports the growth of anaerobes.

Because of the varying redox conditions and substrate gradients, the MABR biofilms will promote the growth of distinct microbial communities compared with the CAS system. When comparing the overall microbial community structure of the CAS to that of the MABR biofilm, they were clearly distinct from one another and group separately (Figure 4.5A, B). Beta diversity of the CAS and MABR microbial communities was explored using both weighted and unweighted UniFrac distance metrics and it was found that the difference in community composition between the two systems was more pronounced using the weighted UniFrac distance metric, whereas the MABR and aeration tank samples from after the upgrade (MABR + CAS configuration) were less distinct from each other with unweighted UniFrac distances (Figure 4.5A). This result further indicates that the MABR system seeded the downstream CAS aeration tanks with microorganisms that were enriched in the MABR cord biofilm, as the unweighted UniFrac metric only assesses how many shared ASVs there are between samples and not the quantity of each ASV within a sample.

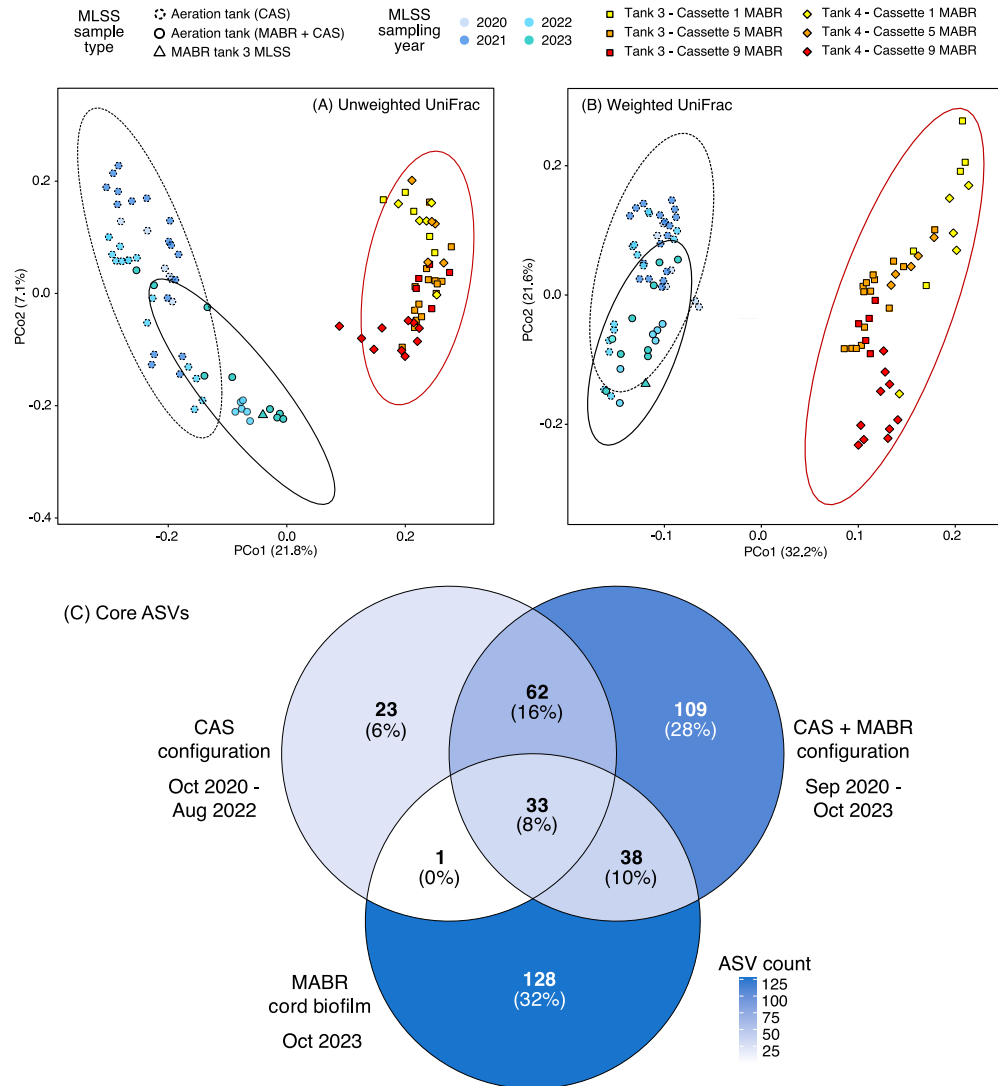


Figure 4.5 Principal coordinates and core taxa analysis revealed shared ASVs between the CAS aeration tank and MABR cord biofilm microbial communities. PCoA was calculated using both the unweighted (A) and weighted (B) UniFrac beta diversity metrics on both CAS and MABR 16S rRNA gene sequencing data rarefied to 11,000 reads per sample. Sample points in the PCoA plots have different shapes and colour based on location and time, with colour of the points representing the sampling year for the MLSS samples. Colour represents the cassette number for MABR samples. The percent variation explained by the principal components for each diversity metric are shown on the axes, with ellipses representing the 95% confidence intervals for sample groups (black dotted line = CAS configuration aeration tank samples, black solid line = CAS + MABR configuration aeration tank samples, red = MABR cord samples from October 2023) (A, B). The Venn diagram shows both the unique and shared core ASVs between aeration tank samples from the CAS only configuration the CAS + MABR configuration, and the October 2023 MABR cord biofilm samples (C). The opacity of colour within the Venn diagram represents the number of ASVs in each of the three groups.

To further quantify the seeding effect of the MABR system, core ASVs present in 70% of samples were compared to identify those shared between groups (CAS pre-upgrade, CAS post-upgrade, phase 4 MABR biofilm). The highest number of unique core ASVs was present in the MABR biofilm (128 ASVs), with 23 core ASVs unique to the CAS aeration tanks before the upgrade and 109 core ASVs unique to the CAS aeration tanks after the upgrade (Figure 4.5C). There were 33 core ASVs shared by all three groups, suggesting these ASVs were ubiquitous in the plant. Only one core ASV was shared exclusively between the MABR and before-upgrade aeration tank samples, whereas 38 core ASVs were shared between the MABR and aeration tank after upgrade, highlighting the seeding effect of the upstream MABR to the aeration tank (Figure 4.5C). Across aeration tank samples from before and after the upgrade 62 core ASVs were shared, representing taxa that were consistently unique to the core microbial community of the CAS system regardless of plant upgrades.

The positive impacts of seeding microorganisms from one biofilm process to downstream CAS mixed liquor has been shown previously in treatment systems with side stream anammox processes (Plaza *et al.*, 2001), and more recently in pilot MABR hybrid systems that supported nitrification activity (Houweling *et al.*, 2018). In this large scale MABR installation, this seeding process was observed not only through the sustained winter nitrification activity observed in the CAS system (Lakshminarasimman and Parker, 2025), but also through the quantification of core microorganisms that were seeded from the MABR biofilm into the CAS aeration tanks (Figure 4.5C). Distinct microbial communities unique to the MABR biofilm and the CAS mixed liquor were also observed, with the same differences in community composition noted in other studies on hybrid MABR-CAS pilot systems (Zhao *et al.*, 2023). To further explore what taxa were distinct within these two processes and determine the microorganisms that were new to the CAS system following the MABR upgrade, 16S rRNA gene sequencing results were further assessed.

4.3.3 16S rRNA gene microbial community profiles of CAS aeration tanks and MABR cords

Across the 3-year sampling of the CAS aeration tanks, most taxa present in the microbial communities of the aeration tank samples were associated with the phylum *Proteobacteria* and *Bacteroidota* (Figure 4.6). Additionally, ASVs associated with *Actinobacteriota* (*Gordonia* – ASV 246, *Intrasporangiaceae* – ASV 22), *Firmicutes* (*Trichococcus* – ASV 5), and *Myxococcota* (*Haliangium* – ASVs 106 and 233) were also present within the activated sludge (Figure 4.6).

Of the *Proteobacteria*, several species were continually detected across the entire sampling period, including *Aquabacterium*, *Aquimonas*, *Rhizobacter*, *Rhodobacter*, *Thermomonas*, *Varivorax*, and *Zoogloea* spp. However, during the summer months of 2021, several of these ASVs were below detectable limits in the aeration tank, and an increase in relative abundances of *Haliangium* spp. and *Comamonadaceae* ASV 83 were observed (Figure 4.6). At this time, the aeration tanks at the plant were undergoing maintenance, which likely explains the observed shifts in the microbial communities. Following this summer period, the aeration tank microbial communities returned to similar relative abundances profiles as observed before the maintenance. The relative abundances of *Bacteroidota* ASVs were less impacted by the maintenance period, with genera such as *Terrimonas* continually detected across all sampling times, including the summer of 2021. Many ASVs associated with the genus *Flavobacterium* were also detected across all seasons, albeit with varying relative abundances for unique ASVs (Figure 4.6). As noted earlier, the beta diversity of samples was strongly correlated with temperature. However, responses of individual ASVs to seasonal temperature changes were diverse, with some showing no change in relative abundance, whereas others did show relative abundance changes corresponding to temperature shifts such as *Nitrosomonas* ASV 20.

Comparing the ASVs present in the CAS aeration tanks before and after the MABR upgrade, many were consistently present despite the change, with some ASVs like *Intrasporangiaceae* ASV 22 showing a higher relative abundance after the upgrade (Figure 4.6). Although, there was some impact on the upstream installation of the MABR system to the microbial community composition of the aeration tanks. Prior to the upgrade, the detection of ASVs from the phyla *Desulfobacterota* and *Nitrospirota* were rare. However, during the phase 4 period of MABR operation, the detection of ASVs from these phyla became consistent, even with the minimum 2.5% relative abundance cutoff (Figure 4.6). When looking at the MABR cord microbial communities from phase 4, ASVs from *Desulfobacterota* and *Nitrospirota* had high relative abundance (Figure 4.7), indicating these ASVs were detected in the aeration tanks because they were relatively abundant in the MABR biofilm. Despite the difference in phylum abundance, the MABR biofilm also had a high relative abundance of ASVs from both *Proteobacteria* and *Bacteroidota*, with some *Firmicutes*, *Actinobacteriota*, and *Myxococcota* much like the aeration tank microbial communities. Similarities between the two systems also included the presence of the same ASVs from the genera *Ferruginbacter*, *Terrimonas*, *Zoogloea*, *Rhodobacter*, *Rhodoferax*, *Rhizobacter*, and *Comamonadaceae*. There were also many ASVs unique to the MABR biofilm, including genera *Chryseolinea*, *Denitratisoma*, *Sulfuritalea*, *Sphaerotilus*, and *Ca. Accumulibacter*, in addition to *Hydrogenophilaceae*-associated ASVs (Figure 4.7). These ASVs appear in the aeration tanks following the upgrade as a result of MABR seeding to the aeration tanks.

Examining microbial community composition of the MABR cord biofilm, variation in ASV relative abundances can be seen between cassette samples along both tank 3 and 4, including higher relative abundances of *Hydrogenophilaceae* in cassette 1 sample profiles (Figure 4.7). Although, many of the ASVs were present across all three cassette sample profiles. There was also minimal variation in microbial community composition across the length of a sampled cord (top-bottom), which has already been explored across all tested phases in this MABR system (Lakshminarasimman, 2024). Additionally, there was high similarity in the cassette microbial communities between tanks 3 and 4, which

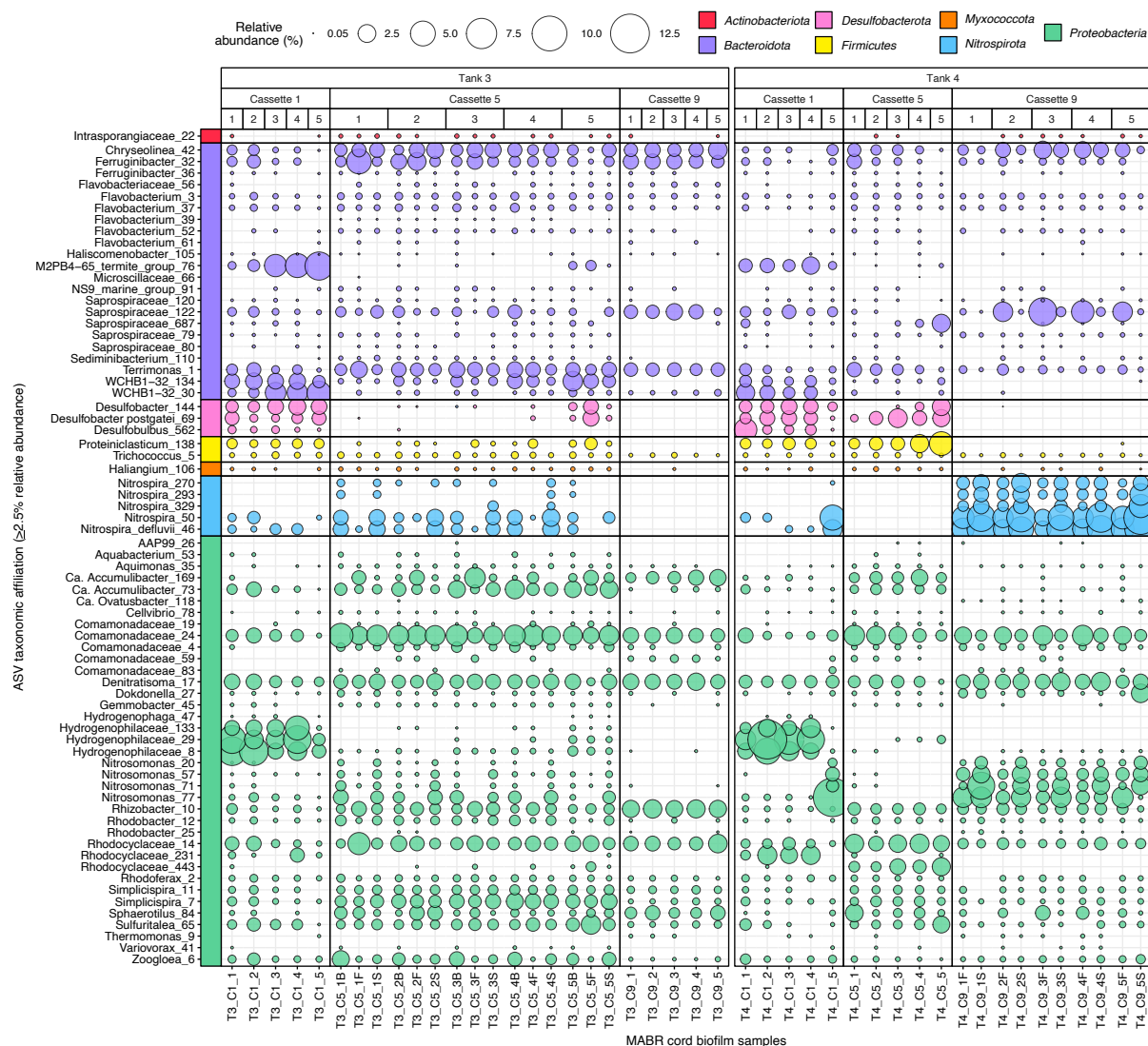


Figure 4.7 Amplicon sequence variant (ASV) profiles of microbial communities from the MABR cord biofilm sampled in October 2023 from cassettes in both tanks 3 and 4. Bubbles represent the relative abundance of each ASV in a particular sample, with colours representing the phylum of each ASV. All ASVs shown were present in at least one sample at $\geq 2.5\%$ RA. Samples are sorted according to the tank, cassette, and section of the cord sampled (1 = top, 2 = top-middle, 3 = middle, 4 = bottom-middle, and 5 = bottom section of MABR cord). Samples collected from tank 3 cassette 5 and tank 4 cassette 9 were also taken from different sides of the cassette in addition to the cord samples collected from the front of each sampled cassette. These are labelled along the x-axis (F = front, S = side, B = back of the cassette).

function in this sampling as biological replicates of the MABR system (Figure 4.7). The only apparent difference between the relative abundance of ASVs between the two tanks was associated with the *Nitrospirota*, which will be discussed further below when discussing the nitrifiers in the plant.

4.3.4 Nitrifiers in the CAS and MABR systems

Over the 3-year sampling of the CAS system, two distinct nitrifiers were detected consistently with 16S rRNA gene sequencing. A single AOB from the genus *Nitrosomonas* was detected, alongside a NOB from the genus *Ca. Nitrotoga*, both before and after the plant upgrade (Figure 4.8). Both of these nitrifiers are commonly found in activated sludge flocs of CAS systems, with *Ca. Nitrotoga* known to be a cold-adapted genus of NOB (Winkler *et al.*, 2013; Liu *et al.*, 2021). Additionally, oc32 ASV 141 associated with *Nitrosomonadaceae* was detected frequently in the aeration tanks, ranging from 0.1-0.2% RA. Although this ASV is taxonomically associated with *Nitrosomonadaceae*, a family of known ammonia-oxidizing bacteria, further data are required to confirm its potential for ammonia oxidation (Prosser *et al.*, 2014). The relative abundance of *Nitrosomonas* follows an evident seasonal trend, with higher relative abundances during warmer seasons compared to a lower RA in colder conditions, correlated positively with temperature. *Ca. Nitrotoga* exhibited less of a seasonal trend in relative abundance, perhaps explained by observed cold adaptability of these bacteria (Liu *et al.*, 2021). These ASVs were below detection limits from Jun – Oct 2021, along with oc32 ASV 141, which coincides with maintenance of the aeration tank during this time frame (Figure 4.8). Aside from these three, there were only a few other nitrifier ASVs that were detected at low abundances in the aeration tanks prior to the upgrade.

Following installation and operation of the MABR system, the number of unique nitrifier ASVs detected in the CAS system increased alongside the nitrifiers previously detected before the upgrade. Several ASVs associated with *Nitrosomonas* spp. appear in aeration tank profiles, in addition to *Nitrosomonas ureae* and *Nitrosomonas mobilis* ASVs, following the plant upgrade (Figure 4.8). From Sep 2022 – Jul 2023 only an increase in AOB ASVs was observed. However, starting in Aug 2023, ASVs

associated with *Nitrospira* spp. were detected in the aeration tank community profiles. The *Nitrospira* ASVs appearing after the upgrade correspond to the same *Nitrospira* ASVs detected in the MABR biofilm in October 2023, once again highlighting the seeding effects from the MABR biomass (Figure 4.9).

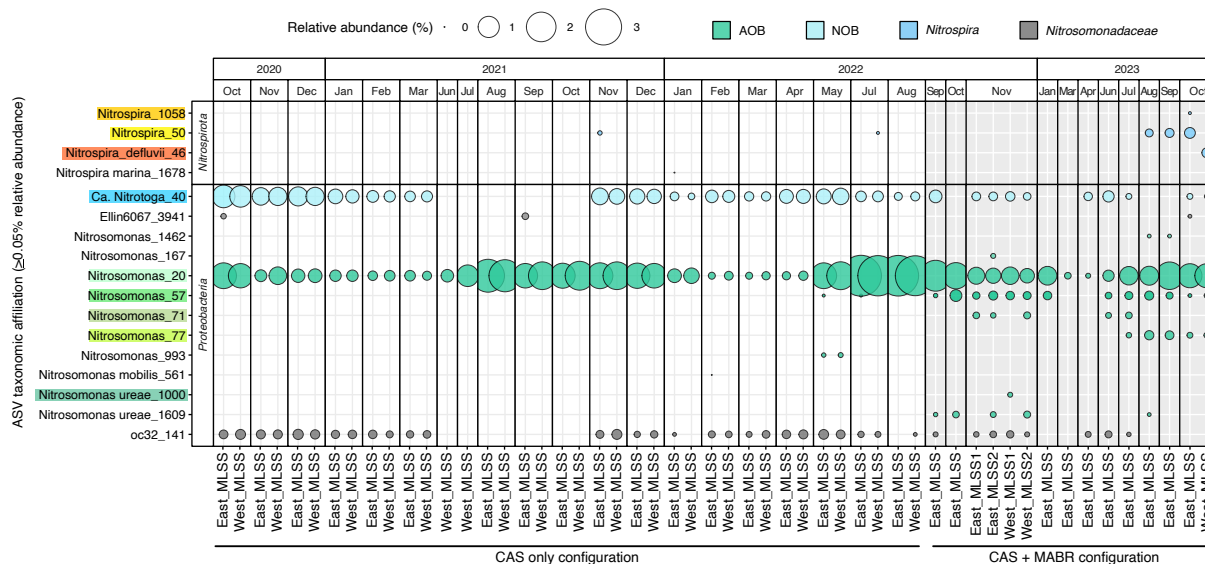


Figure 4.8 Amplicon sequence variants associated with nitrifying taxa in the CAS system aeration tanks. Bubble size represents the relative abundance of each ASV in the collected sample. Colour of the bubbles represents the associated group of nitrifier, including AOB, NOB, *Nitrospira*, and other *Nitrosomonadaceae* ASVs that were not classified with a known AOB genus. Background colour represents the sampling period before the upgrade with the CAS only configuration (white) and after the plant upgrade with the combined CAS and MABR configuration (grey). Each sampling time-point either contains replicate samples from both the east and west aeration basins from the CAS system, or from only one of the basins. The ASVs shown represent only those nitrifying associated taxa that are present in at least one sample at greater or equal to 0.05% relative abundance. Highlighted ASVs shown on the y-axis correspond to the matching nitrifier ASVs also detected in the MABR cord biofilm (Figure 4.9).

In contrast to the number of aeration tank nitrifiers, there was a greater diversity of nitrifiers present in the MABR biofilm. Multiple ASVs associated with *Nitrosomonas* spp. were detected, along with many *Nitrospira* ASVs (Figure 4.9). The same *Nitrologa* ASV was also present in the MABR biofilm, albeit at lower relative abundance, and was the only other NOB-associated genus detected with

16S rRNA gene sequencing alongside *Nitrospira*. The dominant *Nitrosomonas* ASV 20 from the aeration tanks was present in the MABR biofilm, but at lower relative abundance than the other *Nitrosomonas* spp. in the MABR biofilm (Figure 4.9). The variation in AOB and NOB genera between the MABR and CAS systems highlights the niche differences within these two environments. The fixed biofilm interior of an MABR system favors the growth of microorganisms that fall into the category of *K*-strategists, which have slower growth rates and higher specific affinities for their desired substrates, thus allowing them to compete in crowded nutrient-limited environments (Yin *et al.*, 2022). Because of the nutrient concentration gradients found across the MABR biofilm as nutrients diffuse in from the liquid phase, habitable niches for *r*-strategist that have faster growth rates and lower substrate affinities are also present where substrate concentrations are higher, thus providing niches for both groups of organisms. Both *Nitrosomonas* and *Nitrotoga* spp. are generally considered *r*-strategists, whereas *Nitrospira* are known *K*-strategists with low growth rates and high ammonia affinities (Mehrani *et al.*, 2020; Yin *et al.*, 2022). Overall, the distribution of these nitrifiers coincides with their predicted niches, with similar enrichment of *Nitrospira* observed in MABR biofilms (Zhao *et al.*, 2023).

There were inconsistencies in the distribution of nitrifiers across the MABR cassettes in tanks 3 and 4. Cassette 1 samples from tanks 3 and 4 appear similar, despite the bottom section 5 sample from tank 4 - cassette 1 that appears to be an outlier compared with other samples (Figure 4.9). However, cassette 5 from tank 3 contains nitrifier-associated ASVs, in contrast to cassette 5 in tank 4, which shows no detectable *Nitrospira* ASVs and only low relative abundances of *Nitrosomonas* spp., with the opposite observed in cassette 9 samples where high nitrifier abundances were noted in tank 4, but not for tank 3 cassette 9 samples (Figure 4.9). The reason behind the heterogeneity among tanks is unclear, and considering this is one of the first studies to examine the microbial communities of a large full-scale hybrid MABR-CAS system, there are no other studies to corroborate these findings. Further study of this system, including measurements of nutrient concentrations in the mixed liquor along the MABR tanks to determine if there is variation along the length of the tank that may contribute to microbial community

differences across cassettes. In a municipal tertiary treatment system containing trains of rotating biological contactors with fixed biofilm, abundances of nitrifiers were negatively correlated with ammonia concentration, which decreased along the length of the train (Sauder *et al.*, 2012). Although it is possible that similar trends exist along the length of the MABR, this does not address tank profile heterogeneity, as they should be running under comparable operating conditions within the MABR system.

The majority of MABR samples were collected from the front side of the cassettes, however, additional samples were collected from the side and back of cassette 5 (tank 3) and the sides of cassette 9 (tank 4) to look for variation in microbial community composition across cassette faces. For both cassettes 5 and 9, higher relative abundances of nitrifiers were apparent in the biofilm profiles collected from the side and back of the cassettes compared with the front samples (Figure 4.9). This difference might be explained by variation in shearing forces experienced across the cassette, as the front would be exposed more directly to the flow of mixed liquor through the tanks. However, an effort was made to collect the front cord samples from deeper within the cords to minimize potential bias due to excess shearing on the outside layer of the biofilm. Future research should compare samples collected from the other cassettes 5 and 9 from tanks 4 and 3, respectively, to verify whether those samples had lower nitrifier relative abundances or if nitrifiers were present in these cassettes along different sides. Abundances of nitrifiers and nitrification activity was connected to shearing forces and biofilm thickness in this system (Lakshminarasimman, 2024), so it would be worth further exploring these finer scale variations to see if nitrifiers have a further preference for certain areas within the MABR system. In future evaluation, the incorporation of absolute quantification methods would help determine whether changes in relative abundance are representative of absolute abundance differences or a consequence of relative abundance changes of other microorganisms.

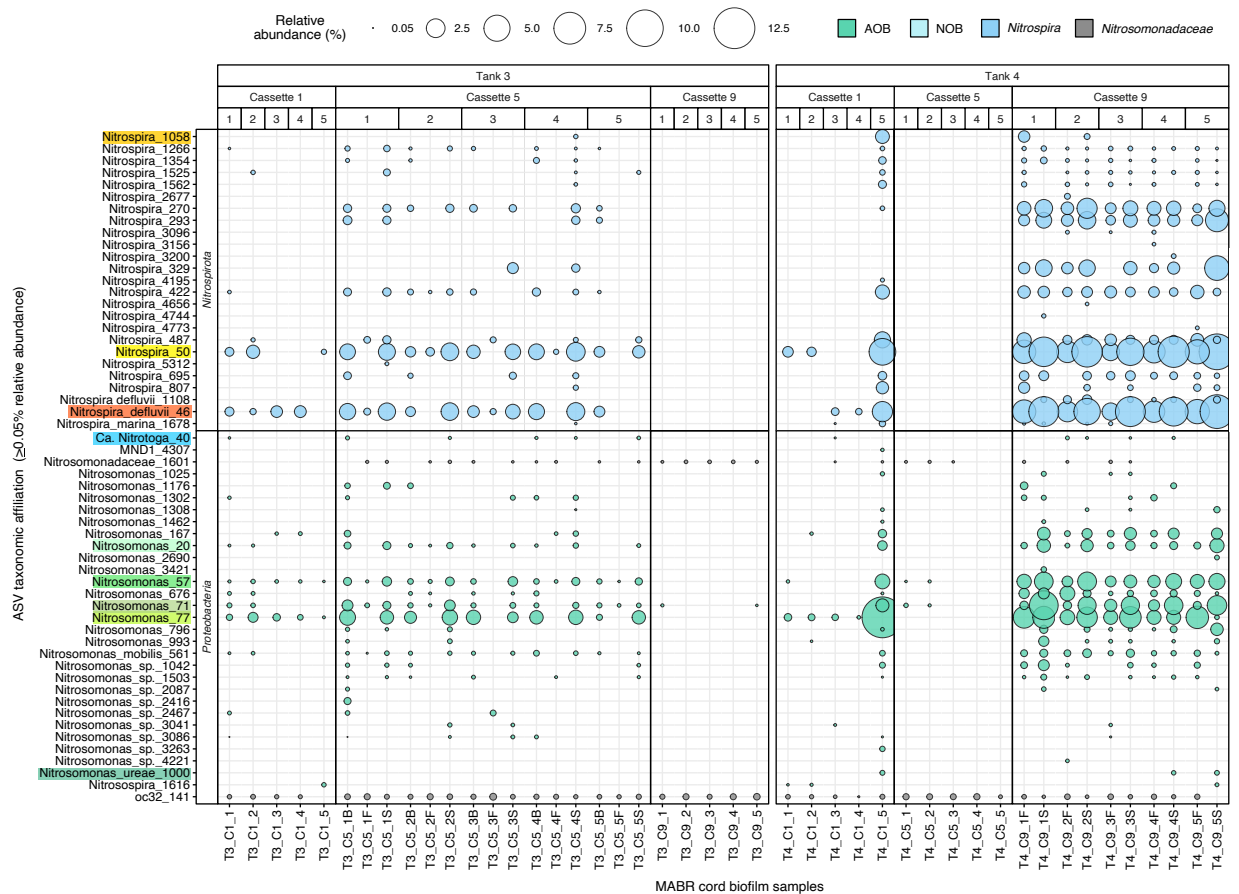


Figure 4.9 Amplicon sequence variants associated with nitrifying taxa in the MABR cord biofilm. Bubble size represents the relative abundance of each ASV in the sample. Colour of the bubbles represents the associated group of nitrifier, including AOB, NOB, *Nitrospira*, and other *Nitrosomonadaceae* ASVs that were not classified with a known AOB genus. Samples are sorted according to the tank, cassette, and section of the cord sampled (1 = top, 2 = top-middle, 3 = middle, 4 = bottom-middle, and 5 = bottom section of MABR cord). Samples collected from tank 3 cassette 5 and tank 4 cassette 9 were also sampled from different sides of the cassette in addition to the cord samples collected from the front of each sampled cassette. These are labelled along the x-axis (F = front, S = side, B = back of the cassette). The ASVs show represent only those nitrifying associated taxa that are present in at least one sample at greater than 0.05% relative abundance. Highlighted ASVs shown on the y-axis correspond to the matching nitrifier ASVs also detected in the CAS aeration tank (Figure 4.8).

4.3.5 Metagenomic profiling of nitrifiers within CAS and MABR system samples

To further explore the functional potential of the microbial communities in the CAS system and the MABR biofilm, samples were selected from before and after the upgrade for metagenomic sequencing. Samples from the CAS east and west aeration tanks collected from October 2020 – March 2021 during the initial monitoring of the plant were included, along with post-upgrade samples from both the CAS and MABR tanks (MLSS), and MABR tank 3 and 4 biofilm cord samples from cassettes 1, 5, and 9 from phase 4 (October 2023), which ran the selected operational parameters for the MABR system.

Profiling of the unassembled metagenomic reads from before the plant upgrade showed similar proportions of AOB and NOB species, as detected with 16S rRNA gene sequencing (Figure 4.10). The AOB species with the largest proportion of hits to *amoA*, *Nitrosomonas* sp. Nm84, also showed similar seasonal patterns as seen in the 16S rRNA gene profiles (Figure 4.7), with decreases in relative abundance during colder months. Unlike 16S rRNA gene profiles, there was a larger diversity of AOB species detected by metagenomic sequencing. More than one species of *Nitrosomonas* was detected in the aeration tanks during preliminary sampling, along with hits taxonomically affiliated to the genus *Nitrospira* in the *amoA* HMM profiles (Figure 4.10). The same was observed for *nxB* HMM hits, where multiple species of *Nitrotoga* NOB were detected, compared to the single ASV present in 16S rRNA gene data (Figure 4.7). Other NOB genera, including *Nitrospina*, *Nitrococcus*, *Nitrobacter*, *Nitrolancea*, and *Nitrospira* were also detected (Figure 4.10). Prior to the upgrade, the *Nitrospira* detected were predominantly associated with canonical NOB, as *Nitrospira amoA* HMM hits were present only in biofilm from January 2021. Following the plant upgrade, October 2023 samples from the aeration tanks showed the same increase in detected AOB species, as observed in the 16S rRNA gene profiling results (Figure 4.10, Figure 4.8). The profiles of MLSS from the MABR were highly similar based on metagenomic profiling, further supporting the results across different methods.

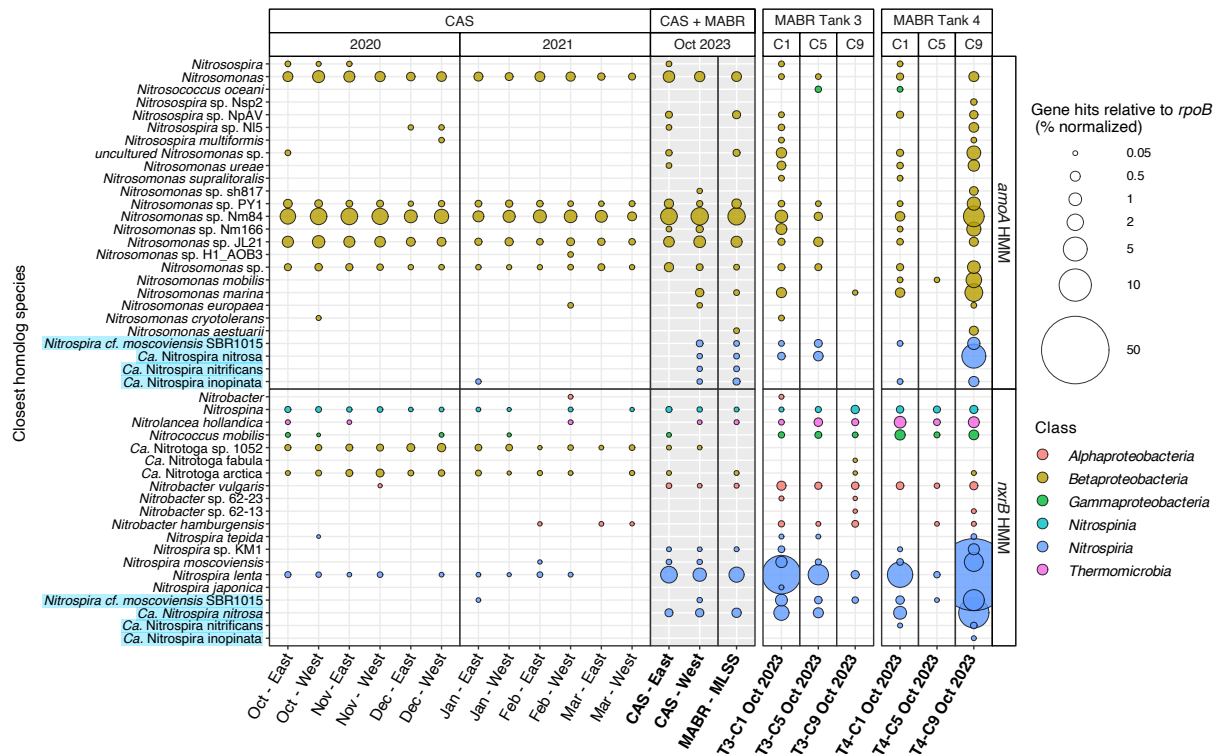


Figure 4.10 Metagenomic profiling using hidden Markov models (HMMs) corresponding to functional genes for ammonia oxidation (*amoA*) and nitrite oxidation (*nrxB*) to characterize nitrifying microorganisms in both the CAS aeration tanks and in the MABR biofilm. Size of the bubbles indicates the HMM gene hits relative to the taxonomic marker gene (*rpoB*), with fill colour indicating the taxonomic class of the detected sequences. The HMM hits were classified at the species level, using NCBI taxonomy. No percent cutoff was used for the results shown, however HMM hits were filtered to display only the closest homolog species detected corresponding to known nitrifying microorganisms, as the *amoA* and *nrxB* HMMs also detect sequences related to methane monooxygenase and nitrate reductase genes, respectively. Bolded sample names on the x-axis indicate samples collected after the plant upgrade. Each sample represents the average HMM hits of the forward and reverse sequence reads.

Because comammox *Nitrospira* do not form a monophyletic group within clade II *Nitrospira*, they cannot be distinguished from canonical NOB using 16S rRNA gene sequences (Pjevac *et al.*, 2017). However, functional profiling can distinguish between canonical NOB and comammox *Nitrospira* based on the presence of either *nrxB* alone or both *amoA* and *nrxB* hits, respectively, to determine which of the *Nitrospira* spp. were representative of either comammox or canonical NOB *Nitrospira*. The HMM profiling with nitrification-specific genes also displays the same pattern of nitrifier distribution between

tanks 3 and 4 that was noted with 16S rRNA gene sequencing results. The highest proportions of nitrifiers associated with both ammonia monooxygenase (*amoA*) and nitrite oxidoreductase (*nxrB*) genes exist in cassette 9 of tank 4, with several species of AOB, NOB, and comammox *Nitrospira* all detected together within the MABR cord biofilm (Figure 4.10). The co-occurrence of these nitrifiers in the MABR biofilm has similarly been observed in studies characterizing the microbial communities in lab-scale reactors (Li and Liu, 2019; Tian *et al.*, 2021), and has been noted across biofilms from different aquatic and wastewater environments (Al-Ajeel *et al.*, 2022). The increased abundance of *Nitrospira* in the MABR biofilm compared to the surrounding mixed liquor was also observed in the same MABR lab study, where *Nitrospira* was highly enriched in the biofilm vs. the activated sludge inoculum (Zhao *et al.*, 2023).

Based on the observed preference of comammox *Nitrospira* for fixed biofilms (Zhao *et al.*, 2022), one would expect to find them in the MABR biofilm, as discussed previously regarding *r*- and *K*-strategists. The occurrence of multiple strains of comammox *Nitrospira* is also characteristic and observed in many other biofilm systems used for water treatment (Spasov *et al.*, 2020). Three of the comammox *Nitrospira* spp. present in the MABR biofilm, namely *N. inopinata*, *N. nitrosa*, and *N. nitrificans*, have been isolated in co-culture during their discovery in 2015 (Daims *et al.*, 2015; van Kessel *et al.*, 2015). The other detected *Nitrospira* cf. *moscoviensis* SBR1015 is associated with two metagenomes from drinking water distribution systems (Wang *et al.*, 2017), and has been identified in aquarium biofilters (McKnight and Neufeld, 2024), however, further characterization of this strain in enrichment cultures is needed.

The sampled MABR supports a wide range of nitrifying microorganisms, with the biofilm providing a range of niches across the counter diffusion gradient of nutrients and oxygen. The presence of this diverse nitrifier community is not only beneficial for the functional redundancy it provides to nitrification processes, but because of the range of nitrogenous substrates that could potentially be used. Particularly, comammox *Nitrospira* can use organic nitrogen substrates like urea, cyanate, and guanidine has been demonstrated as an energy and nitrogen source for *Nitrospira inopinata* (Koch *et al.*, 2019;

Palatinszky *et al.*, 2024). The process upgrade has enriched the previously scarce comammox *Nitrospira*, increasing the range of nitrogenous substrates that could potentially be degraded by the resident nitrifiers. Additionally, comammox *Nitrospira*, along with AOB, are capable of micropollutant biotransformation and degradation, giving them additional important roles in the wastewater treatment systems (B. Wang *et al.*, 2021; Zhou *et al.*, 2021). Studies have shown that comammox *Nitrospira* and AOA have better removal rates for some antibiotic contaminants when compared to AOB species (Zhou *et al.*, 2021).

No AOA were detected within the MABR biofilm, and nor were they detected in the aeration tanks. However, AOA are known inhabitants of many wastewater treatment systems (Al-Ajeel *et al.*, 2022). Most studies of MABR microbial communities have focused on identifying the abundances of AOB and NOB in MABR biofilms. One study, however, detected the AOA *Ca. Nitrososphaera* in MABR biofilm, although the AOA showed low tolerance for elevated ammonia concentrations compared with AOB (Tian *et al.*, 2021). Additionally, mathematical modelling demonstrates that AOA in membrane aerated biofilms, alongside anammox, may be more effective for total nitrogen removal compared with AOB (Liu *et al.*, 2016). As MABR systems become more commonplace in municipal wastewater treatment systems, a future evaluation of nitrifier distribution would help clarify the microbial niche across all nitrifiers within MABR systems.

In contrast to the highly similar overall microbial community profiles of tanks 3 and 4 seen in the 16S rRNA gene data (Figure 4.7), there were clear differences when looking exclusively at the distribution of nitrifiers between the same cassettes (i.e., cassettes 5 and 9) in what could be considered “biological replicate” tanks (Figure 4.9). The lack of ammonia oxidizers detected with both metagenomic and 16S rRNA gene sequencing in cassette 9 of tank 3 contrasts with the high abundance of both AOB and comammox *Nitrospira*, alongside NOB found in cassette 9 of tank 4 (Figure 4.9, Figure 4.10). In the cassette 5 samples, tank 3 had a higher abundance of nitrifiers compared with that of tank 4, where they had a very low relative abundance. The lack of consistency between tanks profiles is most apparent for nitrifiers, whereas denitrification gene profiles in the biofilm showing high consistency between tank 3

and 4 samples across all three cassettes (Figure 4.11). Although, there are some clear inconsistencies in the distribution of non-nitrifying microbial profiles across cassettes, including *Hydrogenophilaceae* and sulfate reducing *Desulfobacter*, which are present at high relative abundances in cassette 1 of tanks 3 and 4 and not detected in cassette 9 biofilm (Figure 4.7). Currently, it is unclear what factors contribute to these uneven distributions. With nitrifiers needed to produce nitrates for denitrification, it is worth testing whether the inconsistent distribution of nitrifiers in cassettes across the tank has any localized effects on nitrification and denitrification rates that may not be observable when monitoring overall nitrogen removal from the system. Seeing as this is the first study to assess the microbial community composition of MABR biofilms in a system at this scale, comparable literature is not available to determine if this pattern of nitrifiers has been previously observed elsewhere. A follow up characterization of the nitrifier distribution in this MABR system after additional periods of operation would be beneficial to determine whether the nitrifier distribution across cassettes homogenized or continued to differ between tanks.

4.3.6 Metagenomic profiling of denitrifiers in the MABR

With denitrification activity and nitrogen removal observed in the MABR during phase 4, the microbial community was characterized using metagenomic profiling with HMMs for associated denitrification genes, including periplasmic (*napA*) and membrane-bound (*narG*) nitrate reductase, copper-containing (*nirK*) and heme-containing cd_1 (*nirS*) nitrite reductases, nitric oxide reductase (*norB*), and nitrous oxide reductase (*nosZ*). The HMM hits revealed a large diversity of taxa, with the top 15 genera in each sample for individual HMMs belonging to *Pseudomonadota* (*Proteobacteria*) (Figure 4.11). Most of the HMM hits across denitrification genes showed consistent results between tanks and also across cassettes, suggesting that denitrifiers were ubiquitous within the MABR system regardless of location. However, there were several genera with higher gene abundances exclusively detected in cassettes 1 (e.g., *Sulfuritortus*). Overall, more consistency was observed in denitrification gene profiles

between tanks than for nitrification gene profiles, indicating a variation in nitrifier distribution across the MABR system that was not seen for denitrifiers.

Several dominant denitrifying taxa detected amongst the HMM hits were also present in the 16S rRNA gene profiles at >2.5% relative abundance in the MABR biofilm, including *Zoogloea*, *Simplicispira*, *Rhodoferax*, *Denitratisoma*, and *Ca. Accumulibacter* (Figure 4.11, Figure 4.7). Of these genera, *Zoogloea*, *Simplicispira*, and *Rhodoferax* were also detected in the aeration tanks before and after the MABR upgrade, with *Denitratisoma* and *Ca. Accumulibacter* only among the genera >2.5% in the aeration tanks after the MABR upgrade (Figure 4.6). Many of the denitrifying genera that were detected with HMM hits were not present at >2.5% RA in the 16S rRNA gene profiles, including *Hyphomicrobium*, *Bradyrhizobium*, *Inhella*, *Dechloromonas*, *Acidovorax*, *Thiothrix*, and *Ca. Competibacter* (Figure 4.11). All of these genera mentioned above had HMM hits corresponding to a complete denitrification pathway from nitrate to dinitrogen gas, however, not all gene hits were present in the top 15 genera shown and were instead present but below the cut-off.

Even though many complete denitrifiers were identified with metagenomic profiling, several of the top 15 genera did not represent either denitrifiers, or bacteria with complete denitrification pathways. Several genera, including *Nitrospira*, *Nitrobacter*, and *Nitrosomonas* had sequence hit to the *nirK* HMM (Figure 4.11). The presence of *nirK* genes is ubiquitous across these nitrifiers, with *Nitrosomonas* capable of growing in anoxic conditions reducing nitrite to dinitrogen gas (Bock *et al.*, 1995; Schmidt *et al.*, 2004). *Nitrobacter* can also perform anaerobic respiration with nitrate and nitrite, which can produce nitrous oxide (Bock *et al.*, 1988). For comammox *Nitrospira*, the expression of *nirK* has been observed when grown under oxic conditions, resulting in the production of nitric oxide; however, the full function of *nirK* and nitric oxide production in a metabolic context is not understood (Kits *et al.*, 2019). Additionally, the genus *Sulfuritalea* had a high proportion of HMM hits to *napA* and *nirS*, but did not possess the full denitrification pathway based on HMM profiling (Figure 4.11). A freshwater lake isolate from the same genus, *S. hydrogeniovorans*, was capable of growing under denitrification conditions on H₂

and sulfur compounds, converting nitrate to nitrogen gas (Kojima and Fukui, 2011); however, full denitrification capabilities may vary among species.

A range of different microorganisms capable of complete denitrification were detected across the MABR biofilm samples. These denitrifiers were also diverse in their ability to facilitate carbon and phosphorus removal. Both *Ca. Accumulibacter* and *Dechloromonas* are known as phosphate-accumulating organisms (PAOs) or denitrifying PAOs (DPAOs), with the former often identified as the dominant PAOs found in activated sludge communities (Flowers *et al.*, 2009). *Ca. Competibacter* is a known glycogen-accumulating organism (GAO) and has been shown in co-culture experiments to cooperate with *Ca. Accumulibacter* for enhanced denitrification and P removal (Rubio-Rincón *et al.*, 2017). Other denitrifiers in the biofilm could use a range of carbon and other substrates for energy, in conjunction with denitrification. *Rhodoferrax* is known to oxidize a variety of carbon substrates, along with possessing sulfur oxidation pathways, and has been previously identified as a denitrifier in activated sludge systems (McIlroy *et al.*, 2016). Several *Denitratisoma* spp. use 17- β -oestradiol (E2), which is a common contaminant excreted by both humans and livestock, as both a carbon and energy source (Fahrbach *et al.*, 2006). Additionally, *Acidovorax* can denitrify using acetate and also hydrogen (Ginige *et al.*, 2005), with *Hyphomicrobium* known to conduct methylotrophic denitrification (Baytshtok *et al.*, 2009), and *Inhella* a known facultative anaerobe that accumulates polyhydroxybutyrate (PHB) (Chen *et al.*, 2012). *Zoogloea* species are well-known denitrifiers, also known for a role in biofilm and biofloc formation in WWTP systems (Wei *et al.*, 2023).

Overall, the MABR system supports complete denitrifiers with metabolic diversity in carbon substrate use and P removal capabilities. The presence of these denitrifiers showed a continuous distribution across the sampled MABR cassettes, with highly similar HMM hits from both tanks, suggesting that within this large-scale system, these bacteria were distributed ubiquitously. This contrasts with the inconsistent distribution of nitrifiers observed from both 16S rRNA gene and metagenomic analyses. The goal of this full-scale MABR system to achieve simultaneous nitrification and

denitrification in this process is supported by the genetic inventory of the microbial community. The process of anammox within MABR systems has also been suggested as part of the denitrification inventory of the biofilm, and bench scale studies have demonstrated partial nitrification coupled with anammox in MABR systems (Li, Li, Sun, *et al.*, 2022; Li, Li, Wang, *et al.*, 2022). In the MABR system explored here, there were four anammox-associated ASVs in the 16S rRNA gene data from biofilm, however, each ASV had less than 10 reads across all samples. This suggests that current operational conditions within this hybrid MABR-CAS system were not optimal to support anammox activity.

4.4 Conclusions

This study evaluated the impact of a full-scale MABR upgrade to a currently existing CAS system in a municipal wastewater treatment plant, to determine how the microbial community of the CAS system changed with an MABR installed upstream. Initial evaluation of the CAS system before the upgrade showed that the microbial community composition was strongly correlated with temperature and contained two dominant nitrifying microorganisms, whose abundances were also influenced by seasonal temperatures. The upgrade to the hybrid MABR + CAS configuration showed increased carbon and nitrogen removal performance, reduced energy consumption, and sustained winter nitrification (Lakshminarasimman and Parker, 2025). Microbial community analysis demonstrated that nitrifiers were seeded from the MABR biofilm into the CAS aeration tanks, which was predicted to enhance winter nitrification, and resulted in increased diversity in nitrifiers in the activated sludge. The biofilm configuration of the MABR increased nitrifier diversity, including comammox *Nitrospira*, along with several *Nitrosomonas* and NOB species, probably increasing the range of nitrogen substrates that can be used by nitrifiers. Although, the distribution of nitrifying microorganisms was not ubiquitous based on the samples collected from cassettes 1, 5, and 9 in both MABR tanks, unlike denitrifiers that were more evenly distributed across the MABR system. Overall, this large-scale MABR-CAS system contains a large inventory of microorganisms capable of carbon, nitrogen, and phosphorus removal. This first microbial community characterization of a system at this scale, supports the process data results that a

hybrid MABR-CAS process at this scale is effective for municipal wastewater treatment. However, additional studies would be valuable to further assess nitrifier distribution in large scale MABRs and determine if further changes can help optimize plant performance.

Chapter 5

Conclusions and future directions

5.1 Nitrification – over a century of understanding

It has been over a century since Sergei Winogradsky first isolated pure cultures of nitrifying microorganisms and identified the existence of chemolithotrophic metabolism, bringing forth the idea of biogeochemical cycling on Earth and the nitrogen cycle (Dworkin and Gutnick, 2012). His discoveries, including first isolation of free-living nitrogen fixing bacteria, paved the way for the field of microbial ecology that exists today. The concept of two-step nitrification performed by aerobic ammonia and nitrite-oxidizing bacteria has been the basis for research over the past century, with much of the literature providing a good understanding of the nitrifiers that have been obtained in pure cultures, including the AOB *Nitrosomonas europaea*, which has been used as a model for ammonia oxidation in many nitrification studies (Radniecki and Lauchnor, 2011).

The scientific understanding of the microorganisms involved specifically in the first step of autotrophic ammonia oxidation remained unchanged until newer DNA sequencing technologies began to emerge. Following the completion of the Human Genome Project, which yielded a nearly complete sequence of the human genome (Venter *et al.*, 2001), the notion of applying the same “shotgun” sequencing technology to environmental samples seemed like a feasible method to perhaps obtain a better understanding of the metabolic potential of microorganisms that were unculturable in the lab. The application of whole-genome shotgun sequencing to DNA samples collected from the Sargasso Sea greatly expanded the knowledge of the ocean’s microbial diversity, with the identification of over 1.2 million novel gene sequences (Venter *et al.*, 2004). Among these novel sequences were the first genetic indications of the existence of ammonia-oxidizing archaea, which were obtained in pure culture the following year from a marine gravel sample (Könneke *et al.*, 2005). The discovery of AOA was the first change in understanding of ammonia oxidation since Winogradsky’s work over a century earlier.

It was only a year later that a publication came out questioning the metabolic division of labour for nitrification (Costa *et al.*, 2006). Although a shortening of long metabolic pathways like complete nitrification can theoretically lead to increased growth rates, it reduced growth yield, as the shortened pathway produces less ATP than the complete pathway. However, the high yield from complete nitrification would be advantageous, albeit only in more oligotrophic environments such as biofilms or microbial aggregates where mixing of biomass and substrates is minimal (Costa *et al.*, 2006). In contrast, environments with high nutrient availability favour microorganisms with faster growth rates that can outcompete slower growing microorganisms (Costa *et al.*, 2006). Almost 10 years after the prediction of comammox, the simultaneous discovery of comammox bacteria by two different groups was perhaps expected, enriched from environments that were predicted to favour comammox metabolism including a deep oil exploration well and recirculating aquaculture system (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Because comammox metabolism happened to exist within a genus known exclusively to contain canonical NOB, *Nitrospira*, it is likely that researchers have previously “detected” comammox *Nitrospira* in the environment without realizing it was distinct from NOB *Nitrospira*, due to the lack of genetic information available on its metabolic capabilities. Of course, with the continued advances and ability to obtain metagenome assembled genomes, detecting comammox *Nitrospira* based on the co-occurrence of ammonia and nitrite oxidation genes has revealed its ubiquity across a range of environments, as discussed in Chapter 1.

A few years prior to the discovery of comammox *Nitrospira* in 2015, experiments from MSc student Natasha Szabolcs in the Neufeld lab at the University of Waterloo, which explored the establishment of both ammonia-oxidizing archaea and bacteria in freshwater biofilters, yielded unexpected results. Despite apparent nitrification activity occurring within some of the experimental aquariums, there were time points during the experiment where neither AOA nor AOB were detected within the biofilters (Szabolcs, 2014). Perhaps the primers used were unable to detect the AOA or AOB present in the biofilters, that the ammonia oxidizers were present but below detectable levels, or that they

existed elsewhere in the aquarium. All these possibilities could not be ruled out at the time, but in hindsight it appears likely that unknown ammonia oxidizers were present in the biofilters.

Shortly after the discovery of comammox *Nitrospira*, unpublished metagenomic results from an office aquarium in the Neufeld Lab, whose biofilter was sampled for the Sauder *et al.* (2011) aquarium biofilter study, revealed a MAG associated with *Nitrospira* and contained both genes for ammonia and nitrite oxidation. Considering the missing nitrifiers from the biofilter establishment experiments and apparent comammox *Nitrospira* MAG present in the office aquarium biofilter, it seemed a logical next step to go back and re-survey the ammonia oxidizers within home aquarium biofilters, with the knowledge of all three existing groups of aerobic ammonia oxidizers.

One of the previous aquarium biofilter establishment experiments that sampled biofilters from three new freshwater home aquariums had only been analyzed for the first 4 weeks of the 12-week experiment (Szabolcs, 2014), providing additional samples to explore the establishment of nitrifying microbial communities in freshwater aquarium biofilters. The original goal of sampling three new home aquarium biofilters during establishment was to determine whether AOA could colonize the biofilters in a conventional home setting, as there were surprisingly low levels of AOA detected in aquarium biofilter establishment experiments that were run in a laboratory setting (Szabolcs, 2014). Following results presented in Chapter 2 revealing the presence of comammox *Nitrospira* in freshwater biofilters, this led to the re-analysis of these experimental home aquarium samples as presented in Chapters 3. These findings now provide additional context to previous understanding of aquarium nitrification. Prior to the work in Chapters 2 and 3, the distribution and role of comammox *Nitrospira* in home aquarium biofilters was unknown.

As with other engineered water treatment systems, understanding of nitrification in municipal water treatment systems mainly relates to two-step nitrification in the context of autotrophic nitrification. Alongside the discovery of both AOA and comammox *Nitrospira*, recent research has revealed that all three of these groups of ammonia oxidizers can exist in WWTP alongside NOB (Chapter 1). Since

comammox *Nitrospira* can instead operate nitrification as a single step, and even potentially contribute to partial nitrification, this is something that would need to be considered in future modelling of nitrification processes in WWTP systems. Many of the newer technologies for biological nitrogen and nutrient removal from wastewater are biofilm-based, including MABRs (Anjali and Sabumon, 2024), which is an ideal niche for the growth of comammox *Nitrospira*. It will be important to evaluate and consider the potential presence of all known nitrifiers in these systems to obtain a better understanding of their functionality and improve performance.

5.2 Summary of research findings

The research presented in this thesis further highlights the ubiquity of previously overlooked comammox *Nitrospira* in biofilter environments. In Chapter 2 of my thesis, I presented results from a survey of home aquarium biofilters, which revealed that both AOA and comammox *Nitrospira* are among the dominant ammonia oxidizers in freshwater biofilter microbial communities, which further overturns the previously held understanding that AOB and NOB are solely responsible for nitrification in these systems. My results also supported previous findings that comammox *Nitrospira* are not found in marine environments, and that saltwater aquarium biofilters were dominated by either AOA or AOB and marine NOB, with overall microbial communities that were distinct from those of freshwater biofilters. The lack of comammox *Nitrospira* in saltwater aquarium biofilters as seen in Chapter 2 reflects the current literature findings that have not found comammox *Nitrospira* in marine or high salinity environments, although they have been found in brackish aquatic environments (Hüpeden *et al.*, 2020; Liu *et al.*, 2020).

The results presented in Chapter 2 also highlight the diversity of comammox *Nitrospira*, specifically belonging to clade A *Nitrospira*, which are present in the freshwater biofilters. Many of the comammox *Nitrospira amoA* gene sequences did not match those currently present in NCBI databases, suggesting there still exists unexplored diversity among comammox *Nitrospira*. Although it was unclear from the results present why AOA might be dominant in some biofilters, while comammox *Nitrospira*

dominate others, the network analysis presented in Figure 2.7 reinforced previous observations that important relationships exist among nitrifiers and other community members. The dynamics between co-existing AOA and comammox *Nitrospira* are not fully understood, but co-cultivation experiments with different ammonia oxidizers are helping to give insight into these relationships. Using enrichments of comammox *Nitrospira* from freshwater lake sediment grown in batch cultivation experiments with AOA belonging to the genus *Nitrosoarchaeum* revealed that comammox *Nitrospira* would always outcompete the AOA, under all tested ammonia concentration conditions (Ghimire-Kafle *et al.*, 2024). It is unsurprising that comammox *Nitrospira* were abundant within freshwater aquarium biofilters, given that conditions within a biofilter align well with their predicted and observed niche preferences for oligotrophic biofilm environments (Costa *et al.*, 2006; Koch *et al.*, 2019; Zhao *et al.*, 2022). Further research is needed to identify mechanistic underpinnings of these relationships within microbial communities and how they may benefit ammonia oxidizers.

Following the revised notion of dominant ammonia oxidizers in freshwater aquarium biofilters, the research presented in Chapter 3 explored the dynamics of microbial community establishment in new home aquarium biofilters and quantified the abundance of all three groups of ammonia oxidizers over the first 12-weeks. Previously the dynamics of nitrifier establishment in home aquarium biofilters has not been assessed. As observed in Chapter 2, comammox *Nitrospira* were present in all three freshwater aquarium biofilters that were included in the study. There were differences in ammonia oxidizer abundance between the three home aquariums, with a delay in the establishment of a nitrifying community observed in Aquarium 3, in contrast to the nitrification activity that reduced the ammonia concentrations below detection limits in Aquariums 1 and 2. Although the reason for the delay of nitrification activity in Aquarium 3 was unclear, the lack of live plants used to decorate Aquarium 3, compared with the live plants present in the other two aquariums, might explain the difference if the plants were acting as a source of inoculant for the biofilter microbial communities. Previous studies have reported comammox *Nitrospira* and AOA in aquaponics systems (Heise *et al.*, 2021) and who have been

specifically detected in the plant-containing hydroponic compartments of these systems (Schmautz *et al.*, 2022), making it plausible that aquatic plants could be carriers for nitrifying microorganisms when added into aquarium systems. Plants as beneficial inoculants is a hypothesis that could be explored with future aquarium experiments. The delayed nitrification activity in Aquarium 3 may have resulted from the presence of compounds inhibitory to nitrification. A variety of compounds, including organic compounds and metals are known to inhibit nitrification (McCarty, 1999). Given that plastic decorations, including artificial plants, were added exclusively to Aquarium 3, which was also inoculated with nitrifying supplements upon establishment, leaves this as a possibility but requires further investigation.

There was a preference for different biofilter carrier materials by AOA, which had a higher abundance on the biofilter beads than on the sponge. The sampling and characterization of two different biofilm carriers was only done for the work presented in Chapter 3, where the experimental set-up controlled for the biofilters used in the experiment. In contrast, to maintain some consistency when sampling across a diversity of different aquarium biofilters in Chapter 2, participants were asked to only bring in samples from either sponge or filter floss material. This also followed the same sampling methods as used in the previous 2011 aquarium biofilter study (Sauder *et al.*, 2011). Since the results from Chapter 2 seem to suggest a niche preference of the AOA for the biofilter beads over sponge, it is worth further assessing the impacts of different biofilm carriers on nitrifying microbial community composition in future studies. Despite variability in microbial community establishment between the three aquariums sampled in Chapter 3, the final dominance of comammox *Nitrospira*, alongside the presence of AOA and AOB in lower abundance, further supports the conclusions from Chapter 2 that comammox *Nitrospira* are dominant ammonia oxidizers in freshwater aquarium biofilters.

On a larger scale of engineered water treatment systems, in Chapter 4, I characterized the microbial communities of a hybrid MABR-CAS system in a municipal WWTP and assessed the impact of an MABR upgrade to the nitrifying microbial communities present in the CAS system. As discussed in Chapter 4, MABR technology seeks to improve upon the issues with conventionally used systems, like

activated sludge, by reducing the energy required for aeration, maintaining nitrification activity even at colder temperatures, and providing conditions desirable for both nitrification and denitrification activity with the use of a counter gradient across the supported biofilm. The data presented in Chapter 4 constitute the first characterization of an MABR system of this size implemented in a full scale municipal system, and complements the work presented by Lakshminarasimman and Parker (2025). The nitrifiers present in the CAS system prior to the upgrade, including *Nitrosomonas* AOB and *Nitrobacter* NOB, exhibited a seasonal abundance and activity pattern, with decreases during colder months. Following the upgrade to the hybrid MABR-CAS system, there was a clear seeding effect from the MABR biofilm into the CAS system, increasing the diversity of the activated sludge microbial community and the diversity of nitrifiers in the system. The MABR nitrifiers were represented by distinct communities compared to the AOB and NOB present in the CAS aeration tanks. Several AOB species previously unobserved in the activated sludge were present in the MABR biofilm, along with *Nitrospira* NOB and comammox *Nitrospira*. The distribution of nitrifiers within the two sampled MABR tanks was inconsistent, which was not observed for denitrifiers. A future assessment of nitrifier distribution within the MABR tanks could be beneficial to clarify if their distribution has any impact on MABR nitrification performance.

Metagenomic analysis provided insight into both the abundance of nitrifiers in the MABR biofilm, and also to the presence of denitrifiers in the system, which were diverse and abundant across the two sampled tanks. It was clear that the biofilm microbial communities were well equipped to perform both nitrification and denitrification in the system, and additional phosphate removal. Although, unlike reports from lab-scale MABR reactors run under low C/N ratio conditions that reported partial nitrification and anammox (Li, Li, Wang, *et al.*, 2022), anammox bacteria were barely detectable in the full-scale MABR, which is likely due to differences in C/N ratios. Overall, characterization of the microbial communities in the plant showed that the new MABR technology had a positive impact in increasing microbial diversity, along with an increased inventory of nitrifiers with a diverse metabolic capability. With comammox *Nitrospira* known to degrade additional nitrogen-containing compounds like

urea and guanidine, which are also found in wastewater, this expands the metabolic inventory of nitrifiers in the system. Along with the increased nitrogen removal performance noted in the plant by Lakshminarasimman and Parker (2025), the results I present here also support the positive effect of one of the first full-scale MABR upgrades in a municipal WWTP.

5.3 Future research directions

The results presented in my thesis have improved understanding of the composition of nitrifying microbial communities in engineered water treatment systems, including both home aquarium biofilters and hybrid MABR systems operating in municipal WWTPs. The first step in understanding these engineered microbial communities is determining composition and metabolic potential of community members, which can be done using amplicon and metagenomic sequencing approaches, respectively. However, it is also important to determine the activity of the different microorganisms. Considering the metabolic versatility of nitrifiers discussed in Chapter 1, and competition among ammonia oxidizers for the same energy source (i.e., ammonia), nitrifiers may exploit different substrates instead of, or in addition to, ammonia within an environment to reduce competitive pressures. Overall, characterization of the activity of nitrifiers within a biofilm will help to determine the roles they play within the environment.

The development of techniques used to study the function of microorganisms in the environment, even at the level of a single cell, have advanced over the years and microbial ecologists have a variety of tools available to help elucidate the physiology within microbial communities (Hatzenpichler *et al.*, 2020). The use of metatranscriptomic and transcript profiling of functional genes would be an effective method to gain insight into the activity of nitrifiers and has been applied in wastewater treatment systems (Yang *et al.*, 2020; Zheng *et al.*, 2021). Beyond assessing transcriptional activity, the use of stable-isotope probing (SIP) allows incorporation of heavy isotopes into the DNA of microorganisms that use the labelled substrates of interest (Radajewski *et al.*, 2000; Neufeld *et al.*, 2007). The use of both DNA-SIP and RNA-SIP have been used already to demonstrate *Nitrospira* activity in groundwater-fed biofilters and

show the active role of comammox *Nitrospira* in acidic soil environments (Gülay *et al.*, 2019; Liu *et al.*, 2023). Metabolomics and proteomics are also techniques that have been implemented in wastewater systems to characterize protein expression and metabolic profiles to identify the activities of microbial community members (Azizan *et al.*, 2020; Q. Liu *et al.*, 2024).

There are still only a few existing enrichments and only one pure culture of comammox *Nitrospira* currently available. Although cultivation efforts have clearly demonstrated the challenge of enriching and isolating these organisms, recent advances in cell sorting and cultivation strategies could help improve cultivation efforts (Hatzenpichler *et al.*, 2020). Increasing the number of comammox *Nitrospira* in enrichments or pure cultures is crucial to improve knowledge of these nitrifiers and their physiology, which will be important for improving wastewater treatment modelling. The recent discovery that comammox *Nitrospira* can grow exclusively on guanidine could potentially be used as a method of enrichment (Palatinszky *et al.*, 2024). Additionally, research has revealed that the use of signaling molecules had positive effects on the growth of comammox *Nitrospira*, which led to its enrichment from anoxic tank sludge (Tang *et al.*, 2024). For future enrichment efforts, the use of quorum sensing molecules in future enrichment attempts may also be beneficial.

5.3.1 Importance for the aquarium industry

Globally, aquariums are a multi-billion dollar industry, with millions of hobbyists world-wide. Products sold to aquarium hobbyists include various designs of biofilters for use with aquariums to help maintain water quality to provide a healthy ecosystem for their resident aquatic organisms. As discussed in Chapter 3, when setting up a new aquarium, it is important for aquarium owners to ensure that a nitrifying microbial community establishes within the biofilter so that ammonia waste from fish does not accumulate to harmful levels. Until now, the understanding of nitrification in the aquarium industry has been based around the two-step nitrification model, including only AOB and NOB. To help accelerate the establishment of nitrifying communities, companies have marketed supplement products containing live AOB and NOB to inoculate biofilters. Considering the results presented in Chapters 2 and 3, it appears

that tested supplements did not contain nitrifiers representative of those found within established aquarium biofilters, which provides an opportunity to develop representative aquarium supplements for both freshwater and marine aquariums. As part of the work presented in Chapter 2, enrichment cultures from freshwater aquarium biofilters containing comammox *Nitrospira* were obtained (Aq-C1 and Aq-C2). Considering these comammox *Nitrospira* originate from freshwater aquariums, the enrichments could be a valuable starting point for development of aquarium bio-supplements that better represent nitrifiers native to aquarium biofilters. As well, continued improvements in the understanding of nitrifier ecology in aquarium and aquaculture biofilms could lead to changes in biofilter design to potentially improve nitrogen removal from these systems, such as modifications to biofilm carrier material to improve nitrifier growth.

5.3.2 Importance for the engineering of wastewater treatment systems

With an evolving understanding of the microorganisms that can be used for nitrogen removal in wastewater treatment systems, there are several perspectives from which this knowledge could be applied. To help design and optimize wastewater treatment system efficacy, the use of modelling is often employed as a management tool (Metcalf and Eddy Inc. *et al.*, 2014). Modelling nitrification and nitrogen removal from wastewater systems is based on measured kinetics parameters for microorganisms relevant to the process being modelled. Models used over the last several decades have centered around two-step nitrification, incorporating AOB and NOB parameters into the equations (Makinia and Zaborowska, 2020). It is only recently that comammox *Nitrospira* have been considered when developing models for nitrification in suspended growth systems (Mehrani *et al.*, 2021). As current studies of microbial communities continue to reveal that comammox *Nitrospira* and AOA also play important roles within these systems, it will be important for future modelling efforts to ensure an incorporation of all nitrifying microorganisms.

From an environmental perspective, greenhouse gas emissions are a major concern for wastewater treatment operations. To reduce nitrous oxide greenhouse gas emissions from nitrifiers, efforts

to increase the abundance of AOA and comammox *Nitrospira* in nitrification systems could be beneficial as they lead to less nitrous oxide production compared to AOB, with both groups lacking the required genes for nitrifier-denitrification (Palomo *et al.*, 2022; Wright and Lehtovirta-Morley, 2023). Goals to improve wastewater management and sustainability have been recently summarized under the NICER paradigm (T. Liu *et al.*, 2024). The acronym outlines the goals including net-zero greenhouse gas emissions, intensifying treatment capacity, cost-effective management, emerging contaminant control, and resource recovery. This review highlights that for nitrification, the implementation of both AOA and comammox *Nitrospira* in wastewater treatment processes can play an important role in meeting NICER goals for wastewater management (T. Liu *et al.*, 2024).

5.4 Outlook

The exciting history of nitrification research serves as a reminder that the microbial world is complex and human understanding of it continues to evolve along with the scientific techniques used for microbiological analysis. Although the work presented in my thesis fills in a knowledge gap in understanding of nitrifiers in engineered water treatment systems, many questions remain unanswered that justify future research into aquarium and wastewater biofilter systems. Overall, the research presented in this thesis has revealed that comammox *Nitrospira* are often the dominant ammonia oxidizer within freshwater home aquarium biofilters both in mature aquarium biofilters and during freshwater biofilter microbial community establishment, which was previously unknown in these particular aquatic systems. My thesis research has also validated the functional microbial potential of novel MABR in the largest known municipal WWTP installation in North America at the time of this research, which confirms the presence of a diverse microbial inventory of nitrifiers and denitrifiers that can support the removal of nitrogen and phosphorus from the wastewater.

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