Enrichment and activity of comammox *Nitrospira* from the rotating biological contactors of a municipal wastewater treatment system

by

Sarah Salim Al-Ajeel

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Author’s declaration

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

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Statement of contributions

Chapter 2 Rachel Beaver prepared samples for high-throughput sequencing. Jackson Tsuji designed the primer set used to target the *cynS* gene of comammox *Nitrospira* MAG069.

Chapter 3 Michelle McKnight assisted with sampling from the RBCs. Taylor Virgin and Ana Fernandez helped with genomic DNA extraction from start- and end-point sampling.

Abstract

Nitrification, the biological conversion of ammonia to nitrate, via nitrite, is an important process in both natural and engineered systems. Nitrification within wastewater treatment plants (WWTPs) is necessary because elevated ammonia concentrations are toxic to fish and can lead to eutrophication of receiving waters. Although the end product of nitrification (*i.e.*, nitrate) also contributes to eutrophication, it is preferred over ammonia because nitrate has no direct oxygen demand and it is often converted to inert dinitrogen by denitrification. Nitrification is mediated by the combined activities of chemolithoautotrophic ammonia and nitrite oxidizers. The recent discovery of *Nitrospira* representatives capable of complete ammonia oxidation (comammox) to nitrate via nitrite has required that previously characterized systems be re-examined for potential contributions of comammox *Nitrospira* to nitrification. Although the abundance and diversity of comammox *Nitrospira* in the rotating biological contactors (RBCs) of a municipal WWTP in Guelph Ontario were recently examined using several cultivation-independent approaches, little is known about the activity of comammox *Nitrospira* in these biofilm-based systems. Additionally, lack of a suitable comammox *Nitrospira* enrichment culture impedes future efforts to explore the biochemical and physiological characteristics of these WWTP nitrifiers.

The aim of this thesis research was to enrich comammox *Nitrospira* from Guelph RBCs and assess their contributions to nitrification within the WWTP biofilm. The enrichment cultures were prepared using a combination of differential size filtration and antimicrobial supplementation. The growth and activity of resulting enrichment cultures were examined using ammonia depletion and nitrate production, as well as end-point PCR targeting the *amoA* genes coding for subunit A of the ammonia monooxygenase of comammox *Nitrospira*, ammonia-

oxidizing bacteria (AOB), and ammonia-oxidizing archaea (AOA). The *amoA*-based phylogeny reveals that the two comammox bacteria grown in enrichment cultures are phylogenetically distinct and represent novel ecotypes, both belonging to clade A sublineage II *Nitrospira*. Culture G4 is an enrichment that contains novel AOA and comammox *Nitrospira* species. In addition to possessing the genetic machinery for complete ammonia oxidation, the comammox *Nitrospira* enriched in G4 also possesses a gene that codes for a cyanase (*cynS* gene). The presence of a cyanase may allow these comammox bacteria to breakdown cyanate to ammonium in ammonia-limited environments. Preliminary results showed that G4 grew with ammonium (from cyanate breakdown), but it remains unclear whether these comammox *Nitrospira* were able to breakdown cyanate biotically using cyanase. The enrichment culture G8 contains a comammox *Nitrospira* representative that is phylogenetically distinct from known comammox *Nitrospira* representatives. Activity assays demonstrated that both enrichment cultures (G4 and G8) grew under ammonia-fed conditions, with complete conversion of ammonia to nitrate. Enrichment cultures will help to further explore physiological characteristics, such as ammonia and nitrite affinities, as well as alternative metabolisms of comammox *Nitrospira* in the RBCs.

In addition to cultivation efforts, my research explored the potential contributions of nitrifiers to RBC biofilm nitrification. Microcosm incubations were established to test the effects of five nitrification inhibitors (*i.e.*, c-PTIO, ATU, DCD, chlorite, and chlorate) on biofilm samples. Additionally, the effects of c-PTIO, ATU, DCD, and simvastatin were investigated on comammox *Nitrospira* enrichment cultures. Results from enrichment culture incubations indicated that comammox *Nitrospira* are inhibited by 10 µM ATU and 10 mM DCD and are insensitive to 100 µM c-PTIO and 8 µM simvastatin. In RBC biofilm suspensions, preliminary evidence suggests that comammox *Nitrospira* were inhibited by ATU, DCD, chlorite, and

possibly chlorate, suggesting that they are likely active members of the nitrifying community in the RBCs. The results of this thesis underline the complexity of the nitrifying community in the RBC biofilm and highlight the need for including comammox *Nitrospira* when evaluating contributions of different nitrifiers to ammonia oxidation. Multiple "omic" approaches, in conjunction with activity assays, will likely be valuable for examining the metabolic versatility and activity of comammox *Nitrospira*, as well as other nitrifiers, in the RBCs. Finally, work presented in this thesis opens new research avenues to explore with enrichment cultures to better characterize the potential metabolic roles for comammox *Nitrospira* in the RBCs, as well as other engineered and natural aquatic environments.

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AMO	Ammonia monooxygenase
amoA	Subunit A of the ammonia monooxygenase gene
amoB	Subunit B of the ammonia monooxygenase gene
amoC	Subunit C of the ammonia monooxygenase gene
Anammox	Anaerobic ammonium oxidation
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
AOM	Ammonia-oxidizing microorganism
ASV	Amplicon sequence variant
ATU	Allylthiourea
AXIOME3	Automation, eXtension, and Integration Of Microbial Ecology 3
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
Ca.	Candidatus
CARD-MAR-FISH	Catalyzed reporter deposition (CARD) microautoradiography-fluorescence *in situ* hybridization (MAR-FISH)
CH_2O_2	Formate
CNO^-	Cyanate ion
Comammox	Complete ammonia oxidation
cynS	Cyanate hydratase gene
DCD	Dicyandiamide
DMPP	3,4-Dimethylpyrazole phosphate
DMSO	Dimethyl sulfoxide
dsDNA	Double-stranded DNA
DNA-SIP	DNA stable-isotope probing
DNRA	Dissimilatory nitrate reduction to ammonium
dNTP	Deoxynucleotide triphosphate
H_2	Hydrogen gas

HAO	Hydroxylamine dehydrogenase
HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
K_m(app)	Apparent half saturation constant
MAG	Metagenome-assembled genome
N_2	Dinitrogen
ND	Not determined
NH_3	Ammonia
NH_4^+	Ammonium ion
NH_4Cl	Ammonium chloride
NO	Nitric oxide
NO_2^-	Nitrite ion
NO_3^-	Nitrate ion
NOB	Nitrite-oxidizing bacteria
NW	Northwest
NXR	Nitrite oxidoreductase
PCR	Polymerase chain reaction
PTIO	2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
QIIME2	Quantitative Insights Into Microbial Ecology 2
qPCR	Quantitative PCR
RBC	Rotating biological contactors
rRNA	Ribosomal RNA
RT	Room temperature
RT-qPCR	Real time quantitative PCR
SCM	Synthetic crenarchaeota medium
sp.	Species
U	Units
VCl_3	Vanadium chloride
WWTP	Wastewater treatment plant

“Curiosity always involves risk. You can’t satisfy your curiosity without accepting some risk… curiosity didn’t kill the cat”

— Haruki Murakami (from Killing Commendatore)

Chapter 1 Introduction and literature review

1.1 Nitrification: Process and key "players"

Nitrification is the biologically mediated conversion of ammonia to nitrate via nitrite. Historically, nitrification was considered a two-step process mediated by two phylogenetically distinct guilds (Figure 1.1), the ammonia- and nitrite-oxidizing bacteria (AOB and NOB, respectively). Ammonia oxidation is generally regarded as the rate-limiting step of nitrification. Aerobic ammonia oxidation was traditionally assumed to be solely performed by AOB belonging

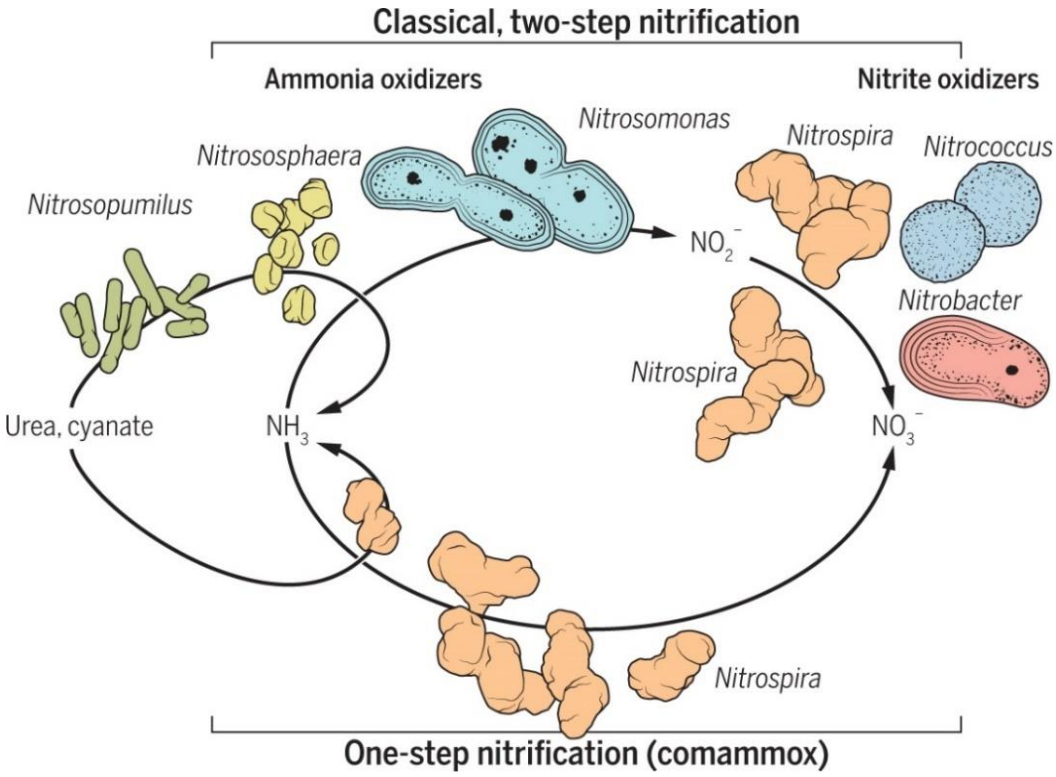

Figure 1.1. The process of nitrification and key "players" (Reproduced from Santoro, 2016). Nitrification is classically considered a two-step process, mediated by two phylogenetically distinct guilds (*i.e.*, ammonia oxidizers and nitrite oxidizers). Comammox refers to the process of complete ammonia oxidation to nitrate by a single microorganism.

to the phylum *Proteobacteria*. However, this understanding was challenged in 2005 with the discovery and cultivation of the ammonia-oxidizing archaeon (AOA) *Nitrosopumilus maritimus*, belonging to the phylum *Thermoproteota* (formerly phylum *Thaumarchaeaota;* Venter *et al.*, 2004; Treusch *et al.*, 2005; Könneke *et al.*, 2005). More recently, two research groups reported the discovery and cultivation of bacteria from the genus *Nitrospira* that are capable of converting ammonium to nitrate aerobically, which is a process called complete ammonia oxidation (comammox; Daims *et al.*, 2015; van Kessel *et al.*, 2015). Another process, where ammonium and nitrite are converted to dinitrogen gas (N_2) can be carried out under anoxic conditions by anaerobic ammonia-oxidizing bacteria (anammox) from multiple genera within the *Planctomycetes* phylum (reviews by Kartal *et al.*, 2013; Stein and Klotz, 2016).

The second step of nitrification, where nitrite is converted to nitrate (Figure 1.1), is carried out by NOB that belong to several phyla, including *Proteobacteria*, *Chloroflexi*, *Nitrospinae*, and *Nitrospirae* (Daims *et al.*, 2001, 2016). Nitrite-oxidizing bacteria transform nitrite to nitrate, which can then be used as a nitrogen source by many microorganisms and eukaryotes (*e.g.*, plants). Therefore, NOB play an important role in regulating nitrification, and the nitrogen cycle by extension (Daims *et al.*, 2016).

Collectively, nitrifiers release electrons and gain energy by the oxidation of inorganic nitrogen compounds (*i.e.*, ammonia or ammonium) to nitrate. Total ammonium (expressed as $NH_3 + NH_4^+$) refers to the two forms of ammonia present in the system: the protonated ammonium ion (NH_4^+) and unprotonated ammonia (NH_3). The two forms are concomitantly present and their relative abundance is pH-dependent (pKa = 9.3). Thus, the availability of ammonia as an energy substrate for AOB, and potentially comammox bacteria and AOA, is largely pH-dependent (Suzuki *et al.*, 1974; Yin *et al.*, 2018; Kits *et al.*, 2019).

1.2 Nitrification in wastewater treatment plants

Untreated municipal wastewater contains relatively high concentrations of nitrogenous compounds, such as ammonia and urea. As a result, wastewater treatment plants (WWTPs) are engineered to promote conversion of nitrogenous compounds, such as ammonia and occasionally nitrate to a less bioavailable form (*i.e.* N_2 gas), before discharge of treated effluent into receiving waters. Consequences of increased nitrogen availability in aquatic ecosystems include eutrophication and associated hypoxia, which increases harmful algal blooms and leads to fish kills. Biological removal of total ammonium in wastewater treatment facilities relies on nitrifying guilds to convert ammonia to nitrate (Figure 1.1). Nitrate is then converted to N_2 gas by denitrification, which is a process that involves sequential reduction of NO_3^- to N_2, or by anammox bacteria, under hypoxic or anoxic conditions. Because nitrous oxide (N_2O; a potent greenhouse gas) is emitted by nitrifiers during the process of ammonia oxidation, nitrification can have both positive and negative impacts on the environment (Qiao *et al.,* 2015).

Because of the importance of nitrifying guilds to wastewater treatment, several studies have examined the phylogeny, abundance, diversity, and potential roles of AOA and AOB in engineered water treatment systems. In contrast, relatively few studies have examined the abundance, biogeography, and activity of comammox bacteria in water treatment systems. Initial studies demonstrated the presence of comammox *Nitrospira* in wastewater treatment systems, often in high abundance and sometimes outnumbering other nitrifier groups (Pjevac *et al.,* 2017; Annavajhala *et al.,* 2018; Xia *et al.,* 2018; Spasov *et al.,* 2020). Given their potential relevance to engineered water treatment systems (*i.e.*, wastewater treatment plants, aquarium biofilters, drinking water systems), comammox bacteria and their potential interactions with other nitrifiers

have been recognized as an important focus for current nitrogen cycle research (Lawson and Lücker, 2018; Mehrani *et al.*, 2020).

1.3 The genus *Nitrospira*

The genus *Nitrospira* forms a monophyletic clade within the *Nitrospirae*, which is a phylum that includes two sister genera, *Nitrospira* and *Leptospirillum*, and the distantly related genus *Thermodesulfovibrio*. Watson *et al.* (1986) isolated and characterized the first *Nitrospira* representative in culture, which was derived from a water sample collected from the Gulf of Maine. Despite their ubiquity, most *Nitrospira* lineages remain recalcitrant to cultivation by traditional methods. Because of this, many members of the genus *Nitrospira* are primarily studied using cultivation-independent methods such as "functional gene" PCR, amplicon sequencing, and metagenomics (Gonzalez-Martinez *et al.*, 2016; Pjevac *et al.*, 2017; Beach and Noguera, 2019; Fujitani *et al.*, 2020; Poghosyan *et al.*, 2020; Spasov *et al.*, 2020; Yang *et al.*, 2020). Metagenomics-based studies have reported the presence of comammox *Nitrospira* in natural and engineered environments, including wastewater and drinking water treatment systems, freshwater systems, aquaculture, lake sediments, and soils (Palomo *et al.*, 2016; Camejo *et al.*, 2017; Pjevac *et al.*, 2017; Wang *et al.*, 2017; Roots *et al.*, 2018; Xia *et al.*, 2018; Poghosyan *et al.*, 2020; Spasov *et al.*, 2020). *Nitrospira* representatives typically associate with other ammonia and nitrite oxidizers in these environments, suggesting that specific physiological adaptations exist for each of the nitrifying guilds (Bartelme *et al.*, 2017; Pjevac *et al.*, 2017; Fowler *et al.*, 2018; Spasov *et al.*, 2020).

The *Nitrospira* genus is divided into six monophyletic sublineages, with clear demarcations at the 16S rRNA gene level (*i.e.*, sequence identities <90%; Figure 1.2). Several members of these sublineages affiliate with specific habitats, with most WWTP-associated *Nitrospira* assigned to sublineages I or II (Daims *et al.*, 2001; Maixner *et al.*, 2006; Mehrani *et al.*, 2020). *Nitrospira* sublineage I is represented by sequences that originate from engineered systems like WWTPs, with *Nitrospira defluvii* being the only cultured representative from this group (Spieck *et al.*, 2006; Nowka *et al.*, 2015). Identified members of *Nitrospira* sublineage I appear to be closely associated to WWTP systems, whereas those linked to *Nitrospira* sublineage II exist in diverse environments and can co-exist with *Nitrospira* from other sublineages (Palomo *et al.*, 2019). *Nitrospira* sublineage II representatives originate from terrestrial, freshwater, and artificial systems (Daims *et al.*, 2001; Altmann *et al.*, 2003; Ushiki *et al.*, 2013). This sublineage contains three NOB isolates: *Nitrospira moscoviensis*, which originated from a heating water system, and both *Nitrospira japonica* and *Nitrospira lenta* BS10, which were detected in activated sludge (Ehrich *et al.*, 1995; Ushiki *et al.*, 2013; Sakoula *et al.*, 2018). Additionally, all known comammox *Nitrospira* species belong to sublineage II (Lawson and Lücker, 2018). *Nitrospira* sublineage III lacks cultivated representatives and currently contains only a small number of sequences, including two from cave systems in the Nullarbor region (Australia; Holmes *et al.*, 2001). Members of *Nitrospira* sublineage IV consist of marine-associated *Nitrospira* that appear adapted to saline conditions and high pH (Watson *et al.*, 1986; Off *et al.*, 2010; Daebeler *et al.*, 2020). Sublineages V and VI are the most recently described *Nitrospira* sublineages. Sublineage V contains several representative sequences and only one cultured representative, *Ca.* Nitrospira bockiana, which originated from a heating system (Lebedeva *et al.*, 2008). *Nitrospira* sublineage VI contains sequences from geothermal water streams and has

one cultured representative, *Nitrospira calida*, which was recovered from a geothermal spring (Lebedeva *et al.*, 2010). Because very few representative species exist as enrichment or pure cultures, the biochemistry and ecophysiology of *Nitrospira* populations remain poorly investigated.

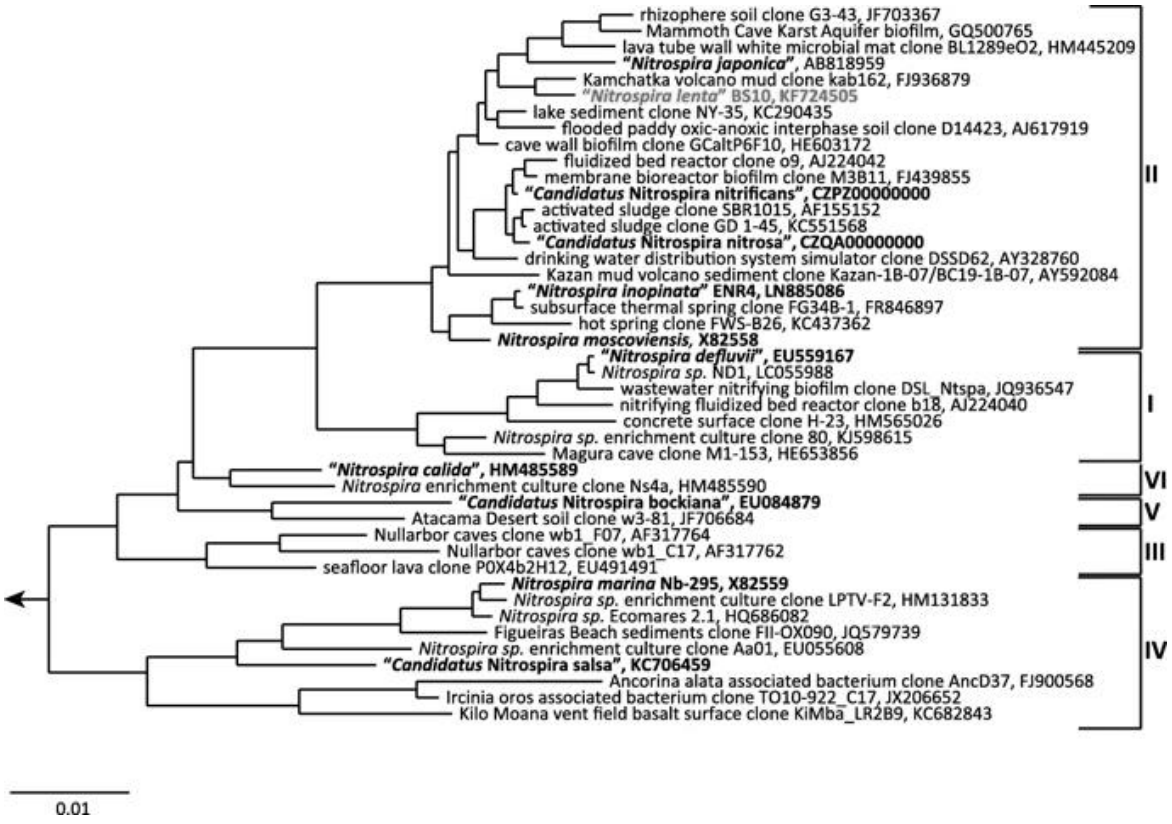

Figure 1.2. 16S rRNA gene-based phylogenetic analysis of selected representatives from the genus *Nitrospira*. The six sublineages of *Nitrospira* are shown with Roman numerals on the right. Bolded *Nitrospira* species are cultured representatives (Reproduced from Sakoula *et al.*, 2018).

1.4 Complete ammonia-oxidizing bacteria (comammox bacteria)

The presence of a microorganism capable of complete ammonia oxidation (comammox) was predicted on the basis of kinetic theory of optimal pathway length (Costa *et al.*, 2006). According to the optimal pathway theory, an organism capable of complete ammonia oxidation would have a higher growth yield and a lower growth rate compared to its competitors. Costa and colleagues argued that comammox bacteria might be found within surface-attached biofilms and in environments that favor microaggregates, where substrate (*i.e.*, ammonia) concentrations are limiting, and conditions favor slow-growing microorganisms. Two studies later reported the discovery and cultivation of three novel *Nitrospira* species capable of complete ammonia oxidation (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Both studies detected comammox bacteria by examining the metagenomes of enrichment cultures obtained from biofilms in oligotrophic environments. The two studies examined *Nitrospira* metagenomic bins encoding gene loci located on the same metagenome-assembled genome (MAG) involved in ammonia and nitrite oxidation (ammonia monooxygenase; AMO and nitrite dehydrogenase; NXR, respectively). Along with chemical analyses, their findings suggested that *Nitrospira* present in their enrichment cultures possessed the genetic repertoire to perform complete ammonia oxidation. This was a surprising finding because members of the genus *Nitrospira* were commonly assumed to be strict NOB (Daims *et al.*, 2001).

Comammox bacteria were overlooked in previous surveys that relied on 16S rRNA gene amplicon sequencing. The genus *Nitrospira* is diverse and widespread in many natural and engineered environments (Pjevac *et al.*, 2017) and, like the 16S rRNA gene, the *nxrA* and *nxrB* genes (genes that encode for subunits A and B of the nitrite oxidoreductase enzyme involved in nitrite oxidation) cannot be used to distinguish between comammox *Nitrospira* and canonical

Nitrospira that are interspersed within *Nitrospira* sublineage II (Daims *et al.*, 2015; van Kessel *et al.*, 2015; Lawson and Lücker, 2018). To distinguish comammox *Nitrospira* from canonical *Nitrospira*, additional evidence from functional gene analyses is required. Ammonia monooxygenase (AMO) is an enzyme responsible for catalyzing the first step of ammonia oxidation (ammonia to hydroxylamine) and is made up of three subunits; AmoA, AmoB, and AmoC, and their respective genes (*i.e.*, *amoA*, *amoB*, and *amoC*) can be found in all aerobic ammonia oxidizers. Compared to AOB and AOA, comammox *Nitrospira* encode a phylogenetically unique *amoA* gene (Daims *et al.*, 2015; van Kessel *et al.*, 2015; Camejo *et al.*, 2017). Because of this, earlier PCR primer sets designed to target the *amoA* genes of AOA and AOB did not amplify the *amoA* genes of comammox *Nitrospira* (Rotthauwe *et al.*, 1997; Treusch *et al.*, 2005; Junier *et al.*, 2008; Meinhardt *et al.*, 2015). The *amoA* genes of comammox *Nitrospira* form two monophyletic clades called clade A and clade B (Daims *et al.*, 2015; van Kessel *et al.*, 2015; Pjevac *et al.*, 2017).

Since the discovery of comammox *Nitrospira*, there have been several primer sets designed to target the *amoA* and *amoB* genes of comammox bacteria (Bartelme *et al.*, 2017; Keene *et al.*, 2017; Pjevac *et al.*, 2017; Fowler *et al.*, 2018; Xia *et al.*, 2018; Beach and Noguera, 2019; Cotto *et al.*, 2020). The widely used primer set developed by Pjevac *et al.* (2017) targets the *amoA* gene of clade A and clade B *Nitrospira* separately, making this primer set suitable for general surveys and quantification of comammox *Nitrospira* in the environment. Other research groups designed specific primer pairs that target clade A comammox *Nitrospira* (Keene *et al.*, 2017; Xia *et al.*, 2018). However, primer sets by Keene *et al.* (2017) and Xia *et al*. (2018) detect and quantify a small proportion of overall comammox *Nitrospira amoA* genes and, more importantly, neither primer set can be used to amplify the *amoA* genes of clade B comammox.

1.5 Comammox *Nitrospira* in wastewater treatment plants

Although many studies have focused on canonical (NOB) *Nitrospira* within WWTP environments (Daims *et al.*, 2001; Mota *et al.*, 2005; Ushiki *et al.*, 2013; Pester *et al.*, 2014; Gruber-Dorninger *et al.*, 2015; Yao and Peng, 2017), primers targeting comammox *Nitrospira* allow for assessments of the diversity and distributions of these nitrifiers as well. Comammox *Nitrospira* were initially found in WWTPs by searching metagenomic databases for MAGs that encode gene clusters involved in the complete ammonia oxidation pathway (*i.e.*, AMO and NXR genes) assigned to the genus *Nitrospira* (Daims *et al.*, 2015; van Kessel *et al.*, 2015; Pjevac *et al.*, 2017). Pjevac *et al.* (2017) reported that comammox *Nitrospira amoA* genes in activated sludge account for approximately 14 – 34% of the total *amoA* gene copy number. Given their relatively high abundance, this finding implies that comammox *Nitrospira* can potentially contribute to nitrification in this wastewater treatment system. Similarly, a study by Roots *et al.* (2019) used qPCR to show that comammox *Nitrospira* were dominant ammonia oxidizers in a nitrification reactor fed with municipal wastewater. Likewise, Spasov *et al.* (2020) used a combination of metagenomics and qPCR to survey the abundance and diversity of ammonia oxidizers in the rotating biological contactors (RBCs; a type of biological treatment system) of a municipal WWTP, showing that these systems are dominated by several comammox *Nitrospira* populations. Earlier work by Sauder *et al.* (2017) employed CARD-MAR-FISH technique to show that *Nitrospira* from the RBCs assimilate labelled bicarbonate. As suggested by Spasov *et al.* (2020), if many of these *Nitrospira* were comammox bacteria, then this implicates comammox *Nitrospira* activity. However, exploration of the relative contribution of the various ammonia-oxidizing microbes (AOMs) to ammonia oxidation activity requires additional methods, such as the use of differential nitrification inhibitors.

Despite the significance of nitrifying guilds in wastewater treatment, few studies have investigated the activity and diversity of comammox *Nitrospira* in WWTPs compared to AOA and AOB. Some evidence suggests that comammox *Nitrospira* contribute directly to WWTP nitrification. To study the functional relevance of comammox *Nitrospira* in a full-scale wastewater treatment plant, Zheng *et al.,* (2019) used reverse transcriptional quantitative PCR (RT-qPCR) to target the *amoA* genes of all nitrifiers present in the activated sludge of a full-scale WWTPs. Their results showed that *amoA* transcript abundance of comammox bacteria was significantly higher than those from AOA and AOB, indicating that comammox *Nitrospira* were the most active members of the nitrifying guilds present in this activated sludge. Similarly, others have used a combination of DNA and RNA stable-isotope probing (DNA-SIP and RNA-SIP, respectively) and 16S rRNA gene amplicon sequencing to identify active nitrifiers in a sand filter community, revealing that *Nitrospira* of sublineage II dominated carbon assimilation in the biofilter (Gülay *et al.,* 2020). Overall, the role of comammox *Nitrospira* in wastewater systems remains largely unexplored. Understanding the contributions of comammox *Nitrospira* to nitrification is particularly relevant from the perspective of biological removal of ammonia from the wastewater.

1.6 Cultivated representatives of comammox *Nitrospira*

Currently, there are four described comammox *Nitrospira* species (Table 1.1), all of which belong to clade A comammox. *Nitrospira* members are considered difficult to cultivate in batch cultures because of their slow growth rates, competitive growth by other nitrifiers, contamination issues, and low biomass yields (Daims and Lücker *et al.,* 2016; Koch *et al.,* 2018).

Table 1.1. Comparison of selected characteristics of cultivated comammox *Nitrospira* representatives

Organism	Source of inoculum	Cultivation status	Ammonium in media (mM)	Inhibitory ammonium concentration (mM)	Apparent ammonia affinity (Km_{app}; nM)	Possible alternate substrate	Cultivation temperature (°C)	Reference
Nitrospira inopinata	Deep oil well	Pure culture	0.5	ND	49–83	Urea	46	Daims *et al.,* 2015
Ca. *Nitrospira* nitrosa	Recirculation aquaculture system	Enrichment culture	0–0.075	ND	ND	Urea	23	van Kessel *et al.,* 2015
Ca. *Nitrospira* nitrificans	Recirculation aquaculture system	Enrichment culture	0–0.075	ND	ND	Urea	23	van Kessel *et al.,* 2015
Ca. *Nitrospira* kreftii	Recirculation aquaculture system biofilter	Enrichment culture	0.01	0.025	~36	Urea	20–22	Sakoula *et al.,* 2020
OTU2-JP-2017 and OTU3-JP-2017	Nitrifying granules	Enrichment culture	0.29–1.77	ND	ND	ND	23	Fujitani *et al.,* 2020
Nitrospira OTU 1,2, 3, and 4	Acidic soil of a tea field	Enrichment culture	<0.40	300	ND	ND	23	Takahashi *et al.*, 2020

ND = not determined.

As a result of this cultivation bottleneck, many physiological and genetic characteristics of comammox *Nitrospira* are inferred from cultivation-independent approaches, such as metagenomics (Camejo *et al.*, 2017; Palomo *et al.*, 2018, 2019; Ushiki *et al.*, 2018; Poghosyan *et al.*, 2019; Spasov *et al.*, 2020; Yang *et al.*, 2020). However, a highly enriched or pure culture is typically required to experimentally validate genomic predictions and acquire detailed information on biochemistry and metabolism. Due to cultivation challenges, knowledge about the required growth parameters, inhibitory compounds, substrate affinity, and the impact of environmental factors on the growth and activity of *Nitrospira* are limited (Koch *et al.*, 2018; Lawson and Lücker, 2018). All currently cultivated comammox *Nitrospira* belong to clade A comammox, with no clade B members isolated yet. Existing comammox *Nitrospira* cultures originate from oligotrophic environments (Table 1.1; Daims *et al.*, 2015; van Kessel *et al.*, 2015). For example, *Nitrospira inopinata*, the only pure comammox *Nitrospira* culture thus far, was derived from biofilm samples from the walls of an oil pipe maintained in hot water (Daims *et al.*, 2015). Similarly, *Candidatus* Nitrospira nitrosa and *Ca*. Nitrospira nitrificans, both currently maintained as enrichment cultures, originate from a trickling filter of a recirculating aquaculture system, where substrate concentrations are also limiting (van Kessel *et al.*, 2015). Although *Nitrospira* coexist with other ammonia and nitrite oxidizers in the same WWTP (Daims *et al.*, 2006; Koch *et al.*, 2014; Roots *et al.*, 2019; Spasov *et al.*, 2020), their growth requirements remain unknown.

Because cultivation protocols differ widely, Nowka *et al.* (2015) proposed a general methodological approach that can be used to isolate and purify *Nitrospira* bacteria. The proposed approach involves a combination of mechanical disruption, density gradient centrifugation, optical tweezers, and cell sorting techniques for cell separation, followed by antibiotic treatment

for inhibition of contaminating heterotrophs (Nowka *et al.,* 2015; Sakoula *et al.,* 2020). However, a major challenge remains to grow sufficient biomass for follow-up experiments.

Recently, Sakoula *et al.* (2020) enriched a novel comammox *Nitrospira* species from an aquaculture biofilter, termed *Ca.* Nitrospira kreftii (Table 1.1). The genome of *Ca.* N. kreftii shows a high similarity to *Ca.* N. nitrificans, in terms of metabolic features. Like *N. inopinata*, *Ca.* N. kreftii shows high apparent ammonia affinity ($Km(app)_{NH3} \approx 0.036$ µM), but unlike *N. inopinata*, *Ca.* N. kreftii seems to have a higher apparent nitrite affinity ($Km(app)_{NO2} \approx 14$ µM for *Ca.* N. kreftii compared to $Km(app)_{NO2} \approx 450$ µM for *N. inopinata*). Taken together, these results suggest that *Ca*. N. kreftii, like other characterized comammox *Nitrospira*, are adapted to substrate-limited environments. Interestingly, *Ca*. N. kreftii is the first reported comammox *Nitrospira* that is inhibited by ammonium concentrations higher than 25 µM (a relatively low ammonium concentration). Inhibition by ammonium at such concentrations further implies that *Ca*. N. kreftii is adapted to oligotrophic environments (Sakoula *et al.*, 2020). Variations in physiological features among comammox *Nitrospira* populations highlights the importance of studying multiple representatives to understand the potential ecological importance and factors involved in the niche differentiation of this ubiquitous group.

Several size-based enrichment strategies have been used to isolate and enrich ammonia-oxidizing microorganisms. For example, Ushiki *et al.* (2013) used a cell-sorting system to remove larger cell aggregates and isolate *Nitrospira*. Cell-sorting uses the shape of micro-aggregates to sort and therefore the size of the micro-aggregates can be used to select for *Nitrospira* micro-colonies. Similarly, size-based selection has been used to isolate and enrich several AOA species, including *Ca*. N. aquarius (Sauder *et al.*, 2018), *Ca.* Nitrosoarchaeum koreensis (Park *et al.*, 2014), and *Nitrosopumilus maritimus* (Könneke *et al.*, 2005). Most

characterized AOB species are larger than 1 µm (in width), therefore size fractionation using various pore size filters can represent a good approach for isolating smaller microorganisms. Like several AOA species, *Nitrospira* cells tend to be small, ranging from 0.2 – 0.5 µm in width (Table1.2). For example, *N. inopinata* has spiral-shaped cells between 0.18 – 0.30 µm in width, making them substantially smaller than AOB and some AOA (Table 1.2). Hence it may be suitable to apply size-based filtration for physical enrichment of comammox *Nitrospira*, especially if other AOA and AOB species present have a larger cell size. As demonstrated in this thesis, size-based selection is a useful technique to use in combination with other traditional enrichment methods or cell sorting.

Table 1.2. Cell morphology and size range of selected nitrifier species

Microorganism	Cell morphology	Cell width (µm)	Reference
Comammox *Nitrospira*			
Nitrospira inopinata	spiral, S-shaped cells	0.18–0.30	Daims *et al.*, 2015
AOA			
Ca. Nitrosotenuis aquarius	slender rod morphology, with thin appendages	~0.30–0.40	Sauder *et al.*, 2018
Ca. Nitrosoarchaeum limnia	rod morphology, with thin appendages	0.19–0.27	Blainey *et al.*, 2011
Ca. Nitrosotenuis uzonensis	rod morphology, with thin appendages	0.20–0.30	Lebedeva *et al.*, 2013
Ca. Nitrosotenuis cloacae	cocci and no appendages	1.0–1.2	Li *et al.*, 2016
Ca. Nitrosocomicus hydrocola	small cocci	~1.3	Sauder *et al.*, 2017
Nitrosocosmicus franklandus	irregular cocci in chains or clusters	~1.1	Lehtovirta-Morley *et al.*, 2016
Nitrosopumilus maritimus	rod morphology	~0.20	Könneke *et al.*, 2005
Nitrososphaera gargensis	small irregular-shaped cocci	~0.90	Hatzenpichler *et al.*, 2008
AOB			
Nitrosomonas eutropha	rod shaped cells (sometimes in chains)	1.0–1.3	Koops *et al.*, 1991
Nitrosomonas nitrosa	spherical-shaped, short, rods	1.3–1.5	Koops *et al.*, 1991
Nitrosomonas sp. JPCCT2	short rods	0.5–0.7	Itoh *et al.*, 2013
Nitrosomonas europaea	oval, rod, shaped cells	1.0–1.2	Meiklejohn, 2009
NOB *Nitrospira*			
Nitrospira lenta	helical shaped cells	0.2–0.3	Nowka *et al.*, 2015
Nitrospira japonica	curved rods	0.3–0.5	Ushiki *et al.*, 2013
Nitrospira moscoviensis	curved rods	0.2–0.4	Ehrich *et al.*, 1995

1.7 Comammox *Nitrospira* metabolic versatility

All characterized comammox *Nitrospira* assimilate inorganic carbon and gain energy by oxidizing either ammonia or nitrite, making them obligate chemolithoautotrophs (Daims *et al.*, 2015; van Kessel *et al.*, 2015; Daims *et al.*, 2016). Nonetheless, several canonical *Nitrospira* were previously shown to use alternate energy substrates, such as formate (CH_2O_2), hydrogen (H_2), and cyanate (CNO^-) (Koch *et al.*, 2014, 2015; Palatinszky *et al.*, 2015). Such metabolic flexibility suggests that members of this genus may occupy distinct niches and their ecological functions may extend beyond nitrogen cycling (Daims *et al.*, 2016). Similarly, genomics-based studies have demonstrated that comammox representatives *N. inopinata*, *Ca*. N. nitrificans, *Ca.* N. nitrosa, and *Ca.* N. kreftii have the genetic machinery needed for ammonia and nitrite oxidation, as well as urea utilization (Daims *et al.*, 2015; van Kessel *et al.*, 2015; Camejo *et al.*, 2017; Palomo *et al.*, 2018; Sakoula *et al.*, 2020). Although ureolytic enzymes and transporters may seem counterintuitive for canonical *Nitrospira*, because they do not use ammonia directly as an energy substrate (although they may assimilate ammonia as a nitrogen source to make amino acids), these NOB convert urea into free ammonia that can be used by ammonia oxidizers, who in turn provide nitrite needed for energy conservation (Koch *et al.*, 2015). The ability to degrade urea may be particularly important in acidic soils, where ammonia is often limiting and urea is the more abundant nitrogen substrate (Burton and Prosser, 2001). Recently, Zhao *et al.* (2021) demonstrated that comammox *Nitrospira* can be enriched using urea or nitrite as the nitrogen source. The authors suggest that although comammox *Nitrospira* lack known nitrite transporters (*i.e.*, comammox *Nitrospira* are incapable of growing solely on nitrite as an energy source), it is possible that other microbial community members may convert nitrite to nitrate, then to ammonia via dissimilatory nitrate reduction to ammonium (DNRA). The authors were also able

to show that urea can specifically enrich for comammox *Nitrospira* and increase nitrification potential of reactor cultures.

In addition to the ability to transport and use nitrite and urea as energy sources, several canonical *Nitrospira* are capable of producing ammonium intracellularly by degrading cyanate. Cyanate forms naturally through urea or cyanide decomposition (Widner *et al.*, 2013; Mooshammer *et al.*, 2020) and can also be produced intracellularly from the dissociation of carbamoyl phosphate, which is a precursor of arginine and pyrimidine (Marina *et al.*, 1999; Purcarea *et al.*, 2003). Canonical *Nitrospira*, like *N. moscoviensis*, can convert cyanate into ammonium and carbon dioxide using cyanate hydratase ("cyanase") (Palatinszky *et al.*, 2015), but this ability is not restricted to canonical *Nitrospira*. For example, the AOA *Nitrososphaera gargensis* is also capable of converting cyanate to ammonium (Palatinszky *et al.*, 2015). More recently, Spasov *et al.* (2020) and Yang *et al.* (2020) reported the presence of cyanase in several comammox MAGs derived from WWTPs. Yang and colleagues (2020) used a combination of metagenomics and metatranscriptomics to show that, despite the detection of cyanase genes in the MAG, no *in situ* transcription of the *cynS* gene was observed. However, the authors did indicate that it is possible that cyanate may be degraded by a yet unknown pathway. The ability to break down cyanate to ammonium could be useful for comammox *Nitrospira* because it would allow them to compete in environments where ammonia may be limiting, and it would also help rid cells of the cytotoxic cyanate (Stark, 1972; Kamennaya *et al.*, 2008).

Lastly, several studies have reported the presence of the genes encoding enzymes needed for hydrogen oxidation in comammox *Nitrospira* genomes (Poghosyan *et al.*, 2019; Spasov *et al.*, 2020; Yang *et al.*, 2020). This was not a surprising finding, given that *N. moscoviensis*, a canonical *Nitrospira*, was shown to oxidize hydrogen instead of nitrite under oxic conditions

(Koch *et al.*, 2014). Overall, these findings reveal the potential metabolic versatility of comammox *Nitrospira*. It appears that some comammox *Nitrospira* may be able to use alternative substrates other than ammonia for energy conservation. Additional studies are still required to validate whether comammox *Nitrospira* can use urea, formate, hydrogen, and cyanate as alternative electron sources for energy conservation.

1.8 Nitrification inhibitors

There are many ways to study the relative contributions of AOA, AOB, and comammox bacteria to nitrification. Generally, researchers have used DNA or RNA stable-isotope probing (SIP) to assess substrate incorporation into biomass associated with ammonia oxidation (Pornkulwat *et al.*, 2018; Wang *et al.*, 2019; Gülay *et al.*, 2020; He *et al.*, 2020). Other groups have employed techniques like microautoradiography-fluorescence *in situ* hybridization (MAR-FISH) for monitoring the uptake of ^{14}C-labelled bicarbonate associated with ammonia oxidation (Hatzenpichler *et al.*, 2008; Sauder *et al.*, 2017). An alternative approach for assessing the relative contributions to nitrification is by measuring rate changes for ammonia depletion and nitrite/nitrate accumulation during the incubation of microcosms with differential inhibitors. Differential inhibitors are often used alone but can also be combined with other methods, like DNA- or RNA-SIP, to indirectly examine nitrifiers actively assimilating bicarbonate in the presence of ammonia from environmental samples (Gülay *et al.*, 2020). It is important to note that AOB, AOA, and comammox bacteria all use ammonia as their energy-generating substrate, yet they use distinct enzyme combinations for oxidizing ammonia (*e.g.*, archaeal genomes lack HAO homologues; Caranto and Lancaster, 2017; Lancaster *et al.*, 2018). Differences in enzymology can allow for differential inhibition of these nitrifying groups.

Ammonia oxidation inhibitors, such as dicyandiamide (DCD), acetylene, and 3,4-dimethylpyrazole phosphate (DMPP) are commonly used for laboratory experiments that assess the activity and relative contributions of ammonia oxidizers to nitrification (Offre *et al.,* 2009; Lehtovirta-Morley *et al.*, 2013; Fu *et al.,* 2019; Obed *et al.,* 2019; Li *et al.,* 2020). General nitrification inhibitors, like DCD and DMPP, are thought to work by inactivation of the ammonia monooxygenase enzyme in AOA, AOB, and comammox *Nitrospira* (Subbarao *et al.,* 2009; Shen *et al.,* 2013; Li *et al.,* 2020). Acetylene is considered a "suicide substrate" that inhibits ammonia oxidation in both AOB and AOA irreversibly (Hynes and Knowles, 1978; Taylor *et al.*, 2010). Other inhibitors, like allythiourea (ATU), octyne, allylsulfide, and organohydrazines, were traditionally used to inhibit ammonia oxidation by AOB, which are inhibited at lower concentrations compared to AOA (Bedard and Knowles, 1989; Neufeld and Knowles, 1999; Wu *et al.*, 2012; Taylor *et al.*, 2015; Sauder *et al.*, 2016). For example, ATU can inactivate the AMO activity of *Nitrosomonas europaea* in pure culture (Hooper and Terry, 1973); inhibition occurs at concentrations between 8 and 80 µM (Ginestet *et al.*, 1998), and reversible chelation of copper in the active site of the ammonia oxygenase enzyme is likely the mechanism (Hyman *et al.*, 1990). Others showed that AOA are less sensitive to inhibition by ATU at concentrations known to inhibit AOB (Hatzenpichler *et al.*, 2008; Taylor *et al.*, 2010; Santoro *et al.*, 2011); higher concentrations (*e.g.*, >100 µM) can inhibit AOA activity (Taylor *et al.*, 2010; Santoro *et al.*, 2011). The reason why AOB and AOA respond differently to inhibition by ATU may be related to differences in sensitivity thresholds of copper-containing proteins like ammonia monooxygenase and nitrite reductase (Gwak *et al.*, 2020). More recently, ATU was also shown to inhibit comammox bacteria at concentrations similar to levels inhibitory to AOB (van Kessel *et al.*, 2015); this is not surprising given that comammox *Nitrospira* share similar ammonia

oxidation machinery as AOB (Palomo *et al.*, 2018). Likewise, preliminary data suggest that comammox *Nitrospira* are also sensitive to octyne at low concentrations (Taylor *et al.*, 2017), which highlights that caution is needed when selecting inhibitors for sample incubations.

To study ammonia oxidation resulting from AOA activity, several groups have previously used nitric oxide scavengers like PTIO to elucidate the relative contributions of AOB and AOA to nitrification in the environment (Shen *et al.*, 2013; Sauder *et al.*, 2016; Fu *et al.*, 2018). Nitric oxide scavengers interfere with the nitric oxide-based electron shuttling used in the hydroxylamine pathway (Jung *et al.*, 2014). Although both AOA and AOB generate nitric oxide as an intermediate in the ammonia oxidation pathway, nitric oxide scavengers, like PTIO, inhibit AOA at low concentration because they exert strict control over the production and uptake of nitric oxide (Shen *et al.*, 2013; Martens-Habbena *et al.*, 2015). As such, AOA are inhibited at lower PTIO concentrations (~18– 30 µM) compared to AOB (>300 µM). Even though comammox *Nitrospira* and AOB share similar ammonia oxidation machinery, the nitric oxide kinetics of *Nitrospira inopinata* are more similar to AOA; both exert strict control over the liberation and consumption of nitric oxide (Kits *et al.*, 2019). As such, it is unsurprising that PTIO inhibits comammox bacteria. Recently, Kits *et al.* (2019) showed that *N. inopinata* cells exert tight control over nitric oxide production and consumption and thus have a lower inhibition threshold by PTIO compared to AOB (*e.g.*, *N. inopinata* was completely inhibited at 33 µM – 100 µM). As a result, PTIO alone can no longer be used as a reliable selective inhibitor of AOA activity.

Other than PTIO, several other AOA-specific inhibitors have been reported. For example, Vajrala *et al.* (2014) showed that the eukaryotic protein synthesis inhibitor cycloheximide can be used to elucidate the relative contributions of AOA and AOB to ammonia oxidation. The authors

cautioned that the effectiveness of cycloheximide as a selective AOA inhibitor may not apply to all AOA lineages and that more research is needed to confirm their findings with other AOA representatives. Others have demonstrated that simvastatin, a compound previously shown to inhibit archaeal cell membrane synthesis, can be used as a selective inhibitor of AOA (Gottlieb *et al.*, 2016; Zhao *et al.*, 2020). They suggest that statins are effective at targeting archaea because they inhibit the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, a crucial enzyme in the cholesterol synthesis pathway used for cell membrane synthesis by archaea. One drawback of using simvastatin for environmental samples is that simvastatin is DMSO soluble. Often, when dealing with environmental samples, higher concentrations of inhibitors are needed to achieve similar effects to those observed in pure cultures. Because of DMSO toxicity, the use of high concentrations of simvastatin may not be a realistic option for environmental samples (Zhao *et al.*, 2020).

To date, there are a few published efforts to explore the effects of nitrification inhibitors on comammox *Nitrospira*. Most studies have focused on the response of comammox *Nitrospira* to commercially available nitrification inhibitors like DCD, DMPP, and ATU within the context of soil nitrification (Obed *et al.*, 2019; Li *et al.*, 2020; Wang *et al.*, 2020). These studies indicate that the abundance and activity of comammox *Nitrospira* was decreased by the addition of nitrification inhibitors. There are no known comammox-specific inhibitors, but Tatari *et al.* (2017) proposed that chlorate may be a useful indicator of activity by comammox bacteria. Chlorate was shown to inhibit nitrite oxidation but not ammonia oxidation activity. Still, its selectivity for inhibition of comammox activity has been questioned (Tatari *et al.*, 2017; Yang *et al.*, 2020). The mechanism of inhibition by chlorate is thought to be due to the conversion of chlorate to chlorite by NXR activity and that chlorite is the actual inhibitor of nitrite oxidation

(Hynes and Knowles, 1983). Thus, the use of chlorate as a differential inhibitor of comammox activity in mixed cultures would require that the process of ammonia oxidation not be affected by the addition of chlorate (Hynes and Knowles, 1983; Tatari *et al.*, 2017). Given tremendous phylogenetic diversity of *Nitrospira* and ammonia-oxidizing archaea, there may be lineage-specific sensitivities to various inhibitors. Preliminary testing of chemical inhibitors against different AOA, AOB, and comammox *Nitrospira* representatives is essential before their use in new environments.

1.9 Research overview

Many WWTPs employ a tertiary wastewater treatment step where nitrogenous compounds (*e.g.*, ammonia and nitrate), as well as other dissolved nutrients, can be removed biologically. One form of tertiary wastewater treatment, as used by the Guelph WWTP, employs rotating biological contactors (RBCs; Figure 1.3) to improve water quality before discharge into receiving waters. The RBCs involve fixed discs that are coated with a nitrifying biofilm. Wastewater enters the RBC system from the secondary clarifier, where ammonia concentrations are relatively low. The wastewater is then fed to one of four treatment trains of the RBCs (northwest, southwest, northeast, and southeast), and the trains are further divided into eight stages.

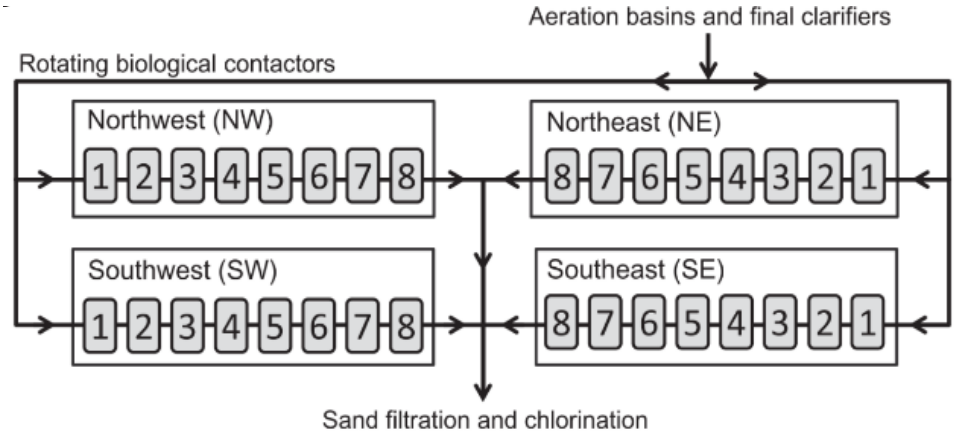

Figure 1.3. The layout of rotating biological contactors (RBCs) at the Guelph WWTP, Ontario, Canada (reproduced from Sauder *et al.*, 2012).

Recent findings demonstrate that comammox *Nitrospira* dominate among the assessed nitrifiers in the RBCs of the Guelph WWTP (Spasov *et al.*, 2020). Despite their high abundance and diversity, the contribution of comammox *Nitrospira* to RBC nitrification remains unknown. The overall goals of my research were to establish an enrichment of comammox *Nitrospira* from the RBCs, using filtration as a size selection method (Chapter 2), and to examine the activity of comammox *Nitrospira* when in mixed communities using differential inhibitors (Chapter 3). The research in this thesis established a protocol for the enrichment of comammox *Nitrospira* that can be tested on samples from other environments (objective 1; Chapter 2) and assessed the effects of various nitrification inhibitors on the relative activity of nitrifiers in the RBCs and in enrichment cultures (objective 2; Chapter 3). Chapter 2 reports on two novel comammox *Nitrospira* enrichments were generated from Guelph's RBCs and the data in Chapter 3 show how nitrification inhibitors can offer a perspective on the possible contributions of comammox *Nitrospira* to the apparently complex nitrification processes in the Guelph RBCs.

Chapter 2 Establishment of novel comammox *Nitrospira* cultures from the RBC biofilm

2.1 Introduction

Ammonia and nitrate can be toxic for fish and cause harmful algal blooms when left untreated in sewage. Nitrification, coupled with denitrification, are key processes for wastewater treatment because the conversion of ammonia and nitrite to nitrate (followed by conversion of nitrate to N_2 gas) helps protect receiving waters and ensure adherence to municipal wastewater guidelines. Ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) were traditionally thought to mediate aerobic ammonia oxidation pathways, whereas nitrite-oxidizing bacteria (NOB) were understood to be solely responsible for the aerobic process of nitrite oxidation. More recently, this model of nitrification was challenged with the identification and cultivation of the first complete ammonia-oxidizing bacteria (comammox) from the genus *Nitrospira* (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Comammox *Nitrospira* can complete both ammonia and nitrite oxidation steps within the same microorganism. Although the distribution and diversity of comammox *Nitrospira* in both engineered and natural systems have been studied through cultivation-independent approaches, enrichment of comammox *Nitrospira* remains a challenge. To fully explore the physiology and metabolism of comammox *Nitrospira*, and to be able to test hypotheses generated by cultivation-independent methods, an enrichment culture or pure cultures are needed.

To date, only one comammox *Nitrospira* enrichment culture exists from a wastewater treatment system (obtained from nitrifying granules; Fujitani *et al.*, 2020). Therefore, this work aimed to enrich comammox *Nitrospira* from biofilm acquired from the rotating biological

contactors (RBCs) of the Guelph municipal WWTP. This was accomplished using filtration and addition of acriflavine (an antimicrobial) to batch cultures. This chapter describes the enrichment of two novel clade A comammox *Nitrospira* species.

2.2 Materials and methods

2.2.1 Sample collection

Biofilm samples were collected with a sterile swab from the northwest train stage 1 (NW1) of RBCs from the WWTP in Guelph, Ontario (Table 2.1). The biofilm material was collected by swabbing multiple surfaces on the contactor several times to make a turbid cell suspension. The area sampled was roughly 15 x15 cm^2. RBC water was also collected from NW1 using wide-mouth polypropylene sampling bottles. The NW1 RBC was chosen for sampling because it was shown previously to have a relatively high comammox *Nitrospira* abundance (Spasov *et al.*, 2020). The collected biofilm was suspended in approximately 500 mL of RBC water and stored on ice until returned to the lab.

Table 2.1. Description of sampling location and associated experiment

Sampling date	Sampling location	Experiment	pH	Ammonia (µM)	Nitrite (µM)	Nitrate (µM)
June 11, 2018	RBC NW1	Preliminary testing for enrichment cultures set up (Chapter 2)	7.54	10.0	22.2	562
December 17, 2018	RBC NW1	Enrichment cultures set up (Chapter 2)	7.20	60.6	BDL	524
November 18, 2019	RBC NW1	Short term activity with inhibitors (Chapter 3)	7.20	23.1	BDL	245

BDL = Below detection limit. Water chemistry measurements were determined based on established protocols (Miranda *et al.*, 2001; Meseguer-Lloret *et al.*, 2002).

2.2.2 Enrichment culture set up

Preliminary cultivation efforts (June 2018) used 5 mL of biofilm material (referred to in section 2.2.1) suspended in either 5 mL RBC water (as medium) or artificial basal salts medium (AOM; the total volume was 10 mL) that was then filtered through a 0.2-µm or 0.45-µm filter (see Table 2.2 and Figure 2.1 for details). The resulting "preliminary" enrichments were incubated at room temperature or 37°C for one month. End-point PCR targeting comammox and AOB *amoA* genes and AOA 16S rRNA genes was used to assess filtration as a selection method for comammox bacteria.

RBC NW1 was sampled again (December 2018) and new "G-series" enrichment cultures were established (Table 2.3) by incubating filtered RBC biofilm with 0.1-µm filtered RBC water or AOM medium.

A 25 mL aliquot of the December 2018 biofilm suspension was gently vortexed to disrupt clumps and then filtered through a 0.45-µm filter into 25 mL of 0.1-µm filtered RBC water. This process was repeated to establish duplicate cultures. Concurrently, another 25 mL of biofilm suspension was gently vortexed prior to filtration through a 0.2-µm filter and into 25 mL of 0.1-µm filtered RBC water, or into 25 mL of AOM medium. Lastly, 25 mL was transferred directly into 25 mL of 0.1-µm filtered RBC water ("unfiltered cell suspensions"; see Figure 2.1 for enrichment culture set up). All G-series cultures were grown in 100 mL borosilicate glass bottles (VWR, Mississauga, Canada) with polypropylene plastic caps (VWR, Mississauga, Canada). Enrichment cultures were supplemented with 0.5 mM ammonium chloride (NH_4Cl) and maintained at room temperature (~22°C) or 37°C in the dark and without shaking.

Table 2.2. Description of the "preliminary" enrichment cultures set up (June 2018)

Culture	Description	Incubation temperature	Growth media
Pr1	0.2-µm filtered suspension	Room temperature (~22°C)	RBC water
Pr2	0.45-µm filtered suspension	Room temperature (~22°C)	RBC water
Pr3	0.2-µm filtered suspension	Room temperature (~22°C)	AOM medium
Pr4	0.45-µm filtered suspension	Room temperature (~22°C)	AOM medium
Pr5	0.2-µm filtered suspension	37°C	RBC water
Pr6	0.45-µm filtered suspension	37°C	RBC water
Pr7	0.2-µm filtered suspension	37°C	AOM medium
Pr8	0.45-µm filtered suspension	37°C	AOM medium

Initial enrichment cultures were 10 mL in volume. See Figure 2.1 for a flowchart of the enrichment cultures set up.

Table 2.3. Description of the "G-series" enrichment cultures set up (December 2018)

Culture	Description	Incubation temperature	Growth media
G1	0.2-µm filtered suspension	Room temperature (~22°C)	RBC water
G2	0.45-µm filtered suspension	Room temperature (~22°C)	RBC water
G3	0.2-µm filtered suspension	37°C	AOM medium
G4	10% (v/v) transfer from G2	Room temperature (~22°C)	AOM medium
G5	0.2-µm filtered suspension	37°C	RBC water
G6	0.45-µm filtered suspension	37°C	RBC water
Gu7	Unfiltered cell suspension	Room temperature (~22°C)	RBC water
Gu8	Unfiltered cell suspension	37°C	RBC water
G8	10% (v/v) transfer from G4 filtered through a 0.8-µm filter	Room temperature (~22°C)	AOM medium
G9	10% (v/v) transfer from G2	37°C	AOM medium
G10	10% (v/v) transfer from G4	30°C	AOM medium

Note that the culture G4 was set up five months after the set up of G2 enrichment culture. G8 was created 7 months after the set up of G4, and G10 was created four months after the set up of G4. See Figure 2.1 for a flowchart of the enrichment cultures set up.

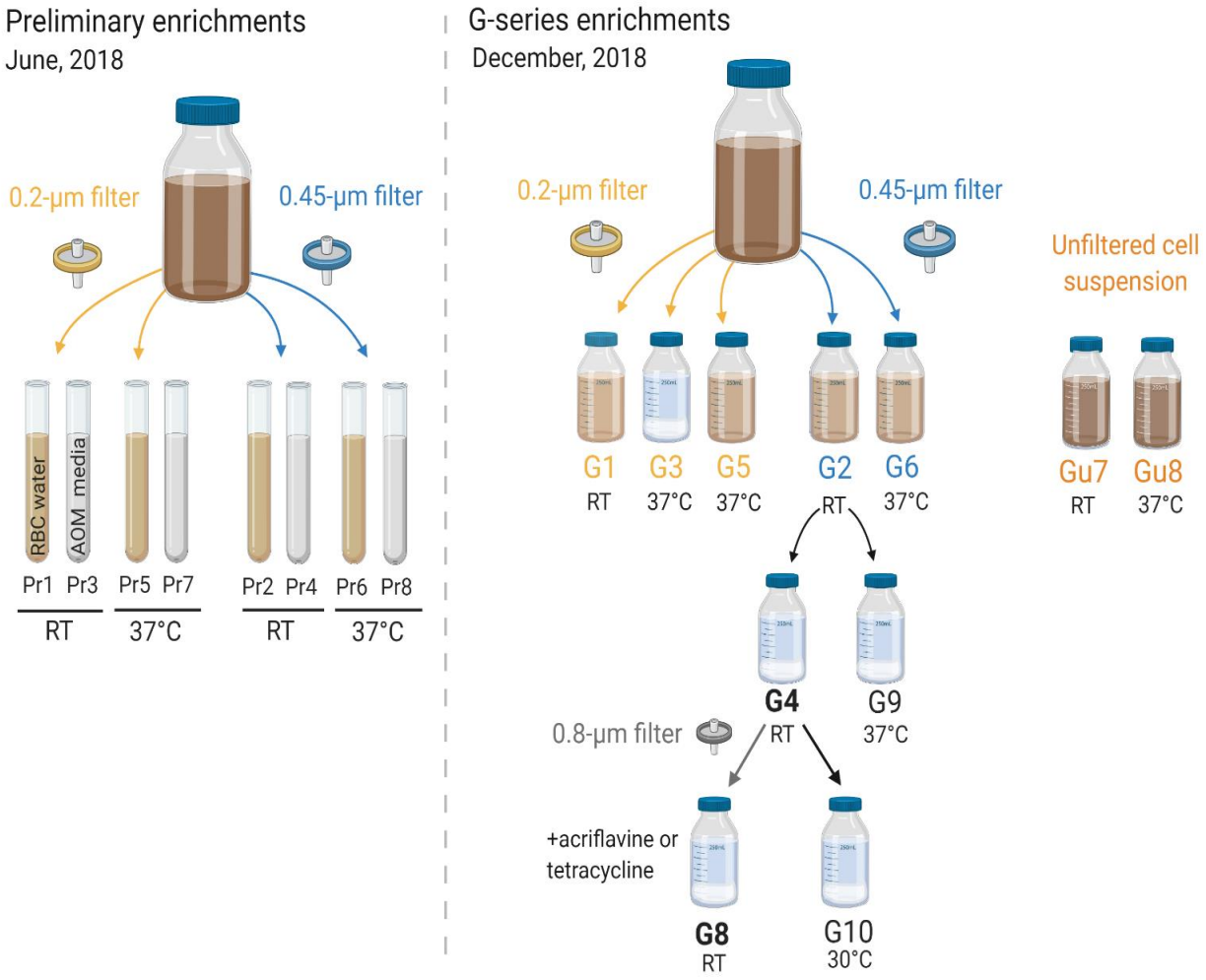

Figure 2.1. Schematic showing "preliminary" and "G-series" enrichment cultures set up. Bottles shown with white precipitate represent incubations in AOM medium. Bottles shown with brown suspension represent incubations in filtered RBC water. Unfiltered suspensions were set up directly from the original suspension. All cultures were set up in duplicate. Bolded cultures (G4 and G8) are the only enrichments that were pursued further. Figure was created with BioRender.com

The cultures were followed for activity using colorimetric assays to monitor the depletion of total ammonium and the production of nitrite and nitrate over time. The presence and absence of *amoA* genes from AOA, AOB, and comammox *Nitrospira* were also monitored using end-point PCR. With respect to G-series cultures, once a comammox *Nitrospira amoA* signal was detected using end-point PCR and the culture was active (*i.e.*, ammonia depletion with nitrate

production), a 1% (v/v) inoculum was transferred into AOM medium, previously used by Daims *et al.* (2015) to grow *Nitrospira inopinata*, and incubated at room temperature in the dark without shaking. The enrichment culture G4 (parent culture for subsequent comammox *Nitrospira* enrichments) was established by transferring 10% (v/v) inoculum from G2 into AOM medium and incubated at room temperature. Briefly, AOM medium is composed of 0.05 g KH_2PO_4, 0.075 g KCl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.58 g NaCl, and 4 g $CaCO_3$ per 1 liter. Additionally, the medium was supplemented with 1 mL trace elements solution and 1 mL selenite-tungstate solution per liter (see Daims *et al.*, 2015 for details). The medium pH was approximately 8.2 after the addition of supplements and was buffered by the excess calcium carbonate present. As noted above, the medium was amended with 0.5 mM NH_4Cl as substrate. Established cultures were maintained in duplicate. Subcultures were named as follows: "G4_sub1" is a 1% subculture derived directly from G4 (parent culture). Similarly, culture "G4_sub2_ammonia" is a sequential subculture two derived from G4 that was supplemented with ammonia. All subcultures apart from the original establishment of G4, G8, G9, and G10 (see Table 2.3) used a 1% (v/v) inoculum.

2.2.3 Enrichment culture purification efforts

To help select against heterotrophic bacteria and AOB, selected G-series enrichment cultures were supplemented with the antimicrobial acriflavine chloride (0.20 µg/mL; Sigma-Aldrich, Oakville, ON, Canada) or the antibiotic tetracycline (30 µg/mL; Sigma-Aldrich, Oakville, ON, Canada) to inhibit the growth of bacteria. Actively growing enrichment cultures that had a detectable comammox *Nitrospira amoA* signal were subcultured (1% inoculum) into fresh AOM medium containing 0.5 mM NH_4Cl and supplemented with 0.20 µg/mL acriflavine or 30 µg/mL tetracycline.

To remove persisting AOA contamination, several experiments were conducted on subcultures of G4 (from G-series). These experiments were conducted in triplicate using 1% (v/v) inocula from G4. For one set of triplicates, cycloheximide, a eukaryotic inhibitor, was added to cultures at 200 µg/mL (Vajrala *et al.*, 2014; Sigma-Aldrich, Oakville, ON, Canada). In another set of triplicates, the cultures were supplemented with a high ammonium concentration (*i.e.*, 30 mM); this concentration was previously shown to be inhibitory to *Ca.* N. hydrocola (formerly called *Ca*. Nitrosocosmicus exaquare) and other AOA representatives (Sauder *et al.*, 2017). In another set of triplicates, a 10% inoculum (v/v) from G4 was first filtered through a 0.8-µm pore size filter to remove relatively large cells.

2.2.4 Total ammonium, nitrite, nitrate concentration measurements

Enrichment culture activities were monitored using colorimetric assays for total ammonium ($NH_3 + NH_4^+$), nitrite (NO_2^-), and nitrate (NO_3^-). All water chemistry measurements were made in duplicate. Concentrations of ammonia, nitrite, and nitrate were calculated by comparing unknown concentration to a standard curve using the SoftMax Pro 6.4 software (Molecular Devices). Ammonia was measured using a previously published protocol (Meseguer-Lloret *et al.*, 2002). Samples were thawed in the fridge, briefly centrifuged to separate any precipitate, and then 100 µL of supernatant was added to a clear bottom 96-well plate, then 100 µL of Nessler's reagent was added to each well. Standards of ammonium chloride were prepared as a dilution series (5 µM to 1000 µM). Plates were incubated in the dark at room temperature for 10 minutes before measuring absorbance at 450 nm using the FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA).

Nitrite concentrations were measured using a colorimetric protocol (Miranda *et al.*, 2001). A 100 µL volume of sample (or standard) was added to clear-bottom 96-well plate, followed by

addition of 100 µL of Griess reagent (*i.e.*, 1:1 mixture of sulphanilamide and naphthylethylenediamine dihydrochloride). Plates were incubated at 37°C for 30 minutes before measuring absorbance at 550 nm using the plate reader.

Nitrate accumulation was determined colorimetrically using an established Griess and vanadium chloride (VCl_3) assay to measure NOx in a sample (Miranda *et al.*, 2001; Schnetger and Lehners, 2014); NOx represents the concentration of nitrite plus reduced nitrate in a sample. Total NOx is measured after reduction using VCl_3 and the nitrate concentration in a sample is calculated as the difference between nitrite and NOx measurements. A 100 µL of sample (or standard) was added to a clear-bottom 96-well plate followed by the addition of 100 µL of the Griess reagent and 100 µL of VCl_3 solution. Plates were incubated at 37°C for 30 minutes before measuring the absorbance at 550 nm using the plate reader. Total nitrogen concentration was calculated as $NH_3/NH_4 + NO_2 + NO_3$.

2.2.5 Genomic DNA extractions, end-point PCR, and Sanger sequencing

Five months following G-series enrichment set up, culture genomic DNA extractions were performed using the DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany). Extractions from biofilm samples were done with the PowerSoil DNA Isolation Kit (Qiagen). The protocols were followed as per the manufacturer's instructions, except that bead-beating with a FastPrep-24 (MP Biomedicals, Santa Ana, CA, USA) was used instead of vortexing, and biofilm samples were extracted from instead of soil. Genomic DNA was quantified on a Qubit fluorometer (Invitrogen, Waltham, MA) using the Qubit 1X dsDNA HS Assay Kit (Invitrogen, Waltham, MA) with 1 µL of sample (total volume 200 µL). Final DNA concentration was adjusted to 1 – 10 ng/µL using Tris + Tween-20 buffer.

Clade A comammox *Nitrospira amoA* genes were detected using equimolar primer mixes of ComaA-244f (A-F) and ComaA-659r (A-R; Pjevac *et al.*, 2017), AOB *amoA* genes were amplified using the primer set amoA-1F and amoA-2R (Rotthauwe *et al.*, 1997), and AOA *amoA* genes were detected using the primer set CrenamoA 23F/CrenamoA 616R (Tourna *et al.*, 2008). All primers, annealing temperatures, and expected product sizes were based on the associated literature protocols (Table 2.4). The PCR products were visualized on a 1% agarose gel and quantified using the Qubit fluorometer and the dsDNA HS Assay Kit (Invitrogen, Waltham, MA), then purified using the ExoSAP-IT (Biolabs MA, USA). ExoSAP-IT eliminates excess primers and nucleotides enzymatically. Briefly, 2.5 µL of ExoSAP-IT Master Mix was mixed with 25 µL of PCR mixture, incubated at 37°C for 40 minutes, 80°C for 15 minutes to inactivate enzymes, then stored at 4°C. Final purified PCR products of comammox *Nitrospira* and AOA were then quantified using Qubit and adjusted to the appropriate concentration before Sanger sequencing at The Center for Applied Genomics (TCAG, Toronto, Canada). The resulting sequences were trimmed for primers and chromatograms visualized using the software Chromas version 2.6.6 (Technelysium Pty Ltd, Brisbane, Australia). The *amoA* gene sequences of comammox *Nitrospira* and AOA species were compared to sequences in Genbank using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990).

Table 2.4. The primer sequences, product size, and annealing temperatures for the primer sets used to quantify AOA, AOB, and comammox *Nitrospira*

Primer set	Sequence (5' – 3')	Product size (bp)	Annealing temperature (°C)	Reference
ComaAF ComaAR	TAYAAYTGGGTSAAYTA ARATCATSGTGCTRTG	415	52	Pjevac *et al.*, 2017
amoA-1F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	491	60	Rotthauwe *et al.*, 1997
CrenamoA23F CrenamoA616R	ATGGTCTGGCTWAGACG GCCATCCATCTGTATGTCCA	628	55	Tourna *et al.*, 2008

2.2.6 Detection of the comammox *Nitrospira* MAG069 *cynS* gene and possible growth on cyanate

In addition to encoding genes for complete ammonia oxidation, Spasov *et al.* (2020) have also reported that the comammox *Nitrospira* bin MAG069 encodes a gene for a cyanase (*cynS* gene). To test enrichment cultures for the presence of *cynS* gene, MAG069 *cynS* gene was used as a template for the new primer set. This new primer set was designed using Primer-BLAST. The resulting primer set was validated against bacterial Refseq representative genomes available on the NCBI database. These primers targeted the *cynS* gene encoded by MAG069 with an expected product size of 184 bp. The primer set did, however, have an unintended template in the *Paenibacillus chitinolyticus* genome, but the false positive product was much shorter than the intended product (*i.e.*, 40 bp). The PCR conditions were selected based on the New England BioLabs' protocol for PCR optimization with *Taq* DNA polymerase (document number: M0273). The calculated melting temperature for the forward primer was 59.8°C, and 60°C for the reverse primer. The optimal annealing temperature for this primer set was determined using

gradient PCR with the following temperature gradient: 53°C, 53.8°C, 55.3°C, 57.6°C, 60.3°C, 62.6°C, 65°C. The optimal annealing temperature for this primer set, which was 60°C, was the lowest tested temperature that did not result in non-specific amplification. Genomic DNA extracted from the RBCs was used a positive control because it contained the *cynS*-encoding MAG069-related comammox *Nitrospira*. The DNA of *Ca*. N. aquarius was used as a negative control. The resulting product was assessed by Sanger sequencing to confirm that the correct product was amplified.

To test its ability to grow on cyanate, duplicate subcultures of G4 were supplemented with either 0.5 mM potassium cyanate, or 0.5 mM ammonium chloride and incubated in the dark. Because cyanate can be converted to ammonium (and carbon dioxide) chemically, abiotic controls with cyanate were also set up. In this instance, aqueous ammonia, nitrite, and nitrate were not quantified colorimetrically; instead, total ammonium was followed via ammonia spot tests (color change when mixed with Nessler's reagent). Estimates of nitrite and nitrate concentrations were determined via color change using nitrite-nitrate strips (MilliporeSigma, MA, USA).

2.2.7 Temperature and ammonia tolerance incubations

To find a suitable growth temperature for G4 enrichments, three incubation temperatures were tested. For temperature experiments, an actively growing G4 enrichment was subcultured (1% transfer) in triplicate into fresh AOM medium supplemented with 0.5 mM NH_4Cl (total volume was 50 mL). The resulting enrichment cultures were incubated at room temperature (~22°C), 30°C, or 37°C. To assess ammonia tolerance on G4 enrichments, an actively growing G4 enrichment was subcultured in triplicate into fresh AOM medium. The resulting enrichments were supplemented with 0.5 mM NH_4Cl (control), 1 mM NH_4Cl, 3 mM NH_4Cl, 10 mM NH_4Cl,

or 30 mM NH_4Cl. All resulting cultures were incubated at room temperature in the dark and without shaking. Ammonia depletion and nitrite/nitrate accumulation were measured over time to assess the activity of nitrifiers in resulting enrichments.

2.2.8 16S rRNA gene amplicon sequencing

The community composition of enrichment cultures was investigated using amplicon sequencing of the V4-V5 region of the 16S rRNA gene. The 16S rRNA gene was amplified using the 515F-Y and 926R primer set (Parada *et al.*, 2016), modified to include six base-pair barcodes used for multiplexing, as well as adapters for binding the flow cell (Bartram *et al.*, 2011). The PCR was performed in triplicate in a UV-treated PCR hood with ISO 5 HEPA filtered air (AirClean Systems, ON, Canada). PCR tubes, tips, bovine serum albumin (BSA), and PCR water were all UV-treated for 15 minutes. The PCR master mix for a 25 μL reaction was composed of: 1X ThermoPol Buffer (New England Biolabs, MA, USA), 0.20 μM forward primer, 0.20 μM reverse primer, 200 μM deoxynucleoside triphosphates (dNTPs), 15 μg BSA, 0.625 U hot start *Taq* DNA polymerase (New England Biolabs), and 1 μL of (1-10 ng/μL) template DNA. The PCR was conducted with a T100 thermal cycler (Bio-Rad) and the following thermocycle conditions: 95°C for 3 minutes (initial denaturation), followed by 40 cycles of 95°C for 30 seconds (denaturation), 50°C for 30 seconds (annealing), 68°C for 1 minute (extension), and a final extension of 68°C for 7 minutes. Several no-template negative controls were included in the 96-well plates to test for potential cross contamination.

Uniquely indexed triplicate PCR amplicons were pooled and then quantified on a 1% agarose gel that was stained with GelRed (Biotium, CA, USA). Based on the relative concentrations of PCR amplicons, product from each 96-well plate was pooled using an equal quantity of PCR amplicon into a single tube. Negative DNA extraction controls and PCR controls were included

in the pool. The pooled PCR amplicons were run on a 1.5% ethidium bromide-stained agarose gel and bands were cut from the agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, WI, USA). The library was denatured and diluted following manufacturer guidelines (Illumina document number: 15039740 v01). The purified pools were quantified using a Qubit fluorometer with the Invitrogen Qubit dsDNA HS Assay Kit, then diluted to 8 pM. The pooled library, which included 15% PhiX control v3 (Illumina Canada, NB, Canada) was sequenced on a MiSeq (Illumina, CA, USA) using a 2×250 cycle MiSeq Reagent Kit v2 as recommended by the manufacturer.

The resulting sequence reads were demultiplexed using the MiSeq Reporter software version 2.5.0.5 (Illumina) and analyzed using QIIME 2 version 2019-10, a pipeline managed by Automation, eXtension, and Integration Of Microbial Ecology 3 (AXIOME3; Min *et al.* 2021). Primer sequences were removed and low-quality sequences were trimmed (after 250 bp for forward and reverse reads) using DADA2 pipeline version 2019.10 (Callahan *et al.*, 2016). Following the removal of low-quality sequences and primers, DADA2 was used to merge the forward and reverse reads (denoise sequences) and dereplicate paired-end reads to generate an amplicon sequence variant (ASV) table. The ASV taxonomy was assigned using a naive Bayes classifier (feature-classifier classify-sklearn) pre-trained with SILVA database release 132 (Quast *et al.*, 2013).

2.2.9 Phylogeny analysis and tree construction

Comammox *Nitrospira amoA* nucleotide sequences from G4 and G8 were compared to clade A and clade B comammox *Nitrospira* cultured representatives and environmental clones. Similarly, the *cynS* gene sequence from G4 was compared to reference sequences from cultured

representatives and environmental samples. Reference sequences used for alignments were acquired from Genbank, and nucleotide sequences were aligned with MUSCLE (Edgar, 2004). For the *amoA*-based tree, evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-3 parameter model; a model that considers the differences in transition and transversion rates and the G+C content bias (Tamura, 1992). For the *cynS*-based tree, the evolutionary history was inferred using the General Time Reversal model; a model that accounts for the different rates of nucleotide substitutions (Tavaré, 1986). In both cases, Gamma distribution was used to model evolutionary rate differences across sites and the most fitting substitution model was selected using the Find Best Nucleotide Model option in MEGAX with the lowest Bayesian information criterion (BIC). The trees were drawn to scale, with branch lengths measured in the number of amino acid substitutions per site. Bootstrap values were calculated based on 500 replicates and are shown above tree branches as circles. All phylogenetic analyses were conducted in MEGAX (Kumar *et al.*, 2018).

Sequences for AOA and *Nitrospira* 16S rRNA genes were compared to reference sequences (acquired from Genbank) of cultured *Nitrospira* and AOA representatives as well as environmental representatives. Nucleotide sequences were aligned using MUSCLE (Edgar, 2004), and the resulting alignments were trimmed so that all sequences had the same length; 409 bp and 411 bp for *Nitrospira* and AOA 16S rRNA genes, respectively. The *Nitrospira* 16S rRNA gene evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-3 parameter model (Tamura, 1992), and the AOA 16S rRNA evolutionary history was inferred using the Kimura 2-parameter model (Kimura, 1980). The Kimura-2 parameter model accounts for the differences in rates of transitions and transversions and that base frequency is constant. In both cases, Gamma distribution was used to model evolutionary rate differences

across sites and the most fitting substitution model was selected using the Find Best Nucleotide Model option in MEGAX with the lowest Bayesian information criterion (BIC). Bootstrap analysis was performed with 500 replicates. All alignments and phylogenetic analyses were conducted in MEGAX (Kumar *et al.*, 2018).

2.3 Results and discussion

2.3.1 Enrichment of comammox *Nitrospira* in batch culture using filtration

Previous information indicates that comammox *Nitrospira* are likely small in size, whereas most AOB and AOA species are greater than 1 µm in width (Table 1.2). Based on end-point PCR, initial tests with filtered RBC biofilm suspensions called "preliminary enrichments" (initiated in June 2018) showed that comammox *Nitrospira* passed through a 0.45-µm filter, whereas AOA did not (Figure 2.2 and Table 2.5). Consequently, filtration with a 0.45-µm filter was selected as a method for comammox *Nitrospira* selection. Initially, it was assumed that the AOA present in the preliminary enrichment cultures were *Ca*. N. hydrocola, a species that dominated Guelph RBCs (Sauder *et al.*, 2017). Preliminary enrichment cultures were generally free of AOB (Figure 2.2). Once filtration was validated as a selection procedure using the preliminary batch cultures, RBC NW1 was sampled again in December 2018 and new "G-series" enrichment cultures were set up.

Five months following the set up of G-series enrichment cultures, genomic DNA extractions were performed followed by end-point PCR targeting comammox *Nitrospira*, AOB, and AOA *amoA* genes. The results showed a comammox *amoA* signal in the enrichment culture G2 (Table 2.6). Culture G2 also had a detectable AOA 16S rRNA gene signal but no detectable AOB *amoA*

signal (Table 2.6). Once comammox *Nitrospira amoA* gene was detected, a 10% (v/v) of inoculum from G2 was transferred from culture grown in RBC water to AOM medium to create G4. The carbonate-based AOM medium provides buffering capacity to ensure that the pH remains stable, in addition to providing a surface for growth given that carbonate concentration exceeds solubility. Most subsequent research was conducted with subcultures from G4.

After transfer into AOM medium, both G4 replicate cultures had detectable comammox *Nitrospira* and AOA *amoA* genes (Table 2.6). G4 oxidized ammonia and generated nitrate without a lag phase (panel A Figure 2.3). After four months of the establishment of G4, a 10% (v/v) of inoculum from G4 was transferred into fresh AOM medium to create culture G10 (see Table 2.6) which was incubated at 30°C. G10 was used to test whether 30°C was a suitable temperature for the growth of G4. Similarly, after seven months of the establishment of G4, a 10% (v/v) of inoculum from G4 was filtered through a 0.8-µm filter and into fresh AOM medium (see Table 2.6). Later subcultures of G8 were supplemented with tetracycline or acriflavine (cultures are called G8_tetracycline or G8_acriflavine, respectively; Table 2.13). Filtration though a 0.8-µm pore size filter was used as a way to eliminate AOA presence in enrichment cultures. Similar to G4, G8 oxidized ammonia and generated nitrate without a lag phase (panel B Figure 2.3). Overall, it can be concluded that comammox *Nitrospira* were present in the G4 and G8 enrichments which grew under oxic conditions in artificial medium provided with ammonia as the only nitrogen and energy source and bicarbonate as the carbon source (Figure 2.3 and Table 2.6).

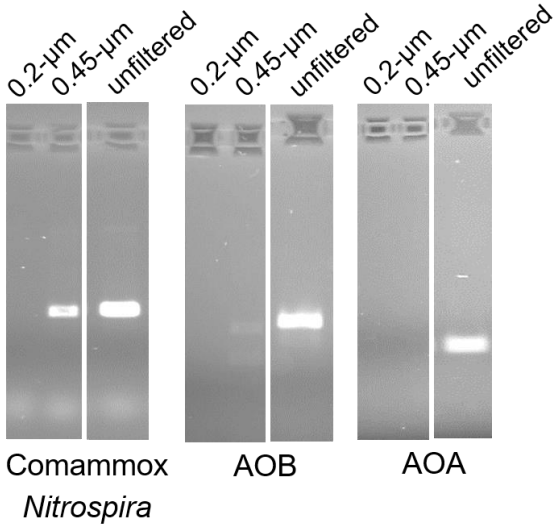

Figure 2.2. End-point PCR targeting the three nitrifying guilds present in preliminary enrichment cultures. Genomic DNA used for PCR was extracted one month following initial inoculation into AOM medium (*i.e.*, Pr3 and Pr4). Unfiltered RBC biofilm was used as positive control for PCR.

Table 2.5. Summary of presence or absence of comammox and AOB *amoA* genes, and 16S rRNA gene of AOA one month after initiating preliminary enrichments

Culture	Description	Presence/ Absence		
		AOA 16S rRNA	AOB *amoA*	Comammox *Nitrospira amoA*
Pr1	0.2-µm filtered suspension in RBC water at RT	–	–	+
Pr2	0.45-µm filtered suspension in RBC water at RT	–	–	+
Pr3	0.2-µm filtered suspension in AOM medium at RT	–	–	–
Pr4	0.45-µm filtered suspension in AOM medium at RT	–	+ (weak)	+
Pr5	0.2-µm filtered suspension in RBC water at 37°C	–	–	–
Pr6	0.45-µm filtered suspension in RBC water at 37°C	+	+	–
Pr7	0.2-µm filtered suspension in AOM medium at 37°C	–	–	+
Pr8	0.45-µm filtered suspension in AOM medium at 37°C	+	–	+

End-point PCR was used to target the *amoA* genes of comammox *Nitrospira* and AOB and the 16S rRNA genes of AOA. (+) represents the presence of *amoA* product, and (–) represents the absence of *amoA* product. RT means room temperature.

Table 2.6. Summary of presence or absence of comammox and AOB *amoA* genes, and 16S rRNA gene of AOA five months after initiating G-series enrichments

Culture	Description	Duplicate 1/Duplicate 2		
		AOA 16S rRNA	AOB *amoA*	Comammox *Nitrospira amoA*
G1	0.2-µm filtered suspension in RBC water (RT)	–/+	–/–	–/–
G2	0.45-µm filtered suspension in RBC water (RT)	+/+	–/–	+/–
G4	10% (v/v) transfer of G2 in AOM medium (RT)	+/+	–/–	+/+
G3	0.2-µm filtered suspension in AOM medium (37°C)	+/–	–/–	–/–
G5	0.2-µm filtered suspension in RBC water (37°C)	–/+	–/–	–/–
G6	0.45-µm filtered suspension in RBC water (37°C)	+/+	–/–	–/–
Gu7	Unfiltered cell suspension in RBC water (RT)	+/+	+/+	–/–
Gu8	Unfiltered cell suspension in RBC water (37°C)	+/+	+/-	–/–
G8	10% transfer of G4 filtered through 0.8-µm filter and grown in AOM medium (RT)	+/+	–/–	+/+
G9	10% (v/v) transfer of G2 in AOM media (37°C)	–/+	–/–	–/–
G10	10% (v/v) transfer of G2 in AOM media (30°C)	+/+	–/–	+/+

Genomic DNA used for PCR was extracted 5 months following initial inoculation into RBC water. (/) distinguishes results from duplicate cultures. (+) represents the presence of 16S rRNA or *amoA* product, and (–) represents the absence of 16S rRNA or *amoA* product. Numbers in brackets represent incubation temperature. RT means room temperature.

The enrichment culture G4 and its subcultures were assessed for ammonia depletion and concomitant nitrite and nitrate accumulation. Cultures typically took between 5 – 14 days to fully oxidize 0.5 mM ammonium to nitrate, with no nitrite accumulation observed (Figure 2.3). Nitrate concentrations in the enrichment cultures were monitored to ensure they remained below 1.5 mM because elevated nitrate concentrations are toxic and can hinder the growth of ammonia oxidizers (Bollmann *et al.*, 2010).

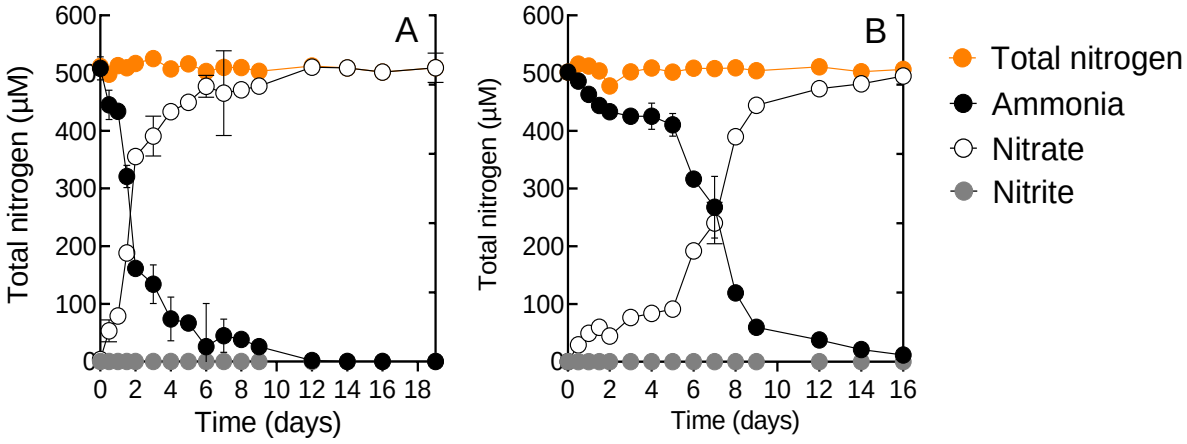

Figure 2.3. Typical water chemistry profile for the cultures G4 (panel A) and G8 (panel B) under ammonia-fed conditions. Both G4 and G8 were incubated at room temperature in the dark, without shaking. Error bars represent the standard deviation of duplicates. For a description of these cultures, see Table 2.3.

To find a suitable growth temperature for comammox *Nitrospira* enrichments, several incubation temperatures were tested, including ambient room temperature, which was used for the initial incubation of enrichment cultures (Preliminary incubations and G-series), 37°C (Preliminary incubations and G-series), and 30°C (G-series only). Ambient room temperature was chosen to reflect the mesophilic temperature range used to operate the RBCs. RBC temperature ranges from 12–21°C (Sauder *et al.*, 2012). A higher incubation temperature (37°C) was chosen based on prior research suggesting that *Nitrospira* may be adapted to higher temperatures (Ehrich *et al.*, 1995; Ushiki *et al.*, 2013; Daims *et al.*, 2015; Courtens *et al.*, 2016). The 37°C incubations resulted in a loss of comammox *amoA* signal from G-series enrichment cultures, implying that comammox *Nitrospira* from the RBCs do not grow at 37°C (Tables 2.6 and 2.7). Based on 16S rRNA gene profiles, the AOA *Ca.* N. aquarius and *Ca.* N. cloacae were enriched at 37°C and no *Nitrospira* were detected (G4 at 37°C; Figure 2.4). This finding is

comparable to reported temperature tolerance reported for these two AOA species (Li *et al.*, 2016 and Sauder *et al.*, 2018). Because comammox *Nitrospira amoA* was not detected in cultures incubated at 37°C, G-series cultures maintained at this incubation condition (*i.e.*, G3, G5, G6, and G9 culture) were not pursued thereafter. Given that 37°C resulted in the inhibition of comammox *Nitrospira* growth, a slightly lower incubation temperature was tested. The 30°C incubation temperature was tested on G4-related enrichment cultures because several NOB *Nitrospira*, like *N. defulvii* and *N. lenta*, have a reported optimal growth temperature close to 30°C (Nowka *et al.*, 2015; Sakoula *et al.*, 2018) and because of the genetic similarity between comammox *Nitrospira* and NOB *Nitrospira*. Based on ribosomal gene profiles, subcultures of G4 that were incubated at 30°C had a higher relative abundance of AOA than *Nitrospira*, suggesting that 30°C favored the growth of AOA over *Nitrospira* (G4_sub1 and G4_sub2 at 30°C in Figure 2.4 and G10 in Table 2.7). Overall, ambient room temperature appeared the best incubation temperature to favor the growth of comammox *Nitrospira* and was, therefore, selected for all further subcultures. This experiment demonstrated that comammox *Nitrospira* derived from RBCs are likely mesophilic and prefer temperatures ranging from 22 – 30°C (although incubation temperatures lower than 22°C were not tested). A lower growth temperature range for this comammox *Nitrospira*, compared to *N. inopinata*, is in agreement with its presence and growth in the RBCs, which are typically maintained at temperatures compatible with mesophilic microbial growth (Sauder *et al.*, 2012).

Table 2.7. Summary of presence or absence of comammox and AOA *amoA* genes in three tested incubation temperatures using end-point PCR

Culture ID	Incubation temperature (°C)	Description	Duplicate 1/Duplicate 2		
			Comammox *Nitrospira amoA*	AOA *amoA*	AOB *amoA*
G4	Room temperature (~22°C)	0.45-µm filtered suspension in AOM medium	++/++	+++/+++	–/–
G10	30°C	0.45-µm filtered suspension in AOM medium	++/++	+++/+++	–/–
G9	37°C	0.45-µm filtered suspension in AOM medium	–/–	+++/+++	–/–

End-point PCR was used to target the *amoA* genes of comammox *Nitrospira*, AOA, and AOB. (/) distinguishes results from duplicate cultures. (+) represents the presence of *amoA* product, and (–) represents the absence of *amoA* product. Numbers in brackets represent incubation temperature. RT means room temperature. Strength of signal is denoted by + (weak), ++ (strong), or +++ (stronger).

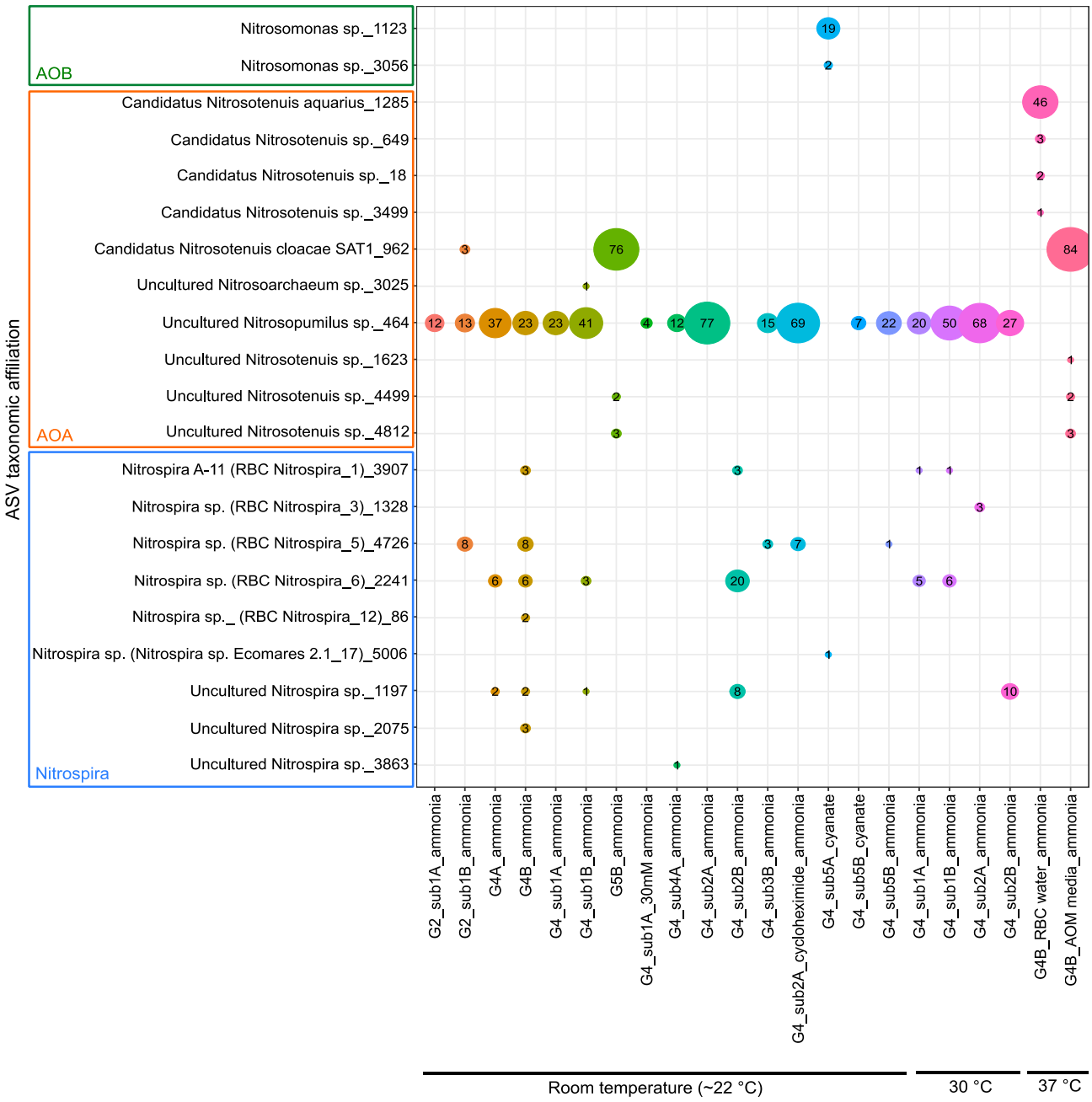

Figure 2.4. Relative abundances of enrichment culture amplicon sequence variants (ASVs) based on 16S rRNA gene amplicon sequencing. Only ASVs assigned to nitrifier genera ≥1% relative abundance are displayed. Numbers shown after the underscore are the ASV number. The numbers in brackets represent *Nitrospira* ASVs previously reported in the RBCs by Spasov *et al.* (2020). The number inside the bubbles represent the relative abundance (%). All cultures shown originate from the same initial culture (*i.e.*, G2) and reflect varying subcultures and/or manipulations of environmental conditions thereafter.

The 16S rRNA gene profile for G4 and its subsequent subcultures (*i.e.*, G4_sub1 to G4_sub5; Figure 2.4) reveals that AOA were present in varying abundances, ranging from 4 – 77% in those enrichment cultures. As predicted originally, AOB were generally absent from G4 and its related subcultures. Several *Nitrospira* ASVs were detected in G4 and its subcultures, and their relative abundances ranged from 1 – 20% (Figure 2.4). The *Nitrospira* ASV 2241 appeared to be enriched from the earlier parent culture (G4), initially present at 6% relative abundance and later at 20% relative abundance. No single *Nitrospira* ASV dominated all cultures. As mentioned earlier, the 16S rRNA gene cannot be used to distinguish between comammox and canonical *Nitrospira*. Additionally, Spasov *et al.* (2020) indicated that most comammox *Nitrospira* genome bins from the RBCs did not have assembled 16S rRNA genes when binned with ATLAS2. As such, ribosomal gene profiles obtained here could not be mapped against previously reported *Nitrospira* MAGs from the RBCs. Nonetheless, it was clear that several *Nitrospira* ASVs co-existed within the cultures at ≥1% relative abundance (Figure 2.4).

A single AOA representative (uncultured *Nitrosopumilus* species; ASV 464) dominated G4 and its subcultures (Figure 2.4). Based on the 16S rRNA gene profiles and end-point PCR, the data suggest that AOA dominated most G4-related cultures and that filtration through a 0.45-µm filter alone was unable to eliminate AOA that originated from the RBC biofilm. This finding is perhaps expected given that the cultured *Nitrosotenuis* representatives like *Ca.* N. aquarius, *Ca.* N. uzonensis, and *Ca.* N. chungbukensis are generally small in size, ranging from 0.2–0.4 µm in width (Lebedeva *et al.*, 2013; Park *et al.*, 2014; Sauder *et al.*, 2018).

2.3.2 Phylogenetic affiliation of *Nitrospira* and AOA species

Phylogenetic analysis of 16S rRNA genes demonstrated that the nine *Nitrospira* ASVs (Figure 2.5) were distinct from one another (87.9 – 99.7% sequence identity). Comparative

analysis (using BLAST) of the 16S rRNA gene revealed that *Nitrospira* ASV 2241 (the *Nitrospira* ASV with the highest abundance) is closely related to other NOB *Nitrospira*: *N. moscoviensis*, *Ca.* N. lenta, and *N. japonica* and is closest to *Nitrospira* RBC_6 (an ASV previously detected in the RBCs; Spasov *et al.* 2020). *Nitrospira* ASV 2075 is closely related to the comammox representatives, *N. inopinata* and *Ca.* N. nitrosa. *Nitrospira* ASV 5006 clustered with *Nitrospira marina* and *Nitrospira* sp. Ecomares 2.1, both reported to be strict marine *Nitrospira* (belonging to sublineage IV). Spasov *et al.* (2020) reported that *Nitrospira* sp. Ecomares 2.1 was found in the RBCs and appeared at 1 – 1.5% of the overall relative abundance. The salinity of Guelph's RBCs is unknown, and the growth media used contains less than 584 mg sodium chloride per liter, therefore it was unclear how these presumed marine associated *Nitrospira* were enriched in cultures. It could be of interest in the future to examine this microorganism more closely because this might be the first clade IV *Nitrospira* from a non-saline environment.

Ten of the ASVs present in the various enrichments at ≥1% relative abundance were classified as AOA (Figure 2.6). Most AOA ASVs were assigned to the genus *Nitrosotenuis* (sequence identity between AOA ASVs is 92.5 – 99.7%). The dominant AOA ASV 464 was assigned as a novel (uncultured) species belonging to the genus *Nitrosopumilus* (Figure 2.4); this ASV was previously detected in the RBC biofilm at a low relative abundance (<0.1%; Spasov *et al*., 2020). Phylogenetically, ASV 464 is associated with Group I.1a Thaumarchaeota and is closely related to cultured *Nitrosopumilus* species isolated from freshwater and saltwater aquariums, WWTPs, Northern Adriatic Sea, and marine sediments (Figure 2.6; Park *et al.*, 2012; Lebedeva *et al.*, 2013; Park, *et al.*, 2014; Li *et al.*, 2016; Sauder *et al.*, 2018; Bayer *et al.*, 2019). The presence of ASVs associated with *Ca.* N. aquarius and *Nitrosopumilus*-like AOA in

WWTPs and aquaculture systems have been reported previously (Mussmann *et al.*, 2011; Bollmann *et al.*, 2014; Sauder *et al.*, 2018). It is important to note that *Ca.* Nitrosocosmicus hydrocola, which was previously reported as the single persistent AOA ASV in the RBC biofilms, was not detected in any of the G4-related enrichment cultures (Figure 2.4). This suggests that filtration of the initial inoculum through a 0.45-µm filter succeeded in removing *Ca.* N. hydrocola, as predicted originally.

Like Guelph's RBCs, the presence of several related *Nitrospira* and AOA in WWTPs was previously reported by numerous groups (Park *et al.*, 2006; Gruber-Dorninger *et al.*, 2015; Roots *et al.*, 2019; Spasov *et al.*, 2020; Zheng *et al.*, 2021). The co-existence of several AOA and *Nitrospira* species suggests that these groups are adapted to different niches. The diversity of both AOA and *Nitrospira* taxa in the enrichment cultures implies that the RBC biofilm offers a valuable habitat to explore the ecology of nitrifiers.

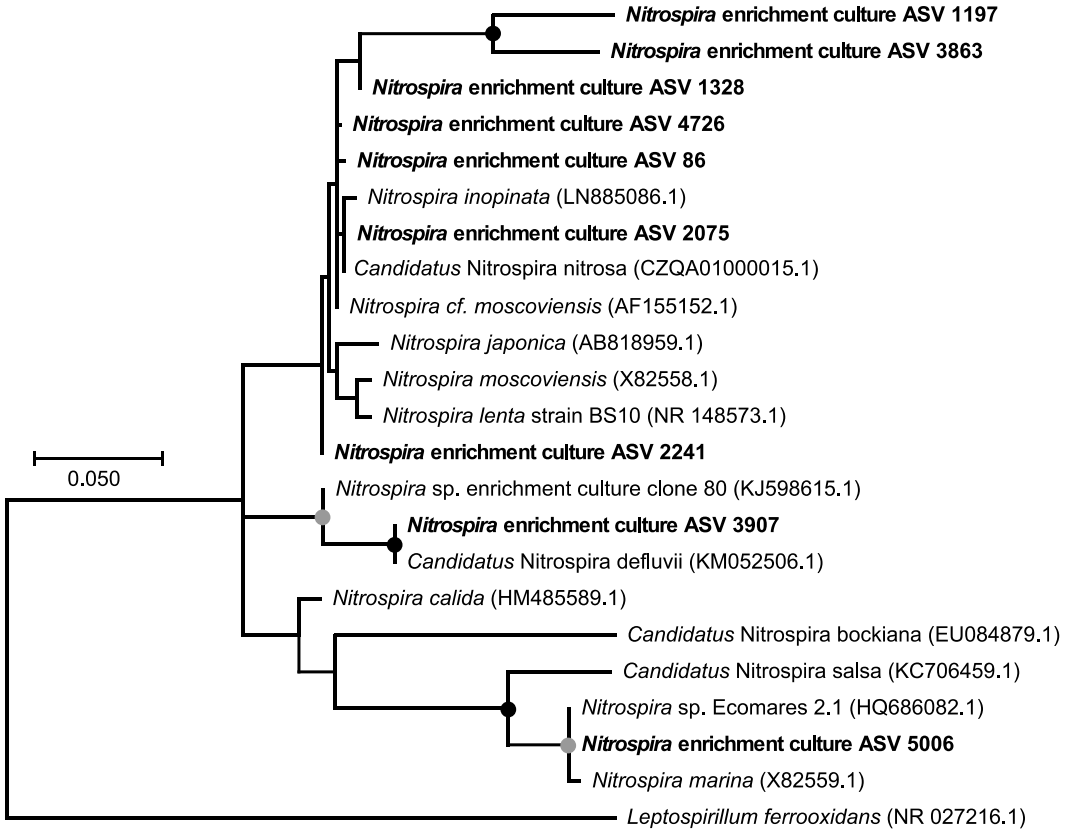

Figure 2.5. Phylogenetic tree showing the relationship of 16S rRNA gene sequences of *Nitrospira* ASVs to reference sequences from the Genbank database. Accession numbers for reference sequences are shown in brackets. Only *Nitrospira* ASVs present in the enrichment cultures at ≥1% are shown. The tree was constructed using maximum-likelihood method based on the Tamura-3 parameter model (Tamura, 1992) with Gamma distribution across sites. Bolded labels are *Nitrospira* sequences from enrichment cultures. A total of 500 bootstrap replicates were used, and values over 60% are shown. Nodes indicated with circles (●) have a bootstrap value >90% and nodes indicated with gray circles (●) have a bootstrap value between 60 – 90%. The scale bar represents 5% nucleotide divergence. The tree was rooted using the 16S rRNA gene of *Leptospirillum ferrooxidans*. Phylogenetic analysis was performed using MEGAX (Kumar *et al.*, 2018).

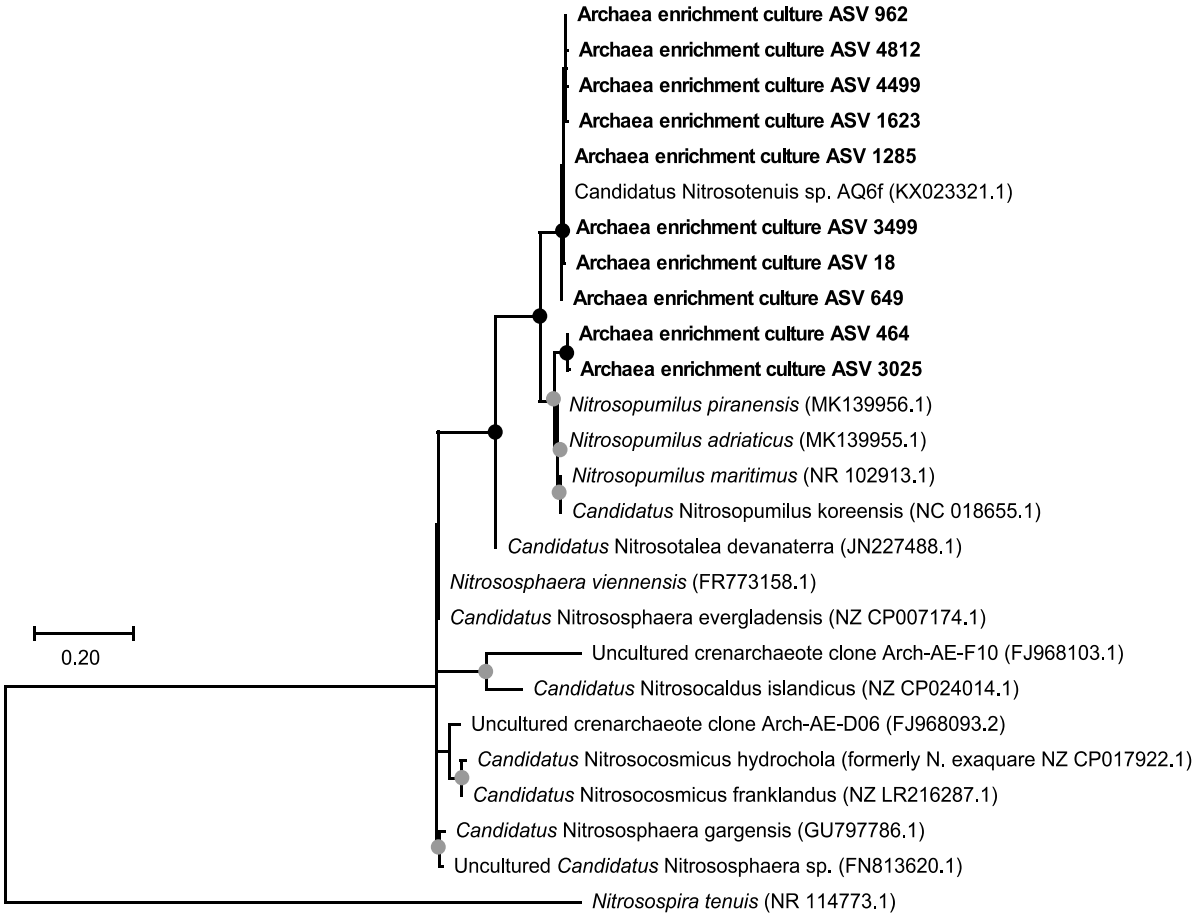

Figure 2.6. Phylogenetic tree showing the relationship of 16S rRNA gene sequences of AOA ASVs to reference sequences from the Genbank database. The tree was constructed using the maximum likelihood method based on the Kimura 2-parameter model with Gamma distribution across sites (Kimura, 1980). Bolded labels are archaea sequences from enrichment cultures. Accession numbers for reference sequences are shown in brackets. Only AOA ASVs present in the enrichment cultures at ≥1% are shown. The tree was constructed using maximum-likelihood method. A total of 500 bootstrap replicates were used, and values over 60% are shown. Nodes indicated with circles (●) have a bootstrap value >90% and nodes indicated with gray circles (●) have a bootstrap value between 60 – 90%. The scale bar represents 2% nucleotide divergence. The tree was rooted using *Nitrosospira tenuis*. Phylogenetic analysis was performed using MEGAX (Kumar *et al*., 2018).

2.3.3 Phylogeny of comammox *Nitrospira amoA* and *cynS* genes and possible growth on cyanate

Sanger sequencing of *amoA* and *amoB* genes that were PCR amplified from cultures G4 and G8 confirmed that the *Nitrospira* observed were comammox *Nitrospira*. The *amoA* and *amoB* gene sequences obtained were very similar to *amoA* and *amoB* sequences of comammox *Nitrospira* MAGs from the RBCs (Spasov *et al*., 2020). The *amoA* sequence derived from G4 was nearly identical to MAG069 *amoA* (99% identical; E-value 6e-123), whereas the *amoB* gene was almost identical to MAG069 *amoB* (97% identical; E-value 8e-122). The *amoA* gene sequence derived from G8 was nearly identical to MAG044 *amoA* gene (99% identical; E-value 4e-140), whereas the *amoB* gene had no significant hits to any of the *amoB* genes encoded by comammox *Nitrospira* MAGs derived from the RBCs by Spasov *et al*. (2020). The *amoA* nucleotide sequences detected in G4 and G8 cultures were assigned to clade A comammox *Nitrospira*. According to the *amoA* phylogeny (Figure 2.7), the G4 *amoA* sequence was closely related to *Nitrospira* bin Group I (MAG069), which is derived from the Guelph RBC metagenomes (Spasov *et al*., 2020). Similarly, the G8 *amoA* sequence was closely related to *Nitrospira* bin Group D (MAG044) (Figure 2.7), which is also derived from the RBC biofilm metagenomes (Spasov *et al*., 2020).

According to Spasov *et al*. (2020), MAG069 and MAG044 encode the genetic repertoire needed for ammonia (the *amo* operon) and nitrite oxidation (*nxrA* gene), but lack the gene that encodes for subunit B of nitrite oxidoreductase enzyme (*i.e.*, *nxrB* is absent). As they indicated, the missing genes are likely due to incomplete genome binning. Despite the missing *nxrB* gene annotations, the metagenomic results imply that MAG069 and MAG044 represent comammox *Nitrospira*. Even though the *amoA* and *amoB* gene sequences were not exact matches to sequences encoded by MAG044, findings from this work support that the *Nitrospira* closely

related to MAG044 is comammox. This finding was generally supported by growing G8 (an enrichment that contains comammox *Nitrospira* as the sole nitrifier) on ammonia, producing nitrate without detectable nitrite accumulation (Figure 2.3). According to metagenomic recruited reads data, MAG044 was present in several RBC trains at abundances ranging from 0.1 – 4.1%, whereas MAG069 was present consistently across RBC trains 1 and 8 at abundances ranging from 0.3 – 11.2% (Spasov *et al*., 2020). Both MAGs represented ideal targets for cultivation because they are phylogenetically unique and have the genetic potential to utilize alternative energy substrates like urea (Spasov *et al*., 2020). Further research should test the use of urea as an alternative way to enrich and select for different comammox *Nitrospira*.

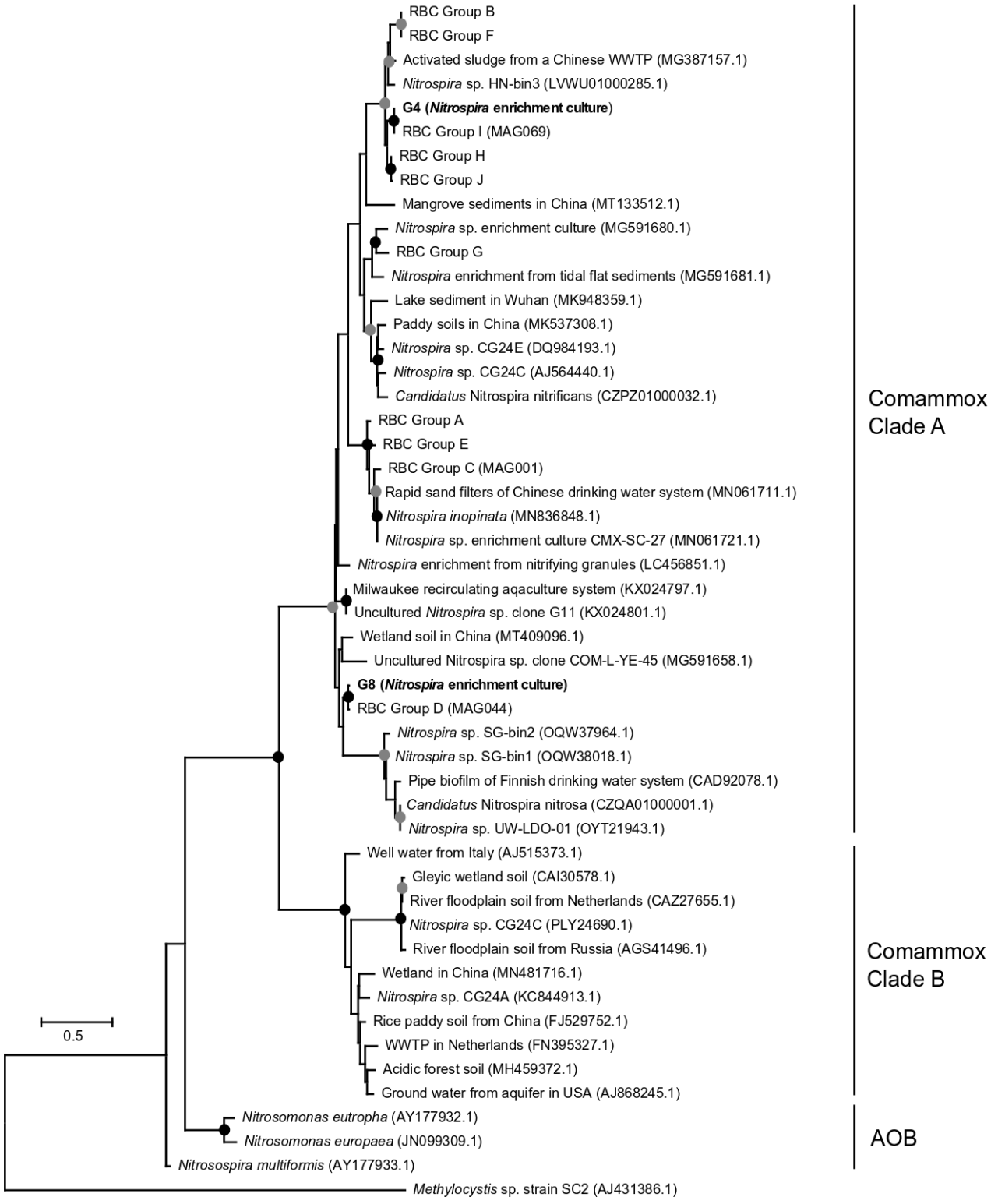
RBC Group B
RBC Group F
Activated sludge from a Chinese WWTP (MG387157.1)
Nitrospira sp. HN-bin3 (LVWU01000285.1)
G4 (Nitrospira enrichment culture)
RBC Group I (MAG069)
RBC Group H
RBC Group J
Mangrove sediments in China (MT133512.1)
Nitrospira sp. enrichment culture (MG591680.1)
RBC Group G
Nitrospira enrichment from tidal flat sediments (MG591681.1)
Lake sediment in Wuhan (MK948359.1)
Paddy soils in China (MK537308.1)
Nitrospira sp. CG24E (DQ984193.1)
Nitrospira sp. CG24C (AJ564440.1)
Candidatus Nitrospira nitrificans (CZPZ01000032.1)
RBC Group A
RBC Group E
RBC Group C (MAG001)
Rapid sand filters of Chinese drinking water system (MN061711.1)
Nitrospira inopinata (MN836848.1)
Nitrospira sp. enrichment culture CMX-SC-27 (MN061721.1)
Nitrospira enrichment from nitrifying granules (LC456851.1)
Milwaukee recirculating aqaculture system (KX024797.1)
Uncultured Nitrospira sp. clone G11 (KX024801.1)
Wetland soil in China (MT409096.1)
Uncultured Nitrospira sp. clone COM-L-YE-45 (MG591658.1)
G8 (Nitrospira enrichment culture)
RBC Group D (MAG044)
Nitrospira sp. SG-bin2 (OQW37964.1)
Nitrospira sp. SG-bin1 (OQW38018.1)
Pipe biofilm of Finnish drinking water system (CAD92078.1)
Candidatus Nitrospira nitrosa (CZQA01000001.1)
Nitrospira sp. UW-LDO-01 (OYT21943.1)
Well water from Italy (AJ515373.1)
Gleyic wetland soil (CAI30578.1)
River floodplain soil from Netherlands (CAZ27655.1)
Nitrospira sp. CG24C (PLY24690.1)
River floodplain soil from Russia (AGS41496.1)
Wetland in China (MN481716.1)
Nitrospira sp. CG24A (KC844913.1)
Rice paddy soil from China (FJ529752.1)
WWTP in Netherlands (FN395327.1)
Acidic forest soil (MH459372.1)
Ground water from aquifer in USA (AJ868245.1)
Nitrosomonas eutropha (AY177932.1)
Nitrosomonas europaea (JN099309.1)
Nitrosospira multiformis (AY177933.1)
Methylocystis sp. strain SC2 (AJ431386.1)
0.5
Comammox Clade A
Comammox Clade B
AOB

Figure 2.7. Phylogenetic tree showing the relationship of *amoA* gene sequences of comammox *Nitrospira* in enrichment cultures to reference sequences from Genbank. Accession numbers for reference sequences are shown in brackets. Bolded labels are G4 and G8 comammox *Nitrospira amoA* nucleotide sequences. The tree was constructed using maximum-likelihood method based on the Tamura-3 parameter model (Tamura, 1992) with Gamma distribution across sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. A total of 500 bootstrap replicates were used. Nodes indicated with circles (●) have a bootstrap value >90% and nodes indicated with gray circles (●) have a bootstrap value between 60 – 90%. Phylogenetic analysis was done using MEGAX (Kumar *et al.*, 2018). The tree was rooted with the *pmoA* gene sequence of *Methylocystis* sp.

In addition to the potential for growth on urea as an energy substrate, MAG069 also encodes a cyanase gene (*cynS*; Spasov *et al.*, 2020). Cyanate can be converted to ammonium by some nitrite oxidizers which can then supply ammonia oxidizers via cross-feeding (Palatinszky *et al.*, 2015). The ability to grow on cyanate was previously demonstrated in one AOA, *Ca.* N. gargensis, and that culture remains the only ammonia oxidizer shown to grow on cyanate (Palatinszky *et al.*, 2015). Other than MAG069 and MAG093 (another *cynS*-encoding *Nitrospira* MAG recovered from the RBCs by Spasov *et al*., 2020) and its closely related sister taxa, *Nitrospira* MAG LK70 has also been identified as a *cynS*-encoding MAG (Yang *et al*., 2020). However, the authors indicate that *in situ* transcription was not observed for the microorganism related to MAG LK70 (Yang *et al.*, 2020). Findings by Spasov *et al.* (2020) and Yang *et al.* (2020) imply that the potential ability to use cyanate for energy might provide comammox *Nitrospira* the advantage to compete with other nitrifiers in environments with low ammonia levels, in addition to detoxifying cyanate produced, for example, during growth on urea or from the intracellular dissociation of carbamoyl phosphate. Figure 2.8 shows that the G4-derived *cynS* gene PCR product is closely related to MAG069-associated comammox *Nitrospira*; this was expected because the used primer set targets the *cynS* gene of MAG069.

For quick screening of the enrichment cultures G4 and G8 for *cynS* gene, the newly designed primer set was used to detect the presence or absence of comammox *Nitrospira* MAG069 *cynS* gene in enrichment cultures. Indeed, as expected, the MAG069 *cynS* gene was detected in cyanate-fed cultures (as well as ammonium-fed G4 cultures) and was undetectable in G8, which corresponded to a MAG without a *cynS* gene (Table 2.10).

To test the ability of G4 to grow on cyanate, duplicate subcultures of G4 were supplemented with 0.5 mM potassium cyanate. If active, comammox *Nitrospira* associated with MAG069 or other canonical *Nitrospira* present in the culture should convert cyanate to ammonium and then to nitrate. Thus, cultures were monitored for total ammonium, nitrite, and nitrate production. Cyanate was initially converted to ammonium in ~1 – 1.5 weeks and then to nitrate (see Table 2.8). Because cyanate can be converted to ammonium and carbon dioxide chemically, abiotic controls were included but abiotic conversion of cyanate to ammonium was slower than the biotic incubation counterpart (Table 2.9). Nitrite and nitrate were generated in the cultures but not in the abiotic controls (Tables 2.8 and 2.9), indicating that most activity observed in cyanate-fed cultures was biological and possibly caused by the activity of *Nitrospira*. Note that culture G8 and its related subcultures, which contain MAG044-related comammox *Nitrospira*, were not tested for growth on cyanate (MAG044 does not encode the *cynS* gene).

During growth on cyanate, ammonia was detected in the G4 culture incubation, presumably available for all nitrifiers to use. Consequently, using cyanate alone as an energy source may be an ineffective way to isolate comammox *Nitrospira* in environments like the RBC biofilm, where several nitrifying guilds co-exist. Nonetheless, these findings provide a glimpse into the potential metabolic versatility of comammox *Nitrospira* in the RBCs.

Table 2.8. Sample cyanate degradation profile for a 0.5 mM cyanate fed enrichment culture

Culture ID	Time (day)	Total ammonium	Nitrite	Nitrate (μM)
G4_sub5B cyanate	1	–	–	–
	3	–	–	–
	5	++	+	<50
	7	+++	+	100
	10	+	–	250
	21	–	–	>500

(+) represents detectable nitrogenous compound, whereas (–) represents the absence of detectable nitrogenous compound. Strength of signal is denoted by + (weak), ++ (strong), or +++ (stronger). Total ammonium concentration was estimated using a spot test via addition of Nessler's reagent. Nitrite and nitrate concentration estimates were determined using nitrite/nitrate test strips. Results in this table are representative of general trends observed with biotic cyanate incubations.

Table 2.9. Sample profile for the abiotic degradation of 0.5 mM potassium cyanate in AOM medium

Culture ID	Time (day)	Total ammonium	Nitrite	Nitrate (μM)
Cyanate in medium (abiotic control)	1	–	–	–
	3	–	–	–
	5	–	–	–
	7	+	–	–
	10	++	–	–
	21	++	–	–
	30	+++	–	–

(+) represents detectable nitrogenous compound, whereas (–) represents the absence of detectable nitrogenous compound. Strength of signal is denoted by + (weak), ++ (strong), or +++ (stronger). Total ammonium concentration was estimated using a spot test via addition of Nessler's reagent. Nitrite and nitrate concentration estimates were determined using nitrite/nitrate test strips. Results in this table are representative of general trends observed with abiotic cyanate incubations.

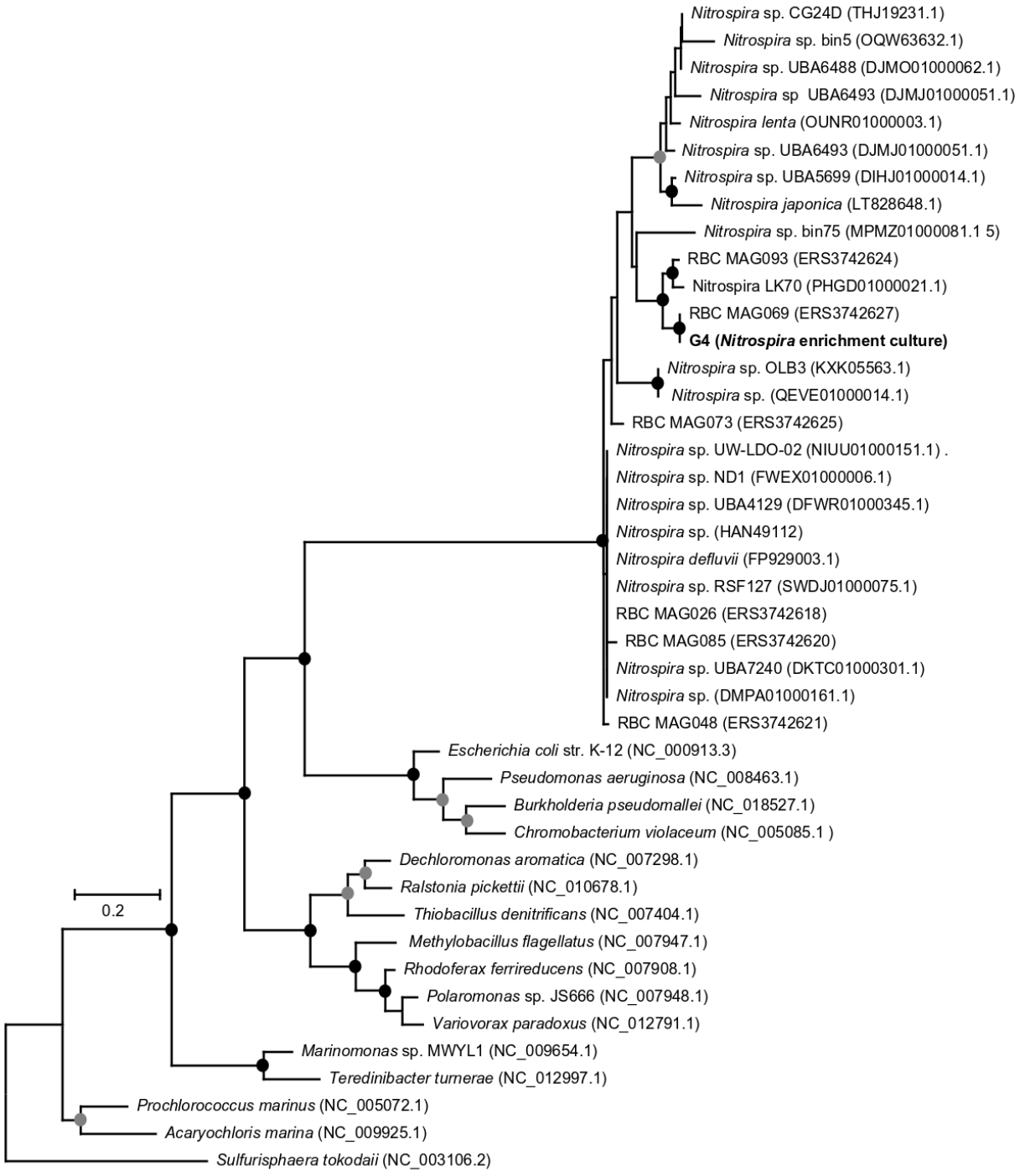
Nitrospira sp. CG24D (THJ19231.1)
Nitrospira sp. bin5 (OQW63632.1)
Nitrospira sp. UBA6488 (DJMO01000062.1)
Nitrospira sp UBA6493 (DJMJ01000051.1)
Nitrospira lenta (OUNR01000003.1)
Nitrospira sp. UBA6493 (DJMJ01000051.1)
Nitrospira sp. UBA5699 (DIHJ01000014.1)
Nitrospira japonica (LT828648.1)
Nitrospira sp. bin75 (MPMZ01000081.1 5)
RBC MAG093 (ERS3742624)
Nitrospira LK70 (PHGD01000021.1)
RBC MAG069 (ERS3742627)
G4 (*Nitrospira* enrichment culture)
Nitrospira sp. OLB3 (KXK05563.1)
Nitrospira sp. (QEVE01000014.1)
RBC MAG073 (ERS3742625)
Nitrospira sp. UW-LDO-02 (NIUU01000151.1) .
Nitrospira sp. ND1 (FWEX01000006.1)
Nitrospira sp. UBA4129 (DFWR01000345.1)
Nitrospira sp. (HAN49112)
Nitrospira defluvii (FP929003.1)
Nitrospira sp. RSF127 (SWDJ01000075.1)
RBC MAG026 (ERS3742618)
RBC MAG085 (ERS3742620)
Nitrospira sp. UBA7240 (DKTC01000301.1)
Nitrospira sp. (DMPA01000161.1)
RBC MAG048 (ERS3742621)
Escherichia coli str. K-12 (NC_000913.3)
Pseudomonas aeruginosa (NC_008463.1)
Burkholderia pseudomallei (NC_018527.1)
Chromobacterium violaceum (NC_005085.1)
Dechloromonas aromatica (NC_007298.1)
Ralstonia pickettii (NC_010678.1)
Thiobacillus denitrificans (NC_007404.1)
Methylobacillus flagellatus (NC_007947.1)
Rhodoferax ferrireducens (NC_007908.1)
Polaromonas sp. JS666 (NC_007948.1)
Variovorax paradoxus (NC_012791.1)
Marinomonas sp. MWYL1 (NC_009654.1)
Teredinibacter turnerae (NC_012997.1)
Prochlorococcus marinus (NC_005072.1)
Acaryochloris marina (NC_009925.1)
Sulfurisphaera tokodaii (NC_003106.2)
0.2

Figure 2.8. Phylogenetic tree showing the relationship of *cynS* nucleotide sequence detected in G4 (bolded) to reference sequences from the Genbank database. Accession numbers for reference sequences are shown in brackets. The tree was constructed using the maximum likelihood method based on the General Time Reversible model (Tavaré, 1986) with Gamma distribution across sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. A total of 500 bootstrap replicates were used. Nodes indicated with circles (●) have a bootstrap value >90% and nodes indicated with gray circles (●) have a bootstrap value between 60 – 90%. Phylogenetic analysis was done using MEGAX (Kumar et al., 2018). The tree was rooted with the *cynS* sequence of *Sulfurisphaera tokodaii*.

Table 2.10. Summary of presence or absence of comammox *Nitrospira cynS* gene in enrichment cultures

Culture ID	Date of establishment	Description of culture	MAG069 *cynS* gene	Possible growth on cyanate?
G4	Dec, 2018	0.45-µm filtered suspension in AOM	++	Yes
G4_sub5B_cyanate	Dec, 2019	Subculture of G4_sub4 fed 0.5 mM potassium cyanate	+++	Yes
G4_sub6B_cyanate	March, 2020	Subculture of G4_sub5 fed 0.5 mM potassium cyanate	+++	Yes
G4_sub7B_ ammonia	June, 2020	Subculture of G4_sub6 fed 0.5 mM ammonia chloride	++	ND
G8	June, 2019	Inoculum (G4) passed through 0.8-µm filter and incubated at room temperature	–	ND
G8_sub2B_ acriflavine	March, 2020	Subculture of G8 with 0.2 µm/ mL acriflavine	+ (weak)	ND
G8_sub1B_tetracycline	April, 2020	Subculture of G8 with 30 µg/ mL tetracycline	++	ND

(+) represents the presence of *cynS* product, and (–) represents the absence of *cynS* product. Strength of signal is denoted by + (weak), ++ (strong), or +++ (stronger). ND = not determined.

2.3.4 Culture purification

Several approaches were tested to eliminate AOA from comammox *Nitrospira* enrichment cultures. One approach was to use the eukaryotic inhibitor cycloheximide. Because archaea share similar protein synthesis machinery to eukaryotes, cycloheximide was previously shown by Vajrala *et al.* (2014) to inhibit the ammonia-oxidizing activity of the AOA *N. maritimus* in pure culture. Cycloheximide was added at the 200 µg/mL, a concentration recommended by Vajrala *et al.* (2014), to triplicate subcultures from the actively growing G4 enrichment (G4_sub1). Cultures were monitored for ammonia, nitrite, and nitrate. Once cultures depleted ammonia and generated nitrate, genomic DNA was harvested and end-point PCR targeting the 16S rRNA and *amoA* genes of AOA was carried out. Based on end-point PCR results, the addition of cycloheximide to enrichment cultures did not reduce the AOA 16S rRNA and *amoA* gene signals detected over the course of the two-month incubation with cycloheximide (Table 2.11). It is important to note that those enrichment cultures were not followed through a second round of subculturing (*i.e.,* G4_sub3_cycloheximide); therefore, it remains unclear whether the addition of cycloheximide was useful at eliminating the AOA in enrichment cultures.

Table 2.11. Summary of presence or absence of AOA 16S rRNA and *amoA* genes in enrichment cultures treated with cycloheximide

Culture ID	Date of establishment	Description of culture	Presence/ Absence	
			AOA *amoA* genes	AOA 16S rRNA genes
G4_sub2A_cyc	July, 2019	Subculture of G4_sub1 supplemented with cycloheximide	++	++
G4_sub2B_cyc	July, 2019	Subculture of G4_sub1 supplemented with cycloheximide	+	+
G4_sub2C_cyc	July, 2020	Subculture of G4_sub1 supplemented with cycloheximide	+	++
G4_sub2B	July, 2020	Subculture of G4_sub1 (control)	+	+

(+) represents the presence of 16S rRNA or *amoA* product. Strength of signal is denoted by + (weak), ++ (strong), or +++ (stronger).

Another strategy used to eliminate AOA was incubation of enrichment cultures with elevated ammonium concentrations. Based on prior research by Sauder *et al.* (2017), *Ca.* N. hydrocola was shown to be completely inhibited at 30 mM ammonium chloride; a relatively high inhibitory ammonium concentration (Hatzenpichler *et al.*, 2008; Sauder *et al.*, 2017). Despite several reports showing high affinity of comammox *Nitrospira* to ammonia, it is unclear whether all comammox *Nitrospira* are adapted to low ammonia environments. Moreover, previous work has shown the presence of comammox *Nitrospira* in the aeration basins of the Guelph WWTP, where ammonia concentrations range from 0.6 – 3 mM (data not shown). As such, several ammonia concentrations were tested on fresh transfers from G4, including 1 mM, 3 mM, 10 mM, and 30 mM. Cultures were monitored for ammonia depletion, and nitrite/nitrate production. Once ammonia depleted (~ 2 months to deplete 30 mM NH_4Cl), genomic DNA was extracted and end-point PCR targeting 16S rRNA and *amoA* genes of AOA (along with comammox

Nitrospira amoA) was carried out (Table 2.12). Comammox *Nitrospira amoA* gene from enrichment cultures (G4_sub2) were detected even after incubation with 30 mM ammonia (although effects of elevated ammonia concentrations on cell growth was untested). A similar high ammonia tolerance by *Nitrospira* was previously observed in high ammonia wastewater by Mota *et al.* (2005). Although comammox *Nitrospira* were not discovered in 2005, the primer set used targets the 16S rRNA genes of *Nitrospira* and therefore it is possible that the researchers were detecting comammox *Nitrospira* (Mota *et al.*, 2005). Regardless, based on end-point PCR results (Table 2.12, Figure 2.4), incubations with high ammonia concentrations did not reduce the AOA 16S rRNA and *amoA* gene signals detected over the course of the two-month incubation. Similar to cycloheximide-treated cultures, incubations with high ammonia concentrations were not followed through a second round of subculturing (*i.e.*, G4_sub3_30 mM); therefore, it remains unclear whether varying elevated ammonia concentrations was useful at eliminating the AOA in enrichment cultures.

Table 2.12. Summary of presence or absence of AOA 16S rRNA and *amoA* genes, and comammox *Nitrospira amoA* gene in enrichment cultures supplemented with high ammonium concentrations

Culture ID	Description of culture	Presence/ Absence		
		AOA *amoA*	AOA 16S rRNA	Comammox *Nitrospira amoA*
G4_sub2A_1mM	Subculture of G4_sub1 supplemented with 1 mM NH_4Cl	+ (weak)	+	++
G4_sub2B_1mM	Subculture of G4_sub1 supplemented with 1 mM NH_4Cl	+ (weak)	+	++
G4_sub2C_1mM	Subculture of G4_sub1 supplemented with 1 mM NH_4Cl	+ (weak)	+	++
G4_sub2A_3mM	Subculture of G4_sub1 supplemented with 3 mM NH_4Cl	++	+	–
G4_sub2B_3mM	Subculture of G4_sub1 supplemented with 3 mM NH_4Cl	+	+	++
G4_sub2C_3mM	Subculture of G4_sub1 supplemented with 3 mM NH_4Cl	++	+	++
G4_sub2A_10mM	Subculture of G4_sub1 supplemented with 10 mM NH_4Cl	++	+	–
G4_sub2B_10mM	Subculture of G4_sub1 supplemented with 10 mM NH_4Cl	++	+	++
G4_sub2C_10mM	Subculture of G4_sub1 supplemented with 10 mM NH_4Cl	++	+	++
G4_sub2A_30mM	Subculture of G4_sub1 supplemented with 30 mM NH_4Cl	++	++	–
G4_sub2B_30mM	Subculture of G4_sub1 supplemented with 30 mM NH_4Cl	++	+	++
G4_sub2C_30mM	Subculture of G4_sub1 supplemented with 30 mM NH_4Cl	++	++	++

(+) represents the presence of 16S rRNA or *amoA* product, and (–) represents the absence of 16S rRNA or *amoA* product. Strength of signal is denoted by + (weak), ++ (strong), or +++ (stronger).

An additional purification strategy tested was a second filtration process using a relatively large pore size to accommodate possible changes in cell size during growth. Most AOA species

range from 0.4 – 2 µm in width (Lebedeva *et al.*, 2013; Li *et al*., 2016; Sauder *et al.*, 2017, 2018), whereas characterized *Nitrospira* species are relatively small, ranging between 0.2 – 0.5 in width (Ehrich *et al.*, 1995; Ushiki *et al*., 2013; Daims *et al*., 2015). Therefore, a filter with a pore size of 0.8-µm might preferentially remove AOA while still allowing the *Nitrospira* present in enrichment cultures to pass through. To test this hypothesis, an inoculum taken from G4, which was gently vortexed to disrupt clumps and then filtered through a 0.8-µm filter and into fresh AOM medium. The resulting filtrate was incubated at room temperature and supplemented with 0.5 mM ammonia (resulting culture is called G8). Cultures were monitored for ammonia depletion and nitrite/nitrate production as a marker for the activity of nitrifier communities. Once ammonia was fully depleted, genomic DNA was extracted and end-point PCR targeting 16S rRNA and *amoA* genes of AOA, as well as the *amoA* gene of comammox *Nitrospira* was performed (Table 2.13). Based on end-point PCR, a relatively weak AOA *amoA* (and 16S rRNA) signal was detected, compared to the comammox *amoA* gene signal, which suggests that filtration through a 0.8-µm filter helped decrease AOA abundance in cultures (Table 2.13 and Figure 2.9). The 16S rRNA gene profiles revealed that cultures that were filtered through a 0.8-µm filter (G8) and its subsequent transfers (G8_sub1 and G8_sub2) had a lower relative abundance of AOA (8.0 – 13%) and increased comammox *Nitrospira* (27 – 29%; Figure 2.9), compared to G4 (inoculum did not undergo a second round of filtration). Additionally, filtration through a 0.8-µm filter was effective at reducing the number of AOA taxa from 10 to 1 (Figure 2.9). To date, the only cultures that remain "AOA-free" are the duplicate cultures filtered through the 0.8-µm filter and supplemented with the antimicrobial acriflavine (*i.e.*, G8_sub2_acriflavine; Table 2.13 and Figure 2.9).

Table 2.13. Summary of presence or absence of AOA 16S rRNA and *amoA* genes, and comammox *Nitrospira amoA* gene in enrichment cultures filtered through a 0.8-µm filter

Culture ID	Date of establishment	Description of culture	Presence/ Absence		
			AOA *amoA*	AOA 16S rRNA	Comammox *Nitrospira amoA*
G8_A	August, 2019	Subculture of G4 filtered through 0.8-µm filter	+ (weak)	+ (weak)	++
G8_B	August, 2019	Subculture of G4 filtered through 0.8-µm filter	+ (weak)	+ (weak)	++
G8_sub2A_acriflavine	March, 2020	Subculture of G8_sub1 supplemented with acriflavine	–	–	++
G8_sub2B_acriflavine	March, 2020	Subculture of G8_sub1 supplemented with acriflavine	–	+	++
G8_sub2A_tetracycline	May, 2020	Subculture of G8_sub1 supplemented with tetracycline	+	+	++
G8_sub2B_tetracycline	May, 2020	Subculture of G8_sub1 supplemented with tetracycline	+	+	++

(+) represents the presence of 16S rRNA or *amoA* product, and (–) represents the absence of 16S rRNA or *amoA* product. Strength of signal is denoted by + (weak), ++ (strong), or +++ (stronger).

Addition of antimicrobials was used to help remove heterotrophs present in G8 and its subcultures. Acriflavine hydrochloride, an antimicrobial and a DNA intercalator that inhibits protein biosynthesis was previously used for the purification of *N. defluvii* (Nowka *et al.*, 2015). In addition, based on genomic data, MAG069 genome encodes a resistance gene against

acriflavine (AcrA/B acriflavine resistance protein) and tetracycline (Spasov *et al*., 2020). Taking advantage of this, enrichment cultures were supplemented with acriflavine or tetracycline. Because acriflavine is effective at eliminating Gram-negative bacteria, it was expected that heterotrophs like *Hyphomicrobium sp*. (ASV 2123), *Sphingobium* sp. (ASV 168), and uncultured *Brevundimonas* sp. (ASV 4571) were eliminated in further subcultures supplemented with acriflavine (G8_sub2_acriflavine and G8_sub1_tetracycline; Figure 2.9). Nonetheless, other putative heterotrophs persisted in enrichment cultures despite filtration and antibiotics, including *Terrimonas* sp. (ASVs 4 and 24; Figure 2.9) and *Sphingorhabdus* sp. (ASV 26; Figure 2.9). In addition, the AOA (ASV 464) present in previous G8 cultures was also removed from acriflavine-treated enrichments, suggesting that acriflavine may be directly removing the AOA or inhibiting the growth of symbiotic heterotrophic bacteria that are associated with AOA in the cultures.

The addition of tetracycline, a broad-spectrum antibiotic, to enrichment cultures was intended to eliminate heterotrophs. Tetracycline use for nitrifier cultures has precedent given that Liu *et al.* (2018) and Yim *et al.* (2006) showed *Nitrospira* relative abundances increase upon addition of 20 – 50 µg/L tetracycline to enrichment cultures. The authors speculated that tetracycline may function as an auto-inducer in the quorum sensing pathway (Yim *et al.*, 2006; Liu *et al.*, 2018). The addition of tetracycline may, therefore, provide a way to help increase the abundance (and biomass) of comammox *Nitrospira* in enrichment cultures. Tetracycline, like acriflavine, was not useful in removing *Dongia* sp. nor *Terrimonas* sp. (ASVs 4, 24, 50, and 78; Figure 2.9) and, given that the tetracycline cultures were established after the acriflavine-supplemented cultures, it appears that tetracycline may have a positive effect on the growth of *Nitrospira* (ASV 1; G8_sub1_tetracycline; Figure 2.9). Additional methods, like the use of optical tweezers (Nowka

et al., 2015) and serial dilutions (Daims *et al.*, 2015; Nowka *et al.*, 2015), will likely be required in conjunction with filtration to eliminate AOA and isolate comammox *Nitrospira*.

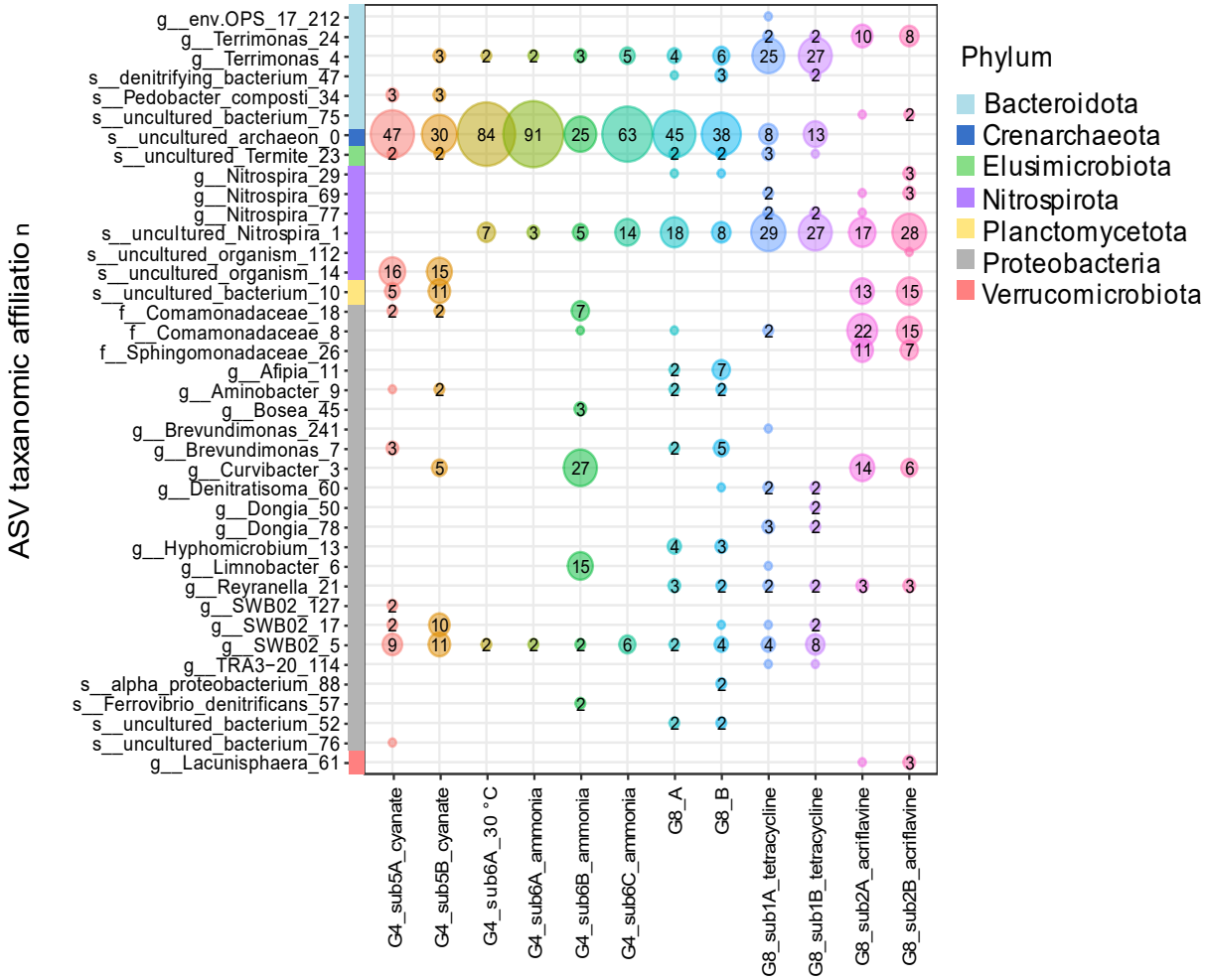

Figure 2.9. Relative abundances of amplicon sequence variants (ASVs) in enrichment cultures based on 16S rRNA gene amplicon sequencing. Only ASVs ≥1% relative abundance are displayed. Numbers shown after the underscore are the ASV number. Letters shown before the ASV label represent the highest taxonomic rank assigned. S= species, g= genus; f= family. The number inside the bubbles represents the percent relative abundance.

2.4 Conclusion

Two enrichment cultures of comammox *Nitrospira* were established by using a combination of filtration (G4 and G8) and antimicrobials (G8 only). The two cultures actively oxidized ammonia to nitrate, with no detectable nitrite (Figure 2.3). Enriched comammox *Nitrospira* (MAG069-related *Nitrospira* and MAG044-related *Nitrospira*) are novel clade A comammox *Nitrospira* ecotypes that cluster with other wastewater comammox *Nitrospira* (Figures 2.4 and 2.6). The comammox *Nitrospira* present in the enrichment culture G8 (MAG044-related comammox *Nitrospira*) grew under tested ammonia-fed conditions at room temperature supplemented with antimicrobials (Figure 2.3 panel B). The growth of the enrichment culture G4 on cyanate provides a glimpse into the potential metabolic versatility of *Nitrospira* in the RBCs, requiring examination. In G4, comammox *Nitrospira* remain in a co-culture status despite the application of several strategies to eliminate the AOA (Figure 2.9). In G8 supplemented with acriflavine (*i.e.*, G8_sub2_acriflavine), detectable comammox *Nitrospira* are ~34% enriched and are the sole ammonia oxidizer present in the culture (Figure 2.9). The AOA present in G4 enrichments is also a novel species, previously detected in the RBCs at less than 0.1% relative abundance (Spasov *et al.*, 2020). It would be interesting in the future to purify and isolate this AOA species and study its physiology, metabolism, and interaction with comammox *Nitrospira* in the enrichment cultures.

Chapter 3 The activity of nitrifiers in enrichment cultures and RBC biofilm suspensions

3.1 Introduction

Comammox bacteria have so far been detected using metagenomics and PCR-based approaches in artificial and natural systems (Palomo *et al.*, 2016; Pinto *et al.*, 2016; Pjevac *et al.*, 2017; Wang *et al.*, 2017, 2020; Spasov *et al.*, 2020), yet the extent of their contribution to nitrification in many of those environments, including wastewater, remains largely unexamined. Efforts to study the activity of comammox *Nitrospira* in wastewater treatment systems have been made by several groups. For example, Zheng *et al.* (2019) used RT-qPCR along with sequencing to show that comammox *Nitrospira* dominated nitrification in eight full-scale WWTPs. Likewise, a combination of metagenomic and metatranscriptomic approaches demonstrated that comammox *Nitrospira* were active in one of the two examined full-scale WWTPs (Yang *et al.*, 2020). Together, these findings provide insight into the activity and contribution of comammox *Nitrospira* to nitrogen cycling. Although methods like RT-qPCR, coupled with sequencing and metatranscriptomics, are valuable and can provide a holistic view of possible activities of nitrifying guilds in engineered environments, they do not assess nitrification activity directly. Differential inhibitors, in combination with qPCR and NH_4^+/NO_2^-/NO_3^- monitoring, can provide an effective means to study the relative activity and contribution of nitrifying guilds in an environment.

Recent findings by Spasov *et al.* (2020) have implicated comammox *Nitrospira* as dominant ammonia oxidizers in Guelph RBCs. Despite the high abundance of comammox *Nitrospira* in these biofilms, their contributions to nitrification remain uninvestigated. To fill this gap, I

examined the effects of known nitrification inhibitors on the activity and abundance of comammox *Nitrospira*. The effects of simvastatin, c-PTIO, ATU, DCD, chlorite, and chlorate were tested on comammox *Nitrospira* enrichment cultures (G4 and G8) and RBC biofilm microcosms, two AOA representatives (*Ca.* Nitrosotenuis aquarius and *Nitrosopumilus maritimus*), and the AOB *Nitrosomonas europaea*.

3.2 Materials and methods

3.2.1 Sample collection

Biofilm samples were collected from RBC NW1 using a sterile swab as in chapter 2 (see section 2.2.1). RBC water used for the biofilm suspension was also collected from the NW1 stage. Several biofilm swabs fully coated with biofilm material were swirled in ~3000 mL RBC water to create three visually turbid cell suspensions. The biofilm suspensions were transported back to the lab on ice until further processing.

3.2.2 Reagents used

The effects of four nitrification inhibitors, including carboxy-PTIO, ATU, DCD, and simvastatin were assessed on reference cultures and the G4 and G8 enrichment cultures. Additionally, five nitrification inhibitors, including c-PTIO, ATU, DCD, chlorite, and chlorate, were tested in RBC cell suspension microcosms. All reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada). Stock solutions were prepared in water (ATU, c-PTIO, chlorite, chlorate, and DCD) or in 100% dimethyl sulfoxide (DMSO; simvastatin) and sterilized by passage through a 0.1-μm pore size PVDF filter (Millipore, Billerica, USA).

3.2.3 Short-term incubation of comammox enrichments and reference cultures with nitrification inhibitors

The effects of the nitrification inhibitors c-PTIO, ATU, DCD, and simvastatin were evaluated on the activity of two aquatic AOA representatives, *N. maritimus* and *Ca.* N. aquarius, the AOB representative *N. europaea* (obtained from the German Collection of Microorganisms and Cell Cultures; DSMZ 28437) and two comammox *Nitrospira* containing enrichment cultures (G4 and G8). The effects of chlorite and chlorate on nitrification by enrichment and reference cultures were not assessed because earlier incubation trials with chlorite and chlorate in the RBC biofilm were inconclusive, and a potential interference of chlorate in nitrite/nitrate quantification was observed in those trials. Incubations were performed in AOM media for G4, G8, *N. europaea*, and *Ca.* N. aquarius, and synthetic crenarchaeota medium (SCM) for *N. maritimus* (Könneke *et al.*, 2005; Daims *et al.*, 2015; Sauder *et al.*, 2018). Ammonia depletion, nitrite and nitrate accumulation were measured over time to assess the activity of nitrifiers.

Due to logistical constraints, only a single effective inhibitor concentration was tested per inhibitor in the experiment (see Figure 3.1 for experimental design). Concentrations used were based on prior research in enrichment cultures or environmental samples (Hooper and Terry, 1973; O'Callaghan *et al.*, 2010; Lehtovirta-Morley *et al.*, 2013; Sauder *et al.*, 2016; Kits *et al.*, 2017; Tatari *et al.*, 2017; Cui *et al.*, 2020; Li *et al.*, 2020). Four nitrification inhibitors were tested in this experiment, including c-PTIO at 100 µM, ATU at 10 µM, DCD at 10 mM, and simvastatin at 8 µM. Simvastatin stock solution (100 µM) was prepared by dissolving simvastatin in 100% DMSO. An aliquot of the simvastatin stock solution was diluted directly in culture flasks to reach the desired simvastatin concentration (8 µM) and a DMSO concentration of 0.1% (v/v) in AOM media. Potential toxicity of DMSO on ammonia-oxidizing activity was assessed by monitoring ammonia depletion and nitrite/nitrate generation in G4 enrichment

cultures supplemented with DMSO at 0.1%. All cultures were incubated in duplicates in the dark without shaking. One mL of culture was sampled (12-hour sampling for the first two days, followed by 24-hour sampling for 16 days) for estimation of activity through changes in the concentration of ammonia, nitrite, and nitrate.

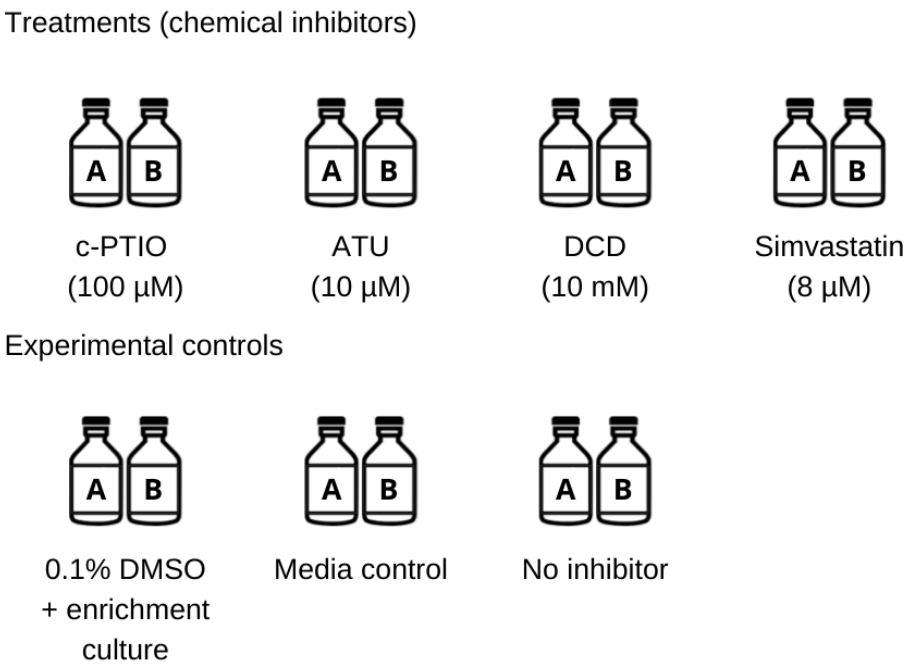

Figure 3.1. Experimental set up of enrichment and reference cultures with differential inhibitors. The letters A and B denote duplicates.

3.2.4 Short-term incubation of RBC biofilm suspensions with nitrification inhibitors

The effects of the nitrification inhibitors c-PTIO, ATU, DCD, chlorite, and chlorate were evaluated on the activity of ammonia-oxidizing guilds present in the biofilm of Guelph's RBCs (see Figure 3.2 for experimental design). Nitrification inhibitors were used in conjunction with filtration, a method shown to eliminate the presence of AOB in the cultures. Sample bottles containing RBC cell suspension were gently vortexed (pre-mixed) to homogenize the cell

suspension before membrane filtration. The entire cell suspension volume (~3000 mL) was filtered through Whatman paper to remove larger aggregates; this suspension was then divided into two volumes of ~1400 mL. One 1400 mL volume was further subdivided into seven volumes of 200 mL, and each 200 mL of cell suspension was passed through a separate 0.45-µm filter. The resulting filtrate was then filtered through a 0.1-µm filter to recover community members smaller than 0.45-µm on the 0.1 µm filter surface ("size-fractionated cell suspension"). The other 1400 mL was split into seven volumes of 200 mL, and each 200 mL of cell suspension was directly filtered through a 0.1-µm filter ("unfractionated cell suspension"). Following fluid passage, the 0.1-µm membrane filters were cut into four sections, with one section immediately frozen for subsequent DNA analysis. The other three sections were used as starting inocula for three cultures. For killed biomass controls, size-fractionated or unfractionated cell suspension was used as starting inoculum and the resulting suspension was then autoclaved twice (at 121 °C for 20 minutes). Incubations with inhibitor were carried out in 30 mL of the AOM medium in 100 mL serum bottles (Hatzenpichler *et al.*, 2008; Daims *et al.*, 2015; Sauder *et al.*, 2017). The medium was supplemented with 0.5 mM ammonium chloride. All bottles were sealed with silicone stoppers for incubation in the dark at room temperature without shaking. All bottles were incubated for eight days with 12-hour sampling intervals for the first four days, followed by a 24-hour sampling interval thereafter. When sampled, 2 mL of each culture was collected and frozen in the -20°C freezer until processed.

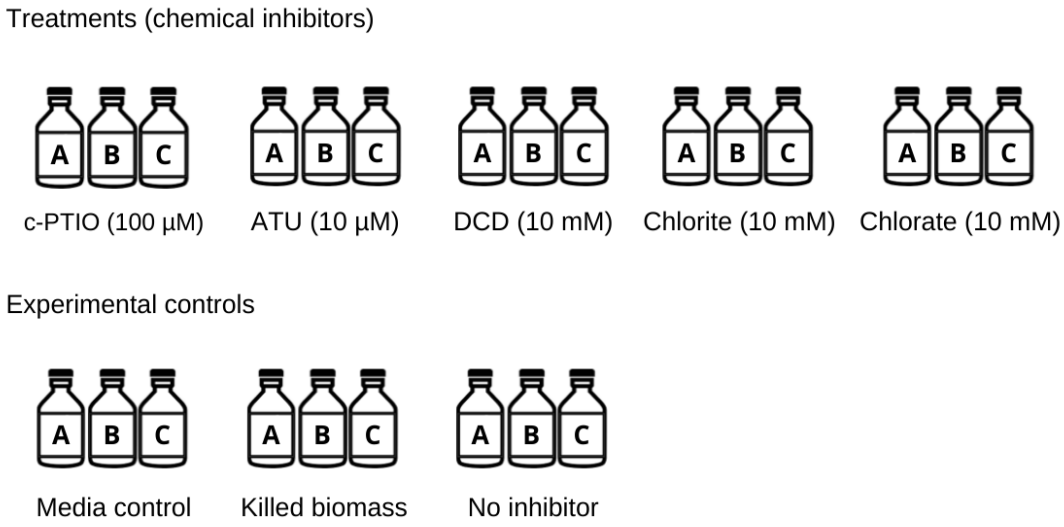

Figure 3.2. Experimental set up of RBC biofilm suspension with differential inhibitors. The letters A, B, and C denote triplicates.

3.2.5 Water chemistry measurements

Ammonia, nitrite, and nitrate measurements were measured for all sampling time points using the methods described in section 2.2.4. Note that background nitrate from the RBC water, which was ~ 200 µM, was not removed from nitrate measurements for microcosms. Both total ammonium and nitrite in the starting RBC biofilm suspension were negligible (< 10 µM).

3.2.6 DNA extractions and quantitative PCR (qPCR)

DNA extractions from filters were done using the Qiagen DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany). DNA concentration was quantified using a Qubit (Invitrogen, Waltham, MA). DNA extractions were performed as described in section 2.2.4. DNA concentration of extracted filter samples was normalized to 1 – 5 ng/µL.

Quantification of clade A comammox *Nitrospira amoA* gene was done using the degenerate primer set ComaA-244f (A-F) and ComaA-659r (A-R) (Pjevac *et al.*, 2017). Quantification of

AOA *amoA* gene was done using CrenamoA 23F/CrenamoA 616R primer set (Tourna *et al.*, 2008), and AOB *amoA* gene was quantified using the primer set amoA-1F/amoA-2R (Rotthauwe *et al.*, 1997) on a CFX96 thermal cycler system (Bio-Rad, Laboratories, Hercules, CA). All primers, annealing temperatures, and expected product sizes were based on the associated literature protocols (see Table 2.4).

The 10-µl reaction mixture contained 5 µL of the 2X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), 0.05 µL of forward and reverse primer at 100 µM, 0.5 µl of BSA at 10 mg/mL, and 4 µl of the template (at a concentration of 1 – 5 ng/µL). For clade A comammox *Nitrospira*, qPCR cycling conditions were: 98°C for 3 minutes, 35 cycles of 98 °C for 30 seconds (denaturation), 52°C for 45 seconds (annealing), 72°C for 60 seconds (extension) followed by a fluorescence read following the extension step. Melt curve analysis was carried out at 65–95°C, in 0.5°C increments. For AOA, qPCR cycling conditions were: 98 °C for 3 minutes, 35 cycles of 98 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 60 seconds. For AOB, qPCR cycling conditions were: 98 °C for 3 minutes, 35 cycles of 98 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 60 seconds. All quantitative PCR amplifications were performed in duplicate. Amplification efficiencies for qPCR runs ranged between 80 – 97% with $R^2 > 0.99$. Statistical analyses using the Student's t-test was performed using the software GraphPad Prism version 9 (GraphPad Prism Software, La Jolla, CA).

3.3 Results and discussion

3.3.1 Effects of nitrification inhibitors on enrichment cultures

Overall, the relative contributions of nitrifiers in the RBCs and enrichment cultures was explored using differential nitrification inhibitors. Major influences of nitrification inhibitors were assessed on bulk nitrification activity. The two enrichment cultures G4 (contained MAG069-associated comammox *Nitrospira*) and G8 (contained MAG044-associated comammox *Nitrospira*) were chosen to assess whether different strains of comammox *Nitrospira* might have varying sensitivities to the same inhibitor.

Carboxy PTIO (c-PTIO) is a nitric oxide scavenger that is widely applied as a selective inhibitor for archaeal ammonia oxidation in pure cultures as well as environmental samples. The addition of 100 µM c-PTIO, a concentration previously shown to inhibit comammox *Nitrospira* (Kits *et al.*, 2019), as well as cultured AOA representatives (Shen *et al.*, 2013; Martens-Habbena *et al.,* 2015; Sauder *et al.,* 2016), did not inhibit ammonia oxidation by *N. europaea* (Figure 3.3). However, c-PTIO inhibited ammonia depletion and nitrite production in the two tested AOA cultures *Ca.* N. aquarius and *N. maritimus* (Figure 3.3). These results are in agreement with previous research that demonstrated the effectiveness of c-PTIO as a selective inhibitor against those two AOA strains (Sauder *et al.,* 2016). Compared to the no inhibitor control, 100 µM c-PTIO resulted in some inhibition (~50% reduction) of ammonia oxidation in G4. This outcome was expected because G4 contained both AOA and comammox *Nitrospira*; therefore, it is likely that the AOA were inhibited by 100 µM c-PTIO. In G8, the addition of 100 µM c-PTIO did not result in the inhibition of ammonia oxidation. Together, these findings suggest that the two comammox *Nitrospira* ecotypes present in G4 and G8 enrichments were insensitive to 100 µM c-PTIO (Figure 3.3). It is important to note that AOB were absent in both enrichment cultures

(Figure 2.9), and therefore, observed activity was attributed to comammox *Nitrospira*. Previous research by Kits *et al.* (2019) demonstrated that the ammonia oxidation activity by *N. inopinata* was completely inhibited at a lower PTIO concentration than the one used here (~63 µM). The authors suggest that the comparable nitric oxide kinetics between *N. inopinata* and AOA make comammox *Nitrospira* more sensitive to inhibition by low concentrations of PTIO (Kits *et al.*, 2019). Here, inhibition of comammox *Nitrospira* by PTIO at 100 µM was not observed, implying that comammox *Nitrospira* present in G4 and G8 may exert less control over the production of nitric oxide compared to *N. inopinata*. This highlights the need to test those inhibitors against several comammox *Nitrospira* representatives before generalizing conclusions.

Allylthiourea (ATU), a copper chelator and an inhibitor of AOB and comammox *Nitrospira* activity, was used at 10 µM, a concentration shown effective against several AOB species (Lehtovirta-Morley *et al.*, 2013; Martens-Habbena *et al.*, 2015). Predictably, ammonia oxidation was completely inhibited in *N. europaea* by ATU and it did not generally affect the activity of *N. maritimus* or *Ca.* N. aquarius (Figure 3.3). Again, these results agree with previous findings by Sauder *et al.* (2016) who showed that in liquid cultures, AOB were sensitive to 10 µM ATU and these two AOA species were not. The effect of 10 µM ATU was differential on G4 and G8 enrichments (Figure 3.3). Complete nitrification inhibition was observed in G8 (similar to *N. europaea*), suggesting that comammox *Nitrospira* were inhibited by 10 µM ATU, as previously demonstrated by van Kessel *et al.* (2015). In G4, the addition of 10 µM ATU resulted in partial nitrification inhibition (~50% reduction) of ammonia oxidation. The most probable explanation is that the comammox *Nitrospira* in G4, like AOB and *N. inopinata*, is inhibited by 10 µM ATU as evidenced by G8, an enrichment culture that lacks AOA and AOB presence.

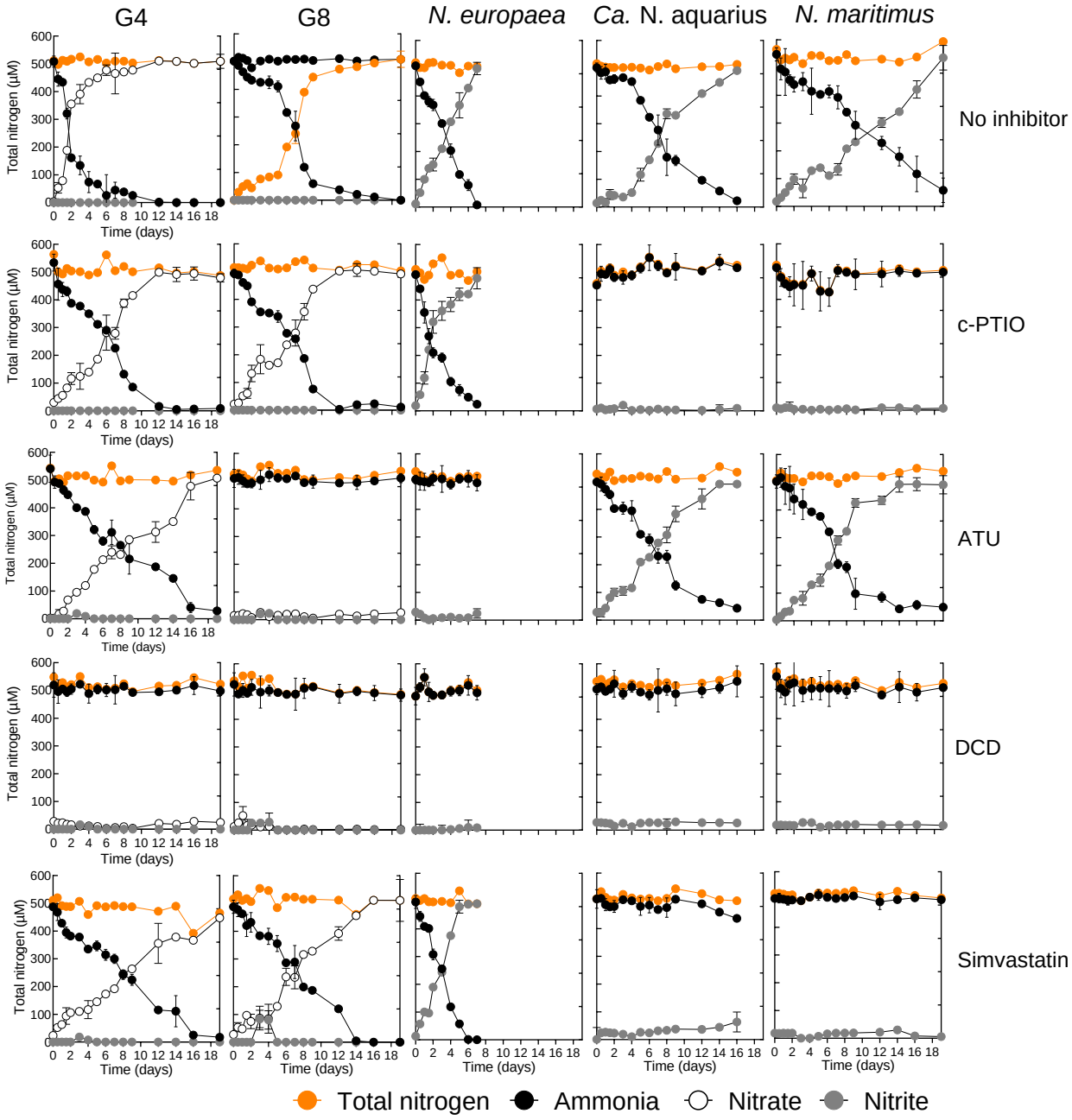

Figure 3.3. The effects of PTIO, ATU, DCD, and simvastatin on the activity of G4, G8, *N. europaea*, *Ca.* N. aquarius, and *N. maritimus*. All incubations were set up using 1% inoculum into fresh AOM media supplemented with 0.5 mM ammonium chloride. Error bars represent the standard deviation of duplicates. Error bars not shown are contained within symbols. Total nitrogen line shown represents total inorganic nitrogen and was calculated as the sum of total ammonium, nitrite, and nitrate concentrations at that sampling time point.

In G4, AOA, as well as several *Nitrospira* ASVs, are present; therefore, the observed activity is likely of the co-existing AOA and NOB *Nitrospira*. ATU is, therefore, only useful as a selective inhibitor for comammox *Nitrospira* in the absence of AOB.

Dicyanamide (DCD), a complete nitrification inhibitor widely used in the agricultural context, was previously shown to inhibit AOA, AOB, and comammox *Nitrospira* (Lehtovirta-Morley *et al.*, 2013; Fu *et al.*, 2018; Li *et al.*, 2020). As expected, 10 mM DCD inhibited nitrification completely in tested reference cultures as well as the two enrichment cultures (Figure 3.3). Thus, DCD is effective in inhibiting chemolithotrophic activity in liquid cultures. Similar conclusions were made by Jung *et al.* (2011), who reported that the activity of the AOA *Ca*. Nitrosoarchaeum koreensis and the AOB *N. europaea* are affected by the addition of 0.5 mM DCD, thereby providing additional evidence for the effectiveness of DCD as a nitrification inhibitor.

Simvastatin was recently shown to be a good selective inhibitor for the activity of AOA in pure cultures and in soil (Zhao *et al.*, 2020). Simvastatin was dissolved in 0.1% DMSO and used at 8 µM, a concentration shown effective for some terrestrial AOA (Zhao *et al.*, 2020). As expected, the ammonia-oxidizing activity of *N. maritimus* and *Ca.* N. aquarius was generally inhibited at 8 µM, whereas *N. europaea* activity was not (Figure 3.3). Although the ammonium depletion and nitrate accumulation rates were not calculated for this experiment, it appears that the addition of simvastatin at 8 µM resulted in ~50% nitrification inhibition in G4 (Figure 3.3). This result indicates that nitrifying activity by AOA was eliminated, and the observed ammonia-oxidizing activity can likely be attributed to comammox *Nitrospira*. Culture G8 showed little to no ammonia oxidation inhibition at 8 µM simvastatin (Figure 3.3). Transient nitrite accumulation along with nitrate production was briefly observed. Nonetheless, it appears that comammox

Nitrospira were generally not inhibited by 8 µM simvastatin. Taken together, these results suggest that the activity observed is likely caused by comammox *Nitrospira* in the enrichment cultures (again, AOB were absent from both enrichment cultures). Even though Zhao *et al.* (2020) did not examine the effects of simvastatin on comammox *Nitrospira*, findings from this study are valuable for the study of comammox *Nitrospira* (and AOB) activity in other environments (*e.g.*, aquaria and drinking water systems). Although effective on AOA species tested here, future studies should test simvastatin against other AOA species to determine whether AOA sensitivity to simvastatin can be generalized. Note that the addition of 0.1% DMSO resulted in some inhibition of activity in G4 compared to the no inhibitor control (Figure 3.4). Overall, the inhibitors used here provide clear evidence for the effectiveness of nitrification inhibitors in determining the relative contributions of nitrifiers in enrichment cultures.

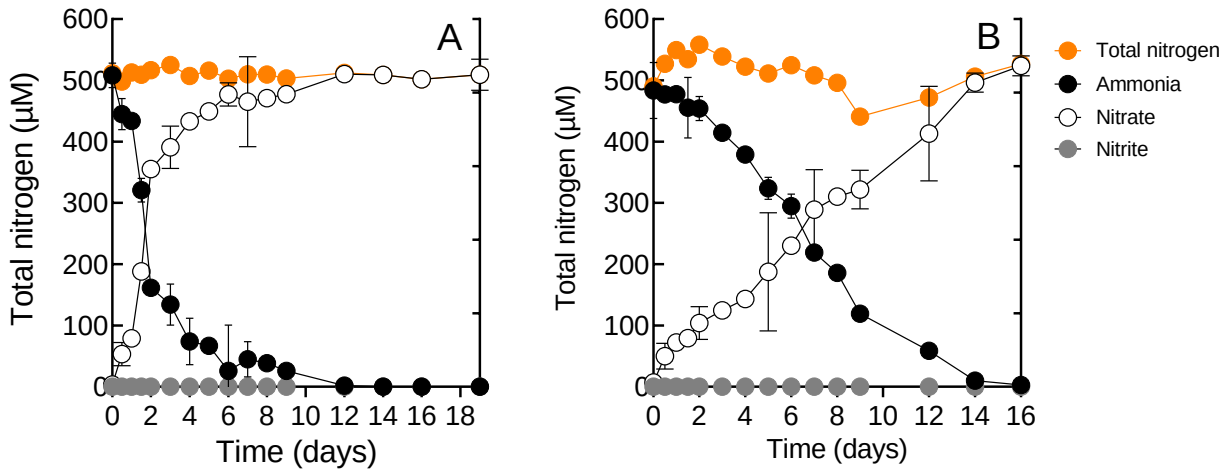

Figure 3.4. The effect of 0.1% DMSO on nitrification activity of G4 (panel B). Panel A shows G4 activity with no inhibitor. Incubation was set up using 1% inoculum into fresh AOM medium supplemented with 0.5 mM ammonium chloride. Error bars represent the standard error of duplicates. Error bars not shown are contained within symbols. Total nitrogen line shown represents total inorganic nitrogen and was calculated as the sum of total ammonium, nitrite, and nitrate concentrations at that sampling time point.

3.3.2 Effects of nitrification inhibitors on RBC biofilm suspensions

To establish whether the inhibition of AOB, AOA, and comammox *Nitrospira* in cultures reflected inhibition in the environment, effects of nitrification inhibitors on growth and activity were examined in microcosms containing size-fractionated or unfractionated cell suspensions. The purpose of the size fractionation was to eliminate AOB and study the relative activity of AOA and comammox *Nitrospira* in the RBC biofilm. It is, however, evident from the qPCR data (Figures 3.6 and 3.7) that filtration through a 0.45-µM filter did not eliminate AOB.

Compared to the no inhibitor control, the addition of 100 µM c-PTIO resulted in partial nitrification inhibition in unfractionated cell suspensions (Figure 3.5). Partial ammonia oxidation may be attributed to AOB or comammox *Nitrospira*, whose activity is not hindered by 100 µM c-PTIO. In addition, the relative proportions of AOB and comammox *Nitrospira amoA* genes remained unchanged throughout the course of the experiment (insignificant change, $p > 0.05$, for raw qPCR *amoA* proportions see Figure 3.7); therefore, it remains unclear whether the activity observed was caused by comammox *Nitrospira* or AOB. In the future, higher effective c-PTIO concentrations (*e.g.*, 300 – 400 µM) need to be tested to ensure complete inhibition of nitrification activity by AOA (Sauder *et al.,* 2016) and possibly comammox *Nitrospira*. The reason why the effective concentration of c-PTIO varies between enrichment culture and biofilm samples is likely due to differences in community composition and abundance of nitrifiers. In size-fractionated cell suspensions (Figure 3.6), ammonia-oxidizing activity was largely inhibited, and the relative proportion of the AOA *amoA* gene, unexpectedly, increased (significant t-test, $p < 0.0001$). This finding implies that c-PTIO may affect AOA activity but not their growth, or that other microorganisms present in the biofilm suspension are more prone to inhibition by the tested c-PTIO concentration, whereas AOA are in biostasis; hence the increase in relative proportions

of the AOA *amoA* gene. The slight increase in nitrate concentration indicates nitrite-oxidizing activity by NOB *Nitrospira* or that some comammox *Nitrospira* were not affected by the c-PTIO concentration used (Figure 3.6). Additionally, the increase in total ammonium concentration observed suggests that other processes like DNRA (conversion of nitrate to ammonia) may have occurred in the size-fractionated c-PTIO-treated cell suspensions (Figure 3.6).

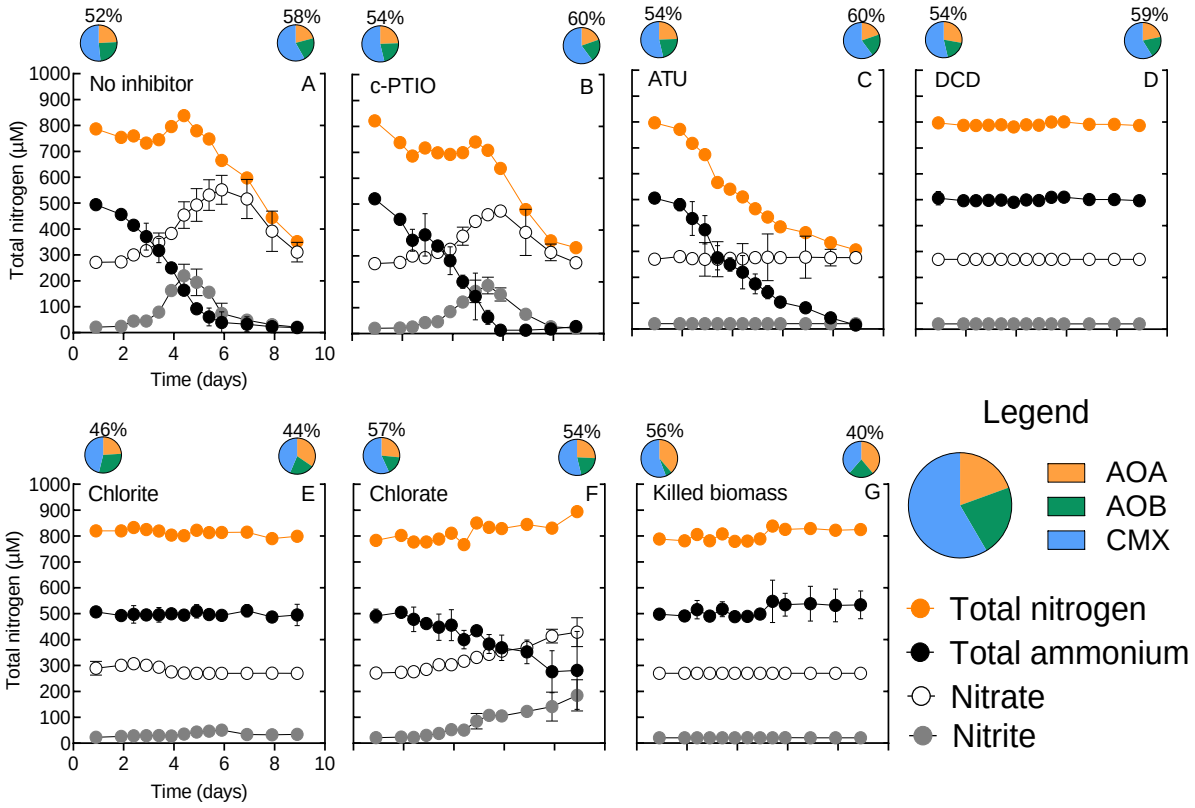

Figure 3.5. Effects of c-PTIO, ATU, DCD, chlorate, and chlorite on the growth and activity of nitrifiers in unfractionated cell suspensions. Error bars indicate standard deviations of triplicates. If not displayed, error bars are contained within symbols. Pie charts shown on top show the relative proportion of AOA, AOB, and comammox *Nitrospira amoA* genes at that time point (starting or end point). Percentages shown on top of the pie chart represent the proportion of comammox *Nitrospira amoA* in that sampling time point. CMX= comammox *Nitrospira*. Total nitrogen line shown represents total inorganic nitrogen and was calculated as the sum of total ammonium, nitrite, and nitrate concentrations at that sampling time point.

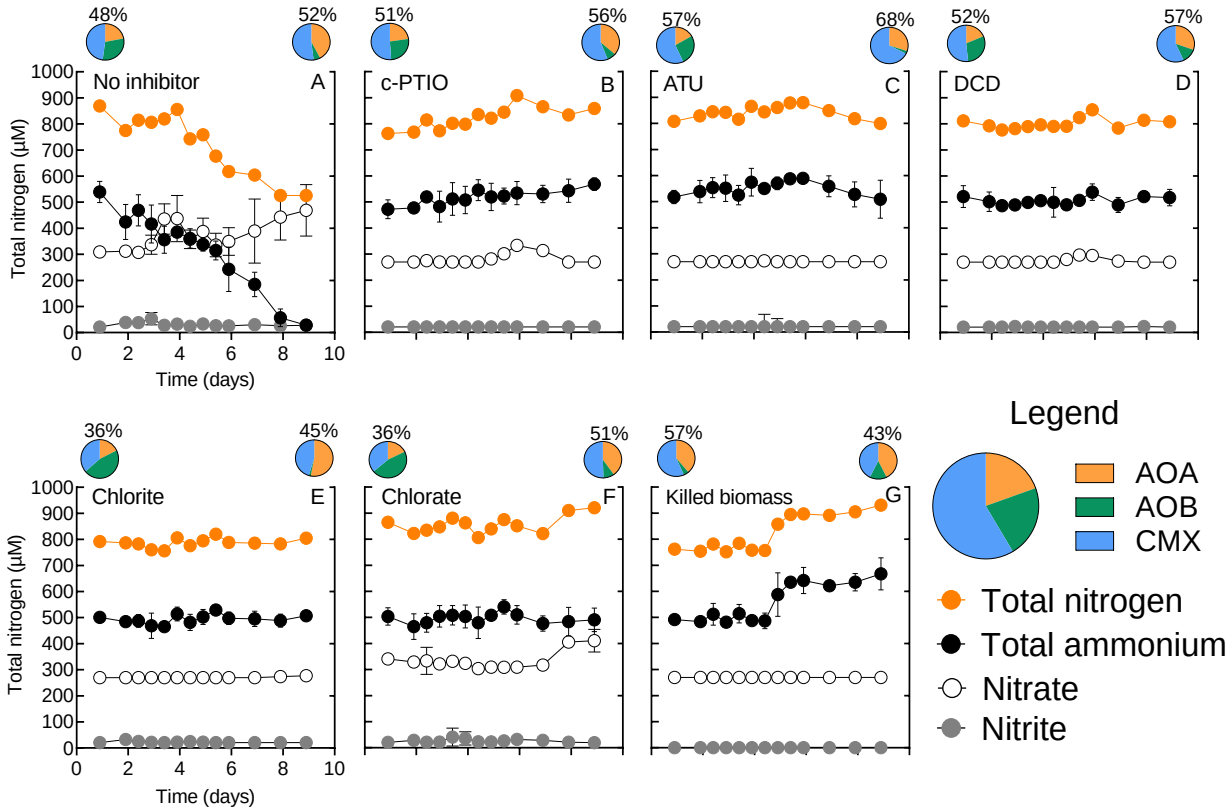

Figure 3.6. Effects of c-PTIO, ATU, DCD, chlorate, and chlorite on the growth and activity of nitrifiers in size-fractionated cell suspensions. Error bars indicate standard deviations of triplicates. If not displayed, error bars are contained within symbols. Pie charts shown on top show the relative proportion of AOA, AOB, and comammox *Nitrospira amoA* genes at that time point (starting or end point). Percentages shown on top of the pie chart represent the proportion of comammox *Nitrospira amoA* in that sampling time point. CMX= comammox *Nitrospira*. Total nitrogen line shown represents total inorganic nitrogen and was calculated as the sum of total ammonium, nitrite, and nitrate concentrations at that sampling time point.

Compared to the no inhibitor control, the addition of 10 µM ATU resulted in ammonia oxidation inhibition in both size-fractionated and unfractionated cell suspensions (Figures 3.5 and 3.6). Counterintuitively, the relative abundance of comammox *Nitrospira amoA* gene increased significantly in both size-fractionated and unfractionated cell suspensions with the addition of 10 µM ATU (t-test, $p < 0.05$). It is unclear why the relative abundance of comammox *Nitrospira amoA* gene increased throughout the experiment, and whether they utilized a different source of energy for growth, or if other microorganisms present in the biofilm suspension are more prone to inhibition by ATU, hence the increase in relative proportions of the comammox *Nitrospira amoA* gene. As an AOB and comammox *Nitrospira* inhibitor, the addition of ATU at 10 µM should not impact ammonia oxidation activity by AOA, yet nitrification was not observed in either size-fractionated or unfractionated cell suspensions. Additionally, in the unfractionated ATU-treated biofilm microcosms (Figure 3.5), the absence of nitrite and nitrate accumulation despite the decline of total ammonium implies that other processes that involve ammonia utilization in the RBC biofilm may have occurred. Taken together, activity and qPCR data imply that AOA in the RBC biofilm may not be participating in ammonia oxidation in the RBCs. This finding is in alignment with previous findings by Mussmann *et al.* (2011), who observed high relative abundance of AOA in WWTPs but demonstrated that the AOA were not participating in ammonia oxidation. For that reason, it is important to not make assumptions about the microorganism's activity based solely on presence and abundance data. Overall, it remains unclear whether the AOA play any role in nitrification and whether they use ammonia for energy conservation in the RBCs.

The chemolithotrophic nitrification inhibitor DCD inhibited nitrification in both the size-fractionated and unfractionated cell suspensions (Figures 3.5 and 3.6). Predictably, the relative

abundance of comammox *Nitrospira*, AOB, and AOA *amoA* gene proportions remained mostly unchanged throughout the course of the experiment. Overall, however, halted activity coupled with no changes in relative abundances of nitrifying guilds, indicates that DCD is a suitable inhibitor for complete inhibition of nitrification by chemolithoautotrophic microorganisms. This general trend is in agreement with prior findings, where DCD was observed to impede the activity of AOA, AOB, and comammox *Nitrospira* in various soil types (O'Callaghan *et al.*, 2010; Zhang *et al.*, 2012; Li *et al.*, 2020).

Chlorate, which is a nitrite oxidation inhibitor, was previously shown to inhibit the activity of nitrite-oxidizing bacteria and possibly comammox bacteria (Hynes and Knowles, 1983; Tatari *et al.*, 2017). Compared to the no inhibitor control, the addition of 10 mM chlorate resulted in the inhibition of ammonia and nitrite oxidation in size-fractionated cell suspensions (Figure 3.6), whereas in unfractionated cell suspensions, the addition of 10 mM chlorate resulted in partial inhibition of ammonia and nitrite oxidation. The slight increase in nitrite concentration observed in the unfractionated cell suspensions suggests ammonia-oxidizing activity by AOB or AOA, whose activity is not expected to be affected by the addition of chlorate. It is also possible that residual (partial) activity by comammox *Nitrospira* occurred and that comammox *Nitrospira* cells were not completely inhibited by 10 mM chlorate. Based on relative gene abundance data, it is unclear which of these scenarios occurred, as the relative proportions of comammox *Nitrospira*, AOA, and AOB *amoA* genes remained unchanged throughout the experiment. In size-fractionated cell suspensions, where ammonia and nitrite oxidation ceased, the increase in the relative AOA *amoA* gene abundance (significant t-test, $p < 0.05$) without activity suggests that the AOA may not be participating in ammonia oxidation or that other microorganisms growing in the biofilm suspension are more sensitive to inhibition by chlorate, thus the increase

in the relative proportion of the AOA *amoA* gene. It is important to note that at higher nitrate concentrations (*e.g.*, > 300 µM), chlorate reacted with nitrite/nitrate products to form an orange colored product, instead of the bright magenta normally observed. Therefore, it is possible that the measured product did not absorb at the same wavelength (*i.e.*, 550 nm) as the non-chlorate, nitrate-containing, samples resulting in interference with nitrite and nitrate measurements. This might explain the slight increase in nitrate observed around day five in size-fractionated and unfractionated samples. Regardless, it appears that chlorate affects ammonia and nitrite oxidation, therefore its selectivity as an inhibitor of nitrite oxidation and comammox *Nitrospira* activity in the RBC biofilm remains questionable.

The proposed mechanism of action for chlorate inhibition is via conversion of chlorate to chlorite by the activity of nitrite oxidoreductase (NXR). As such, chlorite is presumed to be the true nitrite oxidation inhibitor. In addition to testing chlorate against cell suspensions, chlorite was tested against size-fractionated and unfractionated cell suspensions. Like DCD, chlorite inhibited all nitrification in both size-fractionated and unfractionated cell suspensions (Figures 3.5 and 3.6). This was not surprising to observe as chlorite is a strong oxidizing agent with bactericidal activity.

Finally, it is evident that non-nitrification activities were also observed in the size-fractionated and unfractionated cell suspensions. For example, depletion of nitrate in c-PTIO-treated microcosms suggests active denitrification (or anammox) in some flasks (Figures 3.5 and 3.6). Because incubation flasks were not shaken and were generally left undisturbed, anoxic pockets were likely present and could have allowed anammox bacteria to convert ammonia and nitrite to dinitrogen gas. These observations were expected, given the heterogeneity of

community members present in the RBC biofilm, highlighting the complexity of this active system.

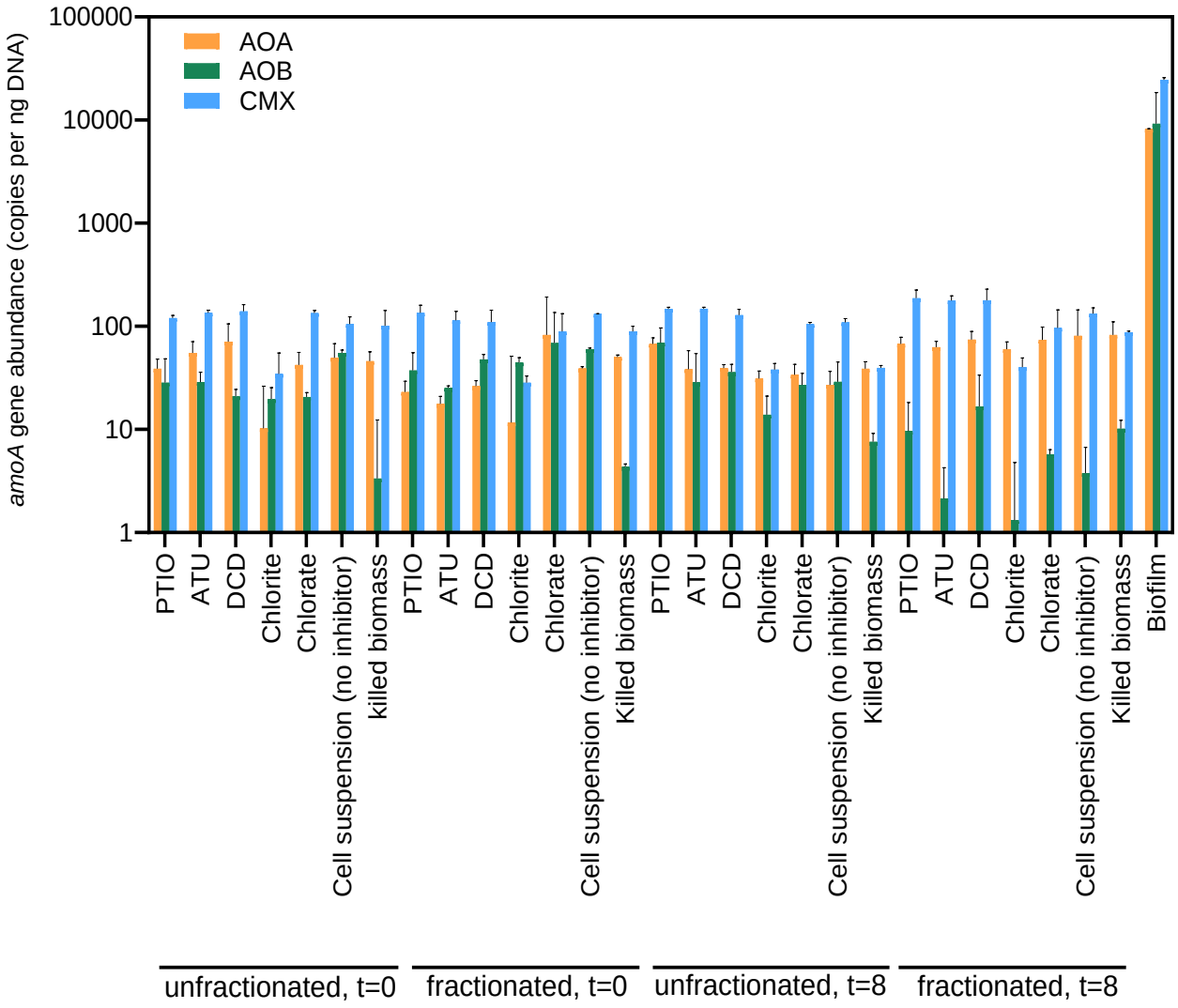

Figure 3.7. qPCR data showing relative proportions of AOB, AOA, and comammox *Nitrospira amoA* genes at time points zero (starting time point) and eight (last time point). Error bars represent the standard error for triplicates. Error bars not seen are contained within the bars. CMX= comammox. *amoA* gene copies were calculated based on the amount of DNA (ng) in the original DNA extract.

3.4 Conclusions

Based on this work, the comammox *Nitrospira* in my enrichments were sensitive to ATU, DCD, and insensitive to the tested c-PTIO concentration (in contrast to earlier findings for *N. inopinata*). Simvastatin inhibited both *Ca.* N. aquarius and *N. maritimus* and was therefore considered a good selective inhibitor for AOA (Figure 3.3). However, as with all other nitrification inhibitors, more testing against many phylotypes and cultured representatives will be necessary to corroborate those findings. Differences in the regulation of nitric oxide production and consumption, as well as reliance on copper (as a co-factor in several enzymes), may have resulted in the observed differences of sensitivity to the same nitrification inhibitor.

In RBC cell suspensions, the tested nitrification inhibitors did not perform comparably in the biofilm samples as in enrichment cultures. Instead, tested nitrification inhibitors highlighted the complexity of the nitrifying community in the RBC biofilm. In this instance, filtration through a 0.45-µm filter was not useful for eliminating AOB. Additionally, inhibitors like c-PTIO, chlorate, and ATU showed partial inhibition in unfractionated cell suspensions (Figure 3.5), likely suggesting the need for using a higher testing concentration in the future (*e.g.*, 300 µM for PTIO and 100 µM for ATU). Because of analytical interference concerns, caution needs to be taken when using chlorate as an indicator of the contribution of comammox *Nitrospira* to nitrification. Nevertheless, based on preliminary evidence shown here, it appears that comammox *Nitrospira* activity was inhibited by several nitrification inhibitors, suggesting that they likely play an active role in the RBCs (Figure 3.6). This work highlights the significance of including comammox bacteria when assessing nitrification in wastewater treatment plants.

Chapter 4 Summary of contributions and future directions

4.1 Summary of thesis contributions

The microbially mediated process of nitrification is an important component of the global nitrogen cycle. As such, ammonia and nitrite oxidizers play a key role in the transformation of nitrogen in natural and engineered environments. Ammonia and nitrite oxidizers ensure that nitrogen compounds (*e.g.*, ammonia and nitrite) do not accumulate in the environment. Wastewater treatment systems, for example, rely on the activity of nitrifiers (and denitrifiers or anammox bacteria) to remove toxic nitrogenous compounds from wastewater prior to discharge in receiving water. Ammonia, which can be found in wastewater, is converted to nitrate via the sequential activity of AOB or AOA, that convert ammonia to nitrite, followed by the activity of NOB, with an important role converting nitrite to nitrate. Alternatively, ammonia can be converted to nitrate by comammox *Nitrospira*. Owing to their recent discovery, comammox bacteria remain largely understudied in engineered environments. Recent work by Spasov *et al.* (2020) showed that comammox *Nitrospira* are the dominant nitrifying guild in the RBCs of the Guelph WWTP (a municipal WWTP). Currently, several cultured representatives of comammox *Nitrospira* exist and information on their nutritional requirements and growth conditions remain limited. Additionally, the activity and possible role of comammox *Nitrospira* in Guelph's WWTP remain uninvestigated. Therefore, the goal of this research was to establish an enrichment of comammox *Nitrospira* and to determine the general growth conditions required to cultivate comammox *Nitrospira* from the RBCs. Additionally, this research examined the possible activity of comammox *Nitrospira* in biofilm (mixed community) and in enrichment

cultures using differential nitrification inhibitors. This work represents a major step toward understanding the growth requirements of comammox *Nitrospira* in laboratory cultures, as well as the broad understanding of their contribution to nitrification in Guelph's RBCs.

In chapter 2, enrichment cultures containing two novel comammox *Nitrospira* ecotypes were obtained from the biofilm of Guelph's RBCs (Figures 2.7 and 2.9). Under ammonia-fed conditions, comammox *Nitrospira* oxidize ammonia to nitrate as in other comammox bacteria (see G8; Figure 2.3). In chapter 3, preliminary findings, based on enrichment cultures and biofilm incubations, suggest that comammox *Nitrospira* are likely active ammonia oxidizers and contribute to nitrification in the RBC biofilm (Figure 3.6). Work presented here can guide future research in exploring the ecology, physiology, and biochemistry of comammox *Nitrospira* in Guelph WWTP, as well as other engineered environments. Research presented in this thesis is built upon on existing body of research that examined nitrification and nitrifiers present in Guelph's RBCs (Sauder *et al.*, 2012, 2016; 2017; Spasov *et al.*, 2020). Along with previous findings, this thesis highlights the significance of the RBCs as a 440,000 m^2 bioreactor that houses multiple ammonia- and nitrite-oxidizing guilds. Given their fixed biofilm design and the ammonia gradient, the RBCs continue to be an important site for studying the ecology and diversity of nitrifiers in engineered systems.

4.1.1 The enrichment of comammox *Nitrospira* from the RBC biofilm

Although several studies have described the presence and distribution of comammox *Nitrospira* in WWTPs (Gonzalez-Martinez *et al.*, 2016; Pjevac *et al.*, 2017; Roots *et al.*, 2019; Zhao *et al.*, 2019; Spasov *et al.*, 2020), cultivation of these bacteria from WWTPs has not been attempted. To date, cultivation remains the main bottleneck in the biochemical and physiological

characterization of comammox *Nitrospira*. Pure and enrichment cultures are valuable because they allow testing of predictions from genomics-based studies, in addition to allowing us to understand the potential roles that microorganisms play in the environment.

The abundance and diversity of comammox *Nitrospira* in the RBCs have raised several questions regarding their ecophysiology and activity. To answer these questions, a comprehensive examination of comammox *Nitrospira* physiology and biochemistry is necessary. Currently there are only a small number of comammox *Nitrospira* strains in enrichment cultures (Table 1.1), and a single comammox *Nitrospira* species available in pure culture (Daims *et al.*, 2015; van Kessel *et al.*, 2015; Kits *et al.*, 2019; Fujitani *et al.*, 2020; Sakoula *et al.*, 2020). The physiological data obtained thus far, including ammonia and nitrite affinity, is solely derived from *Nitrospira inopinata*. In addition to permitting collection of physiological and biochemical data, cultures are useful for studying the effects of chemical inhibitors on the growth and activity of nitrifiers. As suggested by others previously, physiological and activity-related findings from *N. inopinata* may not apply to other comammox *Nitrospira* species; therefore, characterization of several cultured representatives is required before generalization. Given that comammox *Nitrospira* from the RBCs are phylogenetically dissimilar to cultivated comammox *Nitrospira* representatives (Spasov *et al*., 2020), I set out to enrich comammox *Nitrospira* from the RBCs. Additionally, preparing enrichment cultures was important to address my second objective, which assessed the major effects of known nitrification inhibitors on activity of comammox-containing enrichment cultures (cultures used a surrogate to study the activity of comammox *Nitrospira*). To obtain an enrichment culture of comammox *Nitrospira*, I used filtration (through a 0.45-µm filter) as a size selection method for comammox *Nitrospira*. The result was the enrichment of two novel, phylogenetically distinct, comammox *Nitrospira* belonging to clade A

sublineage II *Nitrospira* from the biofilm of the RBCs (Figures 2.7). The first enrichment (G4) is a co-culture of ammonia oxidizers with both AOA and comammox *Nitrospira* co-existing (Figure 2.9). The *Nitrospira amoA* present in G4 is nearly identical to the *amoA* gene encoded by MAG069 (Figure 2.7), which is a MAG previously detected in Guelph RBCs (Spasov *et al.*, 2020). MAG069 has the genetic repertoire for complete ammonia oxidation, as well as cyanate degradation. Preliminary evidence from my work suggests that G4 (which contains MAG069-related comammox *Nitrospira*) may be able to sequentially convert cyanate to ammonium and then to nitrate (Tables 2.8). The second enrichment (G8 with acriflavine supplementation) consists of ~34% enriched comammox *Nitrospira*, with no AOA or AOB present (Figure 2.9). Results from this work demonstrated that under the studied ammonia-fed conditions, comammox *Nitrospira* are likely capable of oxidizing ammonia to nitrate. Overall, this work advances our understanding of the metabolic versatility of comammox *Nitrospira* and their temperature requirement. The new cultures will help to explore further physiological characteristics, such as ammonia and nitrite affinities.

4.1.2 The activity of comammox *Nitrospira* from the RBC biofilm

Most studies to date employ a combination of 16S rRNA gene amplicon sequencing and functional gene targeted qPCR or metagenomics to study the diversity and distributions of comammox *Nitrospira*. More work is needed to focus on the potential activity and role that comammox *Nitrospira* play in the environment. Recent work by Spasov *et al.* (2020) reported the presence of comammox *Nitrospira* in the RBCs of the Guelph WWTP, but their contributions to nitrification remains unknown. To address this gap, qPCR and differential inhibitors were used to assess the nitrification activity of comammox *Nitrospira* in the RBC biofilm (environmental sample) and in two comammox *Nitrospira* enrichment cultures. As such,

I explored the effects of several nitrification inhibitors, like c-PTIO, ATU, DCD, and simvastatin on two comammox *Nitrospira* enrichment cultures (G4 and G8) and on RBC biofilm suspensions. Findings from these experiments suggest that comammox *Nitrospira* are insensitive to 100 µM c-PTIO and are inhibited by 10 µM ATU and 10 mM DCD (Figure 3.3). Additionally, simvastatin was verified as a useful AOA-specific inhibitor, confirming earlier findings by Zhao *et al.* (2020) that demonstrated that simvastatin is a suitable AOA inhibitor (Figure 3.3).

The differential inhibitors c-PTIO, ATU, DCD, chlorite, and chlorate were also tested on biofilm samples. Complete inhibition of activity was observed with the use of DCD and chlorite suggesting that both are effective nitrification inhibitors (Figures 3.5 and 3.6). Other inhibitors like ATU and c-PTIO resulted in partial inhibition of ammonia oxidation in unfractionated RBC cell suspensions (Figures 3.5 and 3.6). Still, based on the inhibitor profiles and qPCR data in size-fractionated and unfractionated cell suspensions, it appears that comammox *Nitrospira* activity was likely inhibited by ATU, DCD, and partially by c-PTIO, suggesting that they are active members in the RBC biofilm (Figures 3.5 and 3.6).

4.2 Future directions

4.2.1 Purification and characterization of comammox *Nitrospira* in G4 and G8

Pure cultures of comammox *Nitrospira* are essential for physiological and biochemical characterization of other *Nitrospira* representatives. Therefore, further work is needed to purify and characterize MAG069-related comammox *Nitrospira* (present in G4) and MAG044-related comammox *Nitrospira* (present in G8). For example, it would be useful to repeat the cycloheximide incubation described in Chapter 2 followed by several serial transfers to fresh media. If cycloheximide indeed inhibits AOA, then the addition of cycloheximide followed by

serial transfers should dilute the AOA over time (dilution to extinction) and aid in the enrichment of comammox *Nitrospira*. Additionally, the use of optical tweezers in conjunction with continuous-feeding bioreactors have previously been successful in the growth of several *Nitrospira* representatives including *Ca.* N. kreftii, *N. defluvii*, *N. lenta*, and *N. japonica* (Ushiki *et al.,* 2013; Nowka *et al.,* 2015; Sakoula *et al.,* 2018, 2020). Nowka *et al.* (2015) highlighted a general protocol for enhancement of *Nitrospira* isolation from the environment, which includes the use of density gradient centrifugation to remove heterotrophs, followed by antibiotic treatment and cell sorting to select for *Nitrospira* cells. A similar approach to the one used by Nowka *et al.* (2015) can be applied to the RBC biofilm to isolate several comammox *Nitrospira*.

Once a pure culture is obtained, the metabolism of comammox *Nitrospira* can be examined more closely. Several genetic studies have indicated that comammox *Nitrospira* may be capable of mixotrophic growth (Daims *et al.*, 2001; Spieck *et al.*, 2006). Based on research by Spasov *et al.* (2020), many comammox *Nitrospira* MAGs have the genes required for urea hydrolysis and transport. Similarly, several MAGs (like MAG069 and MAG093) contain the genetic repertoire for cyanate degradation and hydrogen oxidation. This suggests that comammox *Nitrospira* related to MAG069 and MAG093 may have the genetic capacity to use alternate substrates, especially when ammonia is unavailable. Taken together, the study of alternative metabolisms is of relevance to gain a holistic perspective on the role of comammox *Nitrospira* in the RBCs, and by extension in other engineered environments.

Future work should also focus on elucidating whether comammox *Nitrospira* related to MAG069 can actively transcribe the *cynS* gene under cyanate supplementation. Methods like RT-qPCR, using the newly designed *cynS* primers, can be used to investigate and compare the transcriptional activities of comammox *Nitrospira* in response to cyanate and ammonia

supplementation. This method might be suitable for profiling the potential activity of MAG069 related comammox *Nitrospira* via quantification of *amoA* and *cynS* gene transcripts. For example, a short-term cyanase experiment can concentrate inoculum from an actively growing culture containing MAG069 related comammox *Nitrospira* that is fed either 0.5 mM ammonium chloride or 0.5 mM potassium cyanate (Michael Wagner, personal communication). Strong evidence for cyanase activity being responsible for cyanate use (rather than abiotic conversion of cyanate) is via the concentration of biomass from a culture and showing corresponding higher nitrate production activity in response to cyanate over a short period of time compared to unconcentrated controls. Alternatively, Flow-SIP can be used to study cyanate degradation by comammox *Nitrospira* without side effects caused by cross-feeding (Mooshammer *et al.*, 2020). Comammox *Nitrospira* (related to MAG069) enriched in G4 cultures can be exposed to ^{13}C-labelled bicarbonate in the presence of cyanate. Intermediate metabolites like nitrite and nitrate are continuously removed to ensure no cross-feeding with NOB is occurring, thus demonstrating cyanase activity by comammox *Nitrospira* (Mooshammer *et al.*, 2020).

It may also be of interest to examine the novel AOA species (belonging to the genus *Nitrosopumilus*) present in the G4 enrichment culture. Because there are no known reports of presumably marine-associated *Nitrosopumilus* species detected in non-saline municipal WWTPs, the isolation and characterization of this AOA species could be important in the study of the ecology of ammonia oxidizers generally. Additionally, the possible role of salinity in niche differentiation, which was previously unexplored in the RBCs, requires further investigation.

4.2.2 Further examination of activity by comammox *Nitrospira* in the RBC biofilm

It would be useful to repeat the activity incubation described in Chapter 3 with several modifications. One modification would be to shake the microcosm flasks gently (*e.g.*, 80-100

rpm) to ensure adequate aeration to help minimize the potential for anaerobic processes (*e.g.*, denitrification and anammox), as presumably observed in environmental sample incubations. Additionally, higher inhibitor concentrations may be required for complete nitrification inhibition in environmental samples, as previously recommended by Sauder *et al.* (2015) and Martens-Habbena *et al.* (2015). Simvastatin shows promising results in enrichment cultures and may be useful to use as an inhibitor of AOA activity *in situ*, although its solubility in DMSO might make it problematic for use at higher concentration (*e.g.*, higher than 100 µM). Future research might combine the use of labelled bicarbonate (*e.g.*, DNA- or RNA-SIP) and measure assimilation in the presence of nitrification inhibitors, as previously done by Gülay *et al.* (2019). Alternatively, functional gene transcriptional levels (RT-qPCR targeting *amoA* genes), along with *amoA* gene amplicon sequencing, can be used to assess the activity of nitrifiers in the community, as demonstrated earlier by Zheng *et al.* (2019). Multiple "omic" approaches (*i.e.*, proteomics, transcriptomics, and genomics), combined with activity assays, will likely represent a powerful approach to understanding the metabolic versatility and *in situ* activity of comammox *Nitrospira*, as well as other nitrifiers, in the RBCs. Ultimately, findings from this work advance our understanding of nitrification in the RBCs and the key players involved in this process. Moreover, findings from this work may be generalized to other aquatic systems, like aquaculture systems, where several nitrifying guilds exist (Bartelme *et al.*, 2017). The research presented opens new directions possible to explore with enrichment cultures to better characterize the potential metabolic roles for comammox *Nitrospira* in the RBCs, other engineered and naturally occurring freshwater environments.

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