Nascent nitrifying community response to monochloramine exposure in drinking water

Woo, Yissue

2021

Woo, Y. (2021). Nascent nitrifying community response to monochloramine exposure in drinking water. Doctoral thesis, Nanyang Technological University, Singapore.
<https://hdl.handle.net/10356/153741>

<https://hdl.handle.net/10356/153741>

<https://doi.org/10.32657/10356/153741>

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**NASCENT NITRIFYING COMMUNITY RESPONSE TO
MONOCHLORAMINE EXPOSURE IN DRINKING WATER**

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Interdisciplinary Graduate School

Singapore Centre for Environmental Life Sciences Engineering

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EXPOSURE IN DRINKING WATER

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Interdisciplinary Graduate School

Singapore Centre for Environmental Life Sciences Engineering

A thesis submitted to the Nanyang Technological University in partial fulfilment of the
requirement for the degree of
Doctor of Philosophy

2021 (Year of Submission of Thesis)

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ACKNOWLEDGEMENT

The completion of this thesis would not have been possible without the support and encouragement of my peers in Singapore Centre for Environmental Life Sciences Engineering (SCELSE), and the help of administrative staff members in the Interdisciplinary Graduate Program (IGP, formerly IGS) and SCELSE Graduate Student Office.

I would like to thank my supervisor, Prof. Stefan Wuertz, for the patient guidance and supervision of my scientific and professional training. The strong support and strict critique by my supervisor was instrumental in my growth as a scientist, and as a communicator of scientific research. The disruption caused by the COVID-19 pandemic in the final year of my PhD candidature was incredibly stressful, but Prof. Wuertz was a constant source of support during those periods, with a lot of kind understanding.

Additionally, I am grateful to my TAC committee members, A/Prof. Scott Rice, A/Prof. Cao Bin, Prof. Hans-Curt Flemming and Prof. Veronica Rajal, for their contribution to the discussion of the thesis structure, and their inputs into the interpretation of research data, despite the difficulties in coordinating the schedules of each member. The sum of their ideas and opinions has helped shape the outcome of this thesis.

I would like to especially thank my former supervisor in SCELSE, Dr. Mercedes Cecilia Cruz, for the opportunity to be part of the project that ultimately made this PhD work possible, and for encouraging me to persevere in the enrichment of the drinking water

distribution nitrifiers that were crucial to this thesis. A special acknowledgement to Prof. Ulrich Szewzyk and Dr. Regine Szewzyk for their crucial advice during the enrichment of Ammonia-oxidizing Archaea, as I had struggled with in my first year of candidature. I am thankful for the discussions with my fellow PhD student Sakcham Bairoliya, which were especially engaging and instrumental to some of my experimental designs. A deep-felt thanks to Dr. Ezequiel Santillan, Dr. Samarpita Roy and Ms Hoon Hui Yi for their guidance on the application of amplicon sequence analysis, phylogenetic analysis and fluorescence in-situ hybridization, and great appreciation for Ms Abeed Fatima Binti Mohidin Batcha for her constant assistance in laboratory purchases. The members of the Wuertz Lab in SCELSE were a source of constant support in this PhD journey, with their ideas and critiques, and I am grateful for their wonderful company.

I am very grateful for the company of my friends in SCELSE and in the graduate program, during our course works and teaching duties together. The fun times with fellow students Azean, Soheil, Geraldine, Yu Fen and Kenny made our journey together in this PhD program much more enjoyable.

Finally, I thank NTU, IGP and SCELSE for funding and hosting me during this graduate study, and for giving me room to grow as a scientist. Few students have access to such opportunities, and I will be forever grateful for the trust placed in me.

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ABBREVIATIONS

DWDS: Drinking water distribution systems

AOA: Ammonia oxidizing Archaea

AOB: Ammonia oxidizing Bacteria

NOB: Nitrite oxidizing Bacteria

NOM: Natural organic matter

AMO: Ammonia monooxygenase

HAO: Hydroxylamine oxidoreductase

NXR: Nitrite oxidoreductase

amoA: Ammonia monooxygenase subunit A gene

nxB: Nitrite oxidoreductase beta subunit gene

qPCR: Quantitative polymerase chain reaction

ddPCR: Droplet digital polymerase chain reaction

DMTU: NN'-Dimethylthiourea

ATU: Allylthiourea

SUMMARY

Nitrifiers autochthonous to drinking water distribution systems (DWDS) were enriched in separate ammonia-oxidizing Archaea (AOA), ammonia-oxidizing Bacteria (AOB) and nitrite-oxidizing Bacteria (NOB) enrichment cultures. A strategy was developed to selectively enrich AOA from DWDS biofilms containing both AOA and AOB, while new primers were designed to improve the quantification of the *amoA* and *nxrB* gene copies of AOA/AOB and NOB, respectively, using quantitative PCR and droplet digital PCR. Over several years, individual cultures containing AOA, AOB and NOB were enriched to 51.6 %, 71.2 % and 73.5 %, respectively. The nitrifiers were closely related to *Nitrososphaera viennensis* for AOA, *Nitrosomonas oligotropha* for AOB and *Nitrospira moscoviensis* for NOB. Under planktonic growth conditions, NOB were more resistant to monochloramine than AOA and AOB, and AOB were more resistant than AOA at lower monochloramine concentrations. Monochloramine also inactivated specific nitrifiers experimentally enriched in biofilms, but a small fraction of AOA or AOB cells persisted, likely due to the low concentration of free ammonia in the chloraminated bulk water. The persistence of AOA and AOB in the nitrifying biofilms after continuous exposure to monochloramine could be due to monochloramine cometabolism, where intracellular monochloramine was degraded by ammonia monooxygenase while also performing ammonia oxidation. This study is the first to address the effect of monochloramine on NOB and the regrowth potential of all three types of nitrifiers under planktonic conditions. By directly comparing monochloramine

resistance under planktonic and biofilm conditions in the absence of other nitrifier groups fundamental insights were obtained on their respective roles in DWDS.

1 Introduction

Nitrification is commonly reported in drinking water distribution systems (DWDS) with monochloramine added as a residual disinfectant, which can lead to an increase in nitrite and/or nitrate in the drinking water supplied to the consumer. Nitrification is part of the nitrogen cycle (Fig. 1), where ammonia is oxidized to nitrite by ammonia-oxidizing archaea (AOA) or bacteria (AOB), and nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB). Nitrification can be performed autotrophically, heterotrophically, or in rare cases mixotrophically (Ward, B.B. 2008). The process of treating source water to produce drinking water aims to reduce traces of biologicals and organic materials in the water thus ensuring biological stability during the distribution of treated drinking water to consumers. Autotrophic nitrification would introduce new organic carbon into the system and thus create an environment conducive to biological activities.

The primary effect of nitrification in DWDS is the production of nitrite, and nitrate with the presence of NOB, when free ammonia is available in the bulk water. Both nitrite and nitrate are capable of oxidizing hemoglobin to form methemoglobin, which prevents the binding of oxygen and results in methemoglobinemia (Fan, A.M. et al. 1987). Methemoglobinemia is particularly hazardous to infants under the age of 6 months, but its effects on adults are mild. The World Health Organization determined that the maximum allowable concentration of in drinking water is 1 mg/L $\text{NO}_2\text{-N}$ for nitrite and 11.3 mg/L $\text{NO}_3\text{-N}$ for nitrate. Studies have shown that infant methemoglobinemia is commonly associated with drinking waters that contain nitrite

and/or nitrate above these regulatory limits and are more commonly associated with well waters contaminated by agricultural runoffs (Fan, A.M. and Steinberg, V.E. 1996, Manassaram, D.M. et al. 2007).

While the impacts on human health of nitrites and nitrates produced from nitrification in DWDS during normal operations are minimal, nitrite is very reactive to monochloramine and can increase the rate of decay of the disinfectant in bulk water (Hao, O.J. et al. 1994). Additionally, it has been experimentally demonstrated that autotrophic ammonia oxidation strongly supports the growth of heterotrophic biofilms on surfaces in carbon-limited environments like drinking water, and the inhibition of ammonia oxidation led to the dispersal of the recruited heterotrophic biofilms (Keshvardoust, P. et al. 2019). Therefore, the inhibition of nitrification in DWDS is important to discourage the production of nitrite and the support of heterotrophic biofilm growth in the DWDS, both of which will increase the demand for monochloramine.

The occurrence of nitrification in DWDS employing chloramination for residual disinfection was reviewed by the American Water Works Association, which reported nitrification performed by AOB and NOB (American Water Works Association. 2013). The presence of AOA in DWDS is a more recent discovery, and their contribution to nitrification in DWDS has not yet been extensively reviewed, owing to their lower relative abundance in DWDS and to the difficulty of cultivating them in the laboratory.

Fig. 1 The nitrogen cycle and role of monochloramine. Nitrification is represented by green arrows, blue indicates denitrification, black anaerobic ammonium oxidation (anammox), and the red and purple arrows refer to nitrogen fixation and ammonification, respectively. The grey and yellow arrows refer to the decomposition of monochloramine to release ammonia and the cometabolism of monochloramine coupled with ammonia oxidation respectively.

Ammonia-oxidizing archaea are slow growing microorganisms that require very low concentrations of ammonia. The process of producing drinking water results in limiting concentrations of ammonia in the drinking water, which is favorable for the growth of AOA. Furthermore, the drinking water pipes of DWDS are designed for long service periods, only to be replaced in the event of breakage, which can potentially allow the proliferation of AOA in DWDS to go undetected due to their slow growth rate and rate of ammonia oxidation. These microorganisms have only recently been reported in DWDS, starting with observations in three groundwater treatment plants and the non-chloraminated DWDS in Netherlands, where only AOA were observed in the DWDS connected to the source groundwater, with ammonia concentrations below the detection limit of $<0.05 \text{ mg.L}^{-1} \text{ NH}_4^+$, whereas the remaining 2 DWDS reported higher abundance of AOB over AOA (van der Wielen, P.W.J.J. et al. 2009).

In two full-scale chloraminated DWDS in Ontario, Canada, AOB frequently outnumbered AOA in both DWDS, and AOB showed greater regrowth potential than AOA (Scott, D.B. et al. 2015). Low abundance of AOA was found in some of the five non-chloraminated DWDS in Hungary, where AOB and NOB were more commonly detected (Nagy mate, Z. et al. 2016). Similar findings were reported in a study conducted on two DWDS with and without residual chloramination, where AOA were only quantified in biofilms from non-chloraminated DWDS and AOB only in biofilms from chloraminated DWDS (Waak, M.B. et al. 2019).

More recently, it was reported that lower ammonia concentrations selected for higher AOA abundance over AOB in the biofilms from chlorinated/chloraminated DWDS from

North Dakota and Minnesota, USA, with AOA abundance seemingly more stable than AOB, being less sensitive to temperature fluctuations (Roy, D. et al. 2020). This observation is similar to a study of AOA and AOB in a freshwater lake that revealed niche differentiation between the two types of ammonia-oxidizing microorganisms (AOM); in particular, lower substrate concentrations supported the growth of AOA over AOB (French, E. et al. 2012).

A laboratory study conducted on biofilms cultivated from distribution waters, determined that AOA less sensitive than AOB to inactivation by low concentrations of disinfectant (1.5 and 2.0 mg/L chlorine; 0.05 – 0.1 and 0.3 – 0.4 mg/L chloramine), while higher doses of disinfectant (2.5 mg/L chlorine and 1.5 – 1.6 mg/L chloramine) effectively inactivated AOA and AOB. In addition, higher temperatures were found to decrease the sensitivity of AOA and AOB to inactivation by chloramine (Roy, D. et al. 2020).

Inactivation of AOB and NOB was discussed in the report by the American Water Works Association, describing AOB to tolerate monochloramine better than NOB, and to have better regrowth potential than NOB (American Water Works Association. 2013). Similar to the review of nitrification in DWDS, there is a knowledge gap regarding the inactivation of AOA in drinking water settings.

A study investigating the potential of monochloramine cometabolism by *Nitrosomonas europaea* revealed that hydroxylamine produced by ammonia oxidation can react with monochloramine, to produce ammonia. It was hypothesized that the reaction between monochloramine and hydroxylamine in *Nitrosomonas europaea* cells may be a major

mode of inactivation by monochloramine, by depleting the cells' ability to generate reductants and eventually suffocating them. By supplying exogenous hydroxylamine, the authors were able to resuscitate the model AOB strain (Wahman, D.G. et al. 2014, Wahman, D.G. and Speitel, G.E. 2015).

The phenomenon of monochloramine cometabolism was described as the concurrent breakdown of monochloramine by the enzyme ammonia monooxygenase (AMO), along with ammonia oxidation performed by the enzyme. This mechanism was first proposed by (Woolschlager, J.E., III 2000), then eventually demonstrated in *Nitrosomonas europaea* by (Maestre, J.P. et al. 2013). Cometabolism of monochloramine by AOB, in both pure cultures of *Nitrosomonas europaea* and mixed-nitrifier biofilms, was demonstrated to be possible only at low concentrations of monochloramine in the presence of excess free ammonia (left over from the formation of monochloramine) (Maestre, J.P. et al. 2013, Maestre, J.P. et al. 2016). This phenomenon thus far has only been documented in AOB and not in AOA, and only with an ammonia-monochloramine ratio of 1 or above. Additionally, the AOB studied in those works were allochthonous to the drinking water environment, thus may not truly represent potential monochloramine cometabolism in DWDS.

Singapore's National Water Agency, Public Utilities Board (PUB), uses monochloramine for secondary disinfection in the drinking water supply, replacing chlorine to improve stability of residual disinfection, and to reduce formation of disinfection by-products. In the project 'Initial survey of biofilms as potential refuge for human pathogens and of biofilm-mediated nitrification and denitrification in drinking

water distribution networks' (pub contract no./ref. no. 1150294) undertaken together with PUB, nitrifiers were detected and quantified in the biofilms sampled from the inner pipe walls of 21 random sites of the drinking water distribution system. The study found that the biofilm microbial communities on the inner walls of the drinking water distribution pipes exhibited low microbial diversity and were dominated by AOB when levels of monochloramine were high, whereas at lower monochloramine concentrations the microbial diversity was high and nitrifier communities consisted mostly of AOA and NOB. It was hypothesized that AOB are more tolerant to monochloramine than AOA and NOB, and therefore were responsible for the oxidation of ammonia, liberated by monochloramine decomposition, in sections of the DWDS proximal to the drinking water treatment plants containing higher concentrations of monochloramine. Nitrite generated from ammonia oxidation could then readily react with monochloramine and liberate more ammonia for subsequent use by the nitrifiers (Cruz, M.C. et al. 2020).

According to this hypothesis, the decreased monochloramine concentration in more distal parts of the DWDS would liberate less ammonia through monochloramine decomposition, which is more favorable for the growth of AOA than AOB. The lower rate of ammonia oxidation by AOA could in turn allow for the growth of NOB sensitive to high nitrite concentrations.

Although chloramination disinfection effects on nitrifier dynamics have been explored in controlled laboratory experiments (Roy, D. et al. 2020), using biofilms produced from distribution system waters, studies on individual nitrifiers are currently lacking.

While AOB were presumably more tolerant to monochloramine than AOA, results by Roy et al. (2020) appear to contradict the above hypothesis, with AOA appearing to be less sensitive to chloramine inactivation in general. Additionally, the growth of NOB during monochloramine disinfection remains unexplored.

The aim of this PhD study is to address the knowledge gap in the effects of monochloramine disinfection on specific nitrifier groups present in DWDS, by first enriching nitrifiers from drinking water biofilms in the Singapore DWDS. The study is motivated by the following research questions:

RQ1. What are the identities of the AOA, AOB and NOB enriched from the local DWDS?

Hypothesis: AOB from the DWDS biofilm are hypothesized to be related to *Nitrosomonas oligotropha*, while NOB from the DWDS biofilm is hypothesized to be related to *Nitrospira moscoviensis*. Although AOA from DWDS in published works were identified to be related to the *Nitrosopumilus maritimus* cluster, early data suggested that AOA from the DWDS biofilm should be related to *Nitrososphaera viennensis*.

Rationale: A recent study based in Singapore using 16S rRNA gene amplicon metabarcoding reported the AOB to be of the genus *Nitrosomonas*, the NOB to be of the genus *Nitrospira_sublineage II*, and the AOA to be of the phylum of *Thaumarchaeota* (Cruz, M.C. et al. 2020). *Nitrosomonas oligotropha* is a commonly found oligotrophic AOB in DWDS, and *Nitrospira moscoviensis*, which belong to

Nitrospira_sublineage II, is also commonly found in DWDS. *Nitrososphaera viennensis* was identified from an alignment search of the archaeal *amoA* sequences cloned from biofilm samples obtained from that study.

Approach: Identify the nitrifiers at genus level using 16S rRNA gene amplicon metabarcoding on the DWDS biofilm samples, then sequence the *amoA* and *nxrB* genes to identify the nitrifiers at species level by means of phylogenetic relatedness. Identify a common appropriate growth medium based on the identity of the AOA, then enrich the three nitrifiers in separate cultures. Finally, repeat the 16S rRNA gene amplicon metabarcoding and sequencing of *amoA* and *nxrB* genes to confirm the identity of the three nitrifiers.

RQ2a. Do planktonic AOB have a higher tolerance for monochloramine than AOA and NOB?

Hypothesis: AOB have a higher tolerance for monochloramine disinfection than AOA and NOB.

Rationale: AOB were reportedly observed to be associated with biofilms exposed to higher concentrations of monochloramine, while AOA and NOB were reportedly observed to be associated with biofilms exposed to lower concentrations of monochloramine.

Approach: Model how DWDS nitrifiers tolerate monochloramine using single-nitrifier enrichments, obtained through selective enrichments from DWDS biofilm samples. Introduce monochloramine into diluted nitrifier enrichments and sample for nitrifier and

monochloramine quantification, then split into five small-volume reactor flasks for 24 h of incubation. After 24 h, sample from each flask for nitrifier and monochloramine quantification, then quench the remaining monochloramine. Incubate reactor flasks for 14 d to study recovery of inactivated nitrifiers. After 14 d, sample from each flask for nitrifier quantification.

RQ2b. Do AOB in nitrifying biofilms have a higher tolerance for monochloramine than AOA in nitrifying biofilms?

Hypothesis: AOB in nitrifying biofilms have a higher tolerance for monochloramine disinfection than AOA in nitrifying biofilms, thus more AOB biomass than AOA biomass would be observed in the chloraminated biofilms.

Rationale: Identical to RQ2a.

Approach: Cultivate AOA- or AOB-containing nitrifying biofilms on cement coupons in a CDC biofilm reactor (see Chapter 3.15), then transfer coupons with AOA or AOB biofilms to flow cells. Sample two coupons from the biofilm reactor for each flow cell. Introduce monochloramine into filtered and dechloraminated tap water, then continuously supply the rechloraminated tap water to the flow cells and operate as a flow-through system for 14 d. After 14 d, sample the coupons from the flow cells to quantify the AOM.

2 Literature Review

2.1 Chloramination in drinking water distribution system

Inorganic chloramines, like monochloramine used for secondary disinfection in drinking water, are formed in aqueous solution, via a reaction between hypochlorite and ammonia, in a series of reactions governed by pH and chlorine-to-nitrogen weight ratios (Wolfe, R.L. et al. 1984):

The decrease in pH, and increase in Cl:N ratios in solution, results in the successive chlorination of the ammonia molecule, $\text{NH}_3 \rightarrow \text{NH}_2\text{Cl} \rightarrow \text{NHCl}_2 \rightarrow \text{NCl}_3$. To achieve selective formation of monochloramine, the Cl:N ratio is kept below 5:1 while keeping the pH 6-9. In equation one, the formation of monochloramine is a bi-directional reaction, with an equilibrium constant at 25°C of $1.5 \times 10^{10} \text{ M}^{-1}$. The formation of monochloramine is very fast, able to be produced within seconds at pH 7-9, with a rate of $2.9 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ at 25°C (White, G.C. 1972). Hydrolysis of monochloramine into ammonia and hypochlorite is a slow process that was calculated to require ten hours for 50% hydrolysis of monochloramine, at a rate of $1.9 \times 10^5 \text{ s}^{-1}$ at 25°C (Margerum, D.W. et al. 1979).

The decomposition of monochloramine in clean water may follow the net decay reaction of either equation four or equation five, with the production of nitrogen gas and nitrate respectively. It was found that the nitrogen gas was predominantly produced by monochloramine decay in clean water, meaning that stoichiometrically three molecules of monochloramine will decay to release one molecule of ammonia (Vikesland, P.J. et al. 1998).

The breakdown of monochloramine in water contaminated with natural organic matter (NOM) is of a different nature, with a faster reaction between monochloramine and organic nitrogen species in NOM to liberate ammonia, and a slower reaction between hypochlorite, hydrolyzed from monochloramine, and NOM to produce oxidized NOM (Duirk, S.E. et al. 2005). NOM in bulk water includes soluble microbial products like humic compounds secreted by metabolically active microorganisms, biomass associated products and extracellular polymeric substances found in biofilms. In comparison to the breakdown of monochloramine via reactions with NOM, the autodecomposition of monochloramine (Eq. 4 or 5) occurs at slower rates (Duirk, S.E. et al. 2005).

In very basic solutions, for example the microenvironment of the concrete surface with leaching lime, amination of hydroxide ions by monochloramine can occur quickly to form hydroxylamine (Eq. 6), which will be rapidly aminated by monochloramine to form

the unstable hydroxylhydrazine, which then decomposes further to diamine and then dinitrogen gas and hydrazine. The overall amination of hydroxide by monochloramine is reflected by equation 7 (Anton Hammerl, T.M.K. 2011).

The net reaction between nitrite and monochloramine follows equation 8, where stoichiometrically one molecule each of nitrite and monochloramine will react to produce one molecule of ammonium, nitrate and chloride (Vikesland, P.J. et al. 2001).

The introduction of nitrite into the DWDS can potentially accelerate the release of ammonia through monochloramine decay, when compared to the rate of monochloramine autodecomposition (Hao, O.J. et al. 1994).

2.2 Monochloramine inactivation of nitrifying microorganisms

Monochloramine is a much slower acting chlorine disinfectant compared to hypochlorite, which makes it unsuitable as primary disinfectant. Both forms of chlorine share similar characteristics with the oxidation state of chlorine is +1 in both chloramine and hypochlorite, and with hypochlorite having the redox potential of +1.49 V (WHO, W.H.O. 2000), and monochloramine having a redox potential of -1.48 V in acidic solutions and -0.81 V in basic solutions (Anton Hammerl, T.M.K. 2011).

Monochloramine causes cell injury to microorganisms, through its reaction with amino acids to form organic chloramines, or through oxidation of the sulfhydryl group of cysteine to disulfides states or beyond. The conversion of amino acids to organic chloramines by monochloramine is a slower process than the oxidation of cysteine sulfhydryl groups. In contrast to free chlorine, deamination and decarboxylation does not occur from reactions with monochloramine (Ingols, R.S. et al. 1953).

The oxidation of sulfhydryl groups to disulfide states can be reversible, when the appropriate reducing conditions are introduced, and when the sulfhydryl groups are not oxidized beyond the disulfide state (Jacangelo, J.G. et al. 1987, Watters, S.K. et al. 1989). For cysteine to be oxidized beyond the disulfide state, a residual amount of monochloramine is required to be present, with greater amounts of residual monochloramine resulting in greater proportions of cysteines oxidized beyond the disulfide state. However, the inactivation of microbial cells do not require all cysteines to be oxidized to disulfide states (Jacangelo, J.G. et al. 1987).

The currently accepted major mode of microbial cell inactivation is through the irreversible alteration of heme groups of key microbial enzymes (Ingols, R.S. et al. 1953). Examples of heme containing enzymes are cytochromes, hydroxylamine oxidoreductase (HAO) (Cedervall, P. et al. 2013), nitrite oxidoreductase (NXR) systems (Yamanaka, T. and Fukumori, Y. 1988, Cabello, P. et al. 2009), catalases and peroxidases.

In the ammonia-oxidizing bacteria, hydroxylamine is the intermediate substrate from the oxidation of ammonia to nitrite and can readily react with monochloramine to

release ammonia and chloride, with either nitrite, nitrate, nitrogen gas or nitrous oxide, depending on the reaction pathway (Wahman, D.G. et al. 2014). The removal of hydroxylamine from AOB cells will stall the oxidation of ammonia to nitrite for ATP production, by starving the ammonia monooxygenase (AMO) of electrons contributed by the oxidation of hydroxylamine by HAO. While electron starved, AMO will be unable to oxidize ammonia into the hydroxylamine. Reintroduction of hydroxylamine can rescue AOB from monochloramine induced depletion of hydroxylamine (Wahman, D.G. et al. 2014, Wahman, D.G. and Speitel, G.E. 2015). Although the archaeal analogue to HAO remained unidentified, there is strong evidence that hydroxylamine is also the intermediate substrate of archaeal ammonia oxidation to nitrite (Vajjala, N. et al. 2013). Therefore, AOA may be similarly inactivated by monochloramine through the depletion of intracellular hydroxylamine.

2.3 Ammonia-oxidizing Bacteria in drinking water distribution systems

The reported AOB in DWDS biofilms, chloraminated or not, are related to the *Nitrosomonas oligotropha* cluster, which includes *Nitrosomonas oligotropha* and *Nitrosomonas ureae* (Regan, J.M. et al. 2002, Regan, J.M. et al. 2003), while others were reported to be *Nitrosomonas* -type AOB (Lipponen, M.T. et al. 2004, van der Wielen, P.W. et al. 2009). Both AOB share similar characteristics: Gram-negative non-motile cells; absence of carboxysomes; ability to utilize urea as ammonia source; salt not required for growth; and commonly found in oligotrophic freshwater environments. The differences between the two AOB include *Nitrosomonas oligotropha* being more

sensitive to ammonia concentrations above 50 mM compared to *Nitrosomonas ureae* above 100 mM, its morphology including spherical cells in addition to rod-shaped cells with rounded ends, and its size being smaller at 0.8 - 1.2 by 1.1 - 2.4 μm compared to the size of *Nitrosomonas ureae* of 0.9 - 1.1 by 1.5 - 2.5 μm . *Nitrosomonas oligotropha* has an ammonia oxidation K_s value of 2.4 - 4.2 μM , compared to *Nitrosomonas ureae* of 1.9 - 3.6 μM , which allows for growth in environments with low ammonia concentrations (Koops, H.P. et al. 1991, Koops, H.-P. and Pommerening-Röser, A. 2015). The characterization of *Nitrosomonas oligotropha* was based on six strains and that of *Nitrosomonas ureae* on eight strains (Koops, H.P. et al. 1991).

The rate limiting step of ammonia oxidation in the *Nitrosomonas oligotropha* cluster is governed by the AMO operon with the *amoCAB* arrangement, which exists in three copies in the representative genomes of *Nitrosomonas oligotropha* (*Nitrosomonas* sp. Is79) and *Nitrosomonas ureae* (*Nitrosomonas ureae* Strain Nm10) (Bollmann, A. et al. 2013, Kozłowski, J.A. et al. 2016, Lehtovirta-Morley, L.E. 2018). AMO is a transmembrane enzyme located in the cytoplasmic membrane, and in the case of *Nitrosomonas*, it is in the intracytoplasmic membrane (ICM) folded into stacks along the periphery of the cells (Fiencke, C. and Bock, E. 2006, Ferguson, S.J. et al. 2007). With the AMO located along the ICM in *Nitrosomonas*, the number of catalytic sites for ammonia oxidation to hydroxylamine is larger compared to AOB without ICM, increasing the reaction rate. AMO is a copper dependent enzyme, proven experimentally by the reactivation of extracted AMO from *Nitrosomonas*, while

sensitive to inhibition by metal ions like Co^{2+} , Zn^{2+} , Ni^{2+} and Fe^{2+} , and also sensitive to inhibition by copper chelators like allylthiourea (Ensign, S.A. et al. 1993).

The oxidation of ammonia is completed by the oxidation of hydroxylamine to nitrite, catalyzed by the enzyme HAO, encoded by three copies of the *haoAB* operon in *Nitrosomonas* sp. Is79, and by four copies in *Nitrosomonas ureae* strain Nm10 (Bollmann, A. et al. 2013, Kozłowski, J.A. et al. 2016). HAO is a multi-heme periplasmic enzyme, using the heme P460 as the catalytic site while additional c-type hemes perform electron transfer out of the catalytic site. The critical P460 heme is held in the catalytic pocket, through the heme-binding domain (C-X-X-C) motif, and essentially stabilized for catalysis to occur via a unique covalent linkage to a tyrosine residue in an adjacent subunit (Igarashi, N. et al. 1997, Ferguson, S.J. et al. 2007).

Traditionally, the oxidation of ammonia to nitrite was thought to be a two-step process, involving the oxidation of ammonia to hydroxylamine, then the oxidation of hydroxylamine to nitrite. In the second step, four electrons are released, supplying two for the oxidation of ammonia to hydroxylamine, and the remaining two for cellular respiration. The updated finding strongly suggests a three-step process instead, involving the oxidation of ammonia to hydroxylamine, then the oxidation of hydroxylamine to nitric oxide (NO), and finally the possible enzymatic oxidation of NO to nitrite. If the oxidation of NO to nitrite is non-enzymatic in nature, the oxidation of hydroxylamine would only liberate three electrons, sparing just one for cellular respiration (Caranto, J.D. and Lancaster, K.M. 2017, Lehtovirta-Morley, L.E. 2018). The enzymatic oxidation of NO to nitrite is suspected to be catalyzed by a copper-

containing nitrite reductase (*nirK*), which is present in the genome of *Nitrosomonas oligotropha* and *Nitrosomonas ureae* (Bollmann, A. et al. 2013, Kozlowski, J.A. et al. 2016).

The first step of AMO catalyzed ammonia oxidation to hydroxylamine is performed on the cytoplasmic membrane, which release hydroxylamine into the periplasmic space. In the periplasm, periplasmic HAO will catalyze the oxidation of hydroxylamine to NO, which could be enzymatically oxidized to nitrite by periplasmic nitrite reductase. The electron transport chain for cellular respiration occurs on the cytoplasmic membrane, using quinol to shuttle electrons. Cytochrome *c*₅₅₄ is used to shuttle electrons from the periplasmic space to the electron transport chain (Ferguson, S.J. et al. 2007, Lehtovirta-Morley, L.E. 2018).

AOB like *Nitrosomonas* are considered obligate autotrophs, utilizing the Calvin-Benson-Bassham (CBB) cycle to assimilate carbon dioxide into biomass, via enzymatic carboxylation by ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). In *Nitrosomonas oligotropha* and *Nitrosomonas ureae*, their genome possesses two copies of Form I RubisCO, with *Nitrosomonas sp.* Is79 possessing a Form IA and Form IC RubisCO. Form IA RubisCO is reported to have a higher affinity for carbon dioxide than Form IC RubisCO, indicating that *Nitrosomonas oligotropha* may be less sensitive to fluctuations in carbon dioxide concentrations (Ferguson, S.J. et al. 2007, Bollmann, A. et al. 2013, Kozlowski, J.A. et al. 2016).

2.4 Ammonia-oxidizing Archaea in drinking water distribution systems

The diversity of AOA spans 4 clusters: Group 1.1a *Nitrosopumilus* cluster, Group 1.1a-associated *Nitrosotalea* cluster, Group 1.1b *Nitrososphaera* cluster, and ThAOA group *Nitrosocaldus* cluster. The habitat of AOA ranges from open oceans and coastal waters to soil and freshwater sediments (Hatzenpichler, R. 2012). The major clusters of AOA are the Groups 1.1a and 1.1b.

The AOA reported in The Netherlands' drinking water distribution systems, source water and water treatment plants, and in the Tokyo source water and water treatment plants, are closely related to the Group 1.1a *Nitrosopumilus* cluster, while the Group 1.1b *Nitrososphaera* cluster AOA were not detected (de Vet, W.W. et al. 2009, van der Wielen, P.W.J.J. et al. 2009, Niu, J. et al. 2016, Roy, D. et al. 2020). Unlike AOB in DWDS, AOA in DWDS are poorly identified, with much of the AOA identified originating from raw water, or from the filter media used in the water treatment process.

The Group 1.1a AOA cluster belongs to the *Candidatus Nitrosopumilus* order, which branches into the *Candidatus Nitrosopumilacaceae* and *Candidatus Nitrosotenuaceae* families, with the type genus being *Ca. Nitrosopumilus* (Qin, W., Martens-Habbena, W., Kobelt, J.N. and Stahl, D.A. 2016). *Ca. Nitrosopumilus maritimus* is the type species of Group 1.1a AOA, described to be non-motile slender rods with sizes of 0.17 - 0.22 by 0.5 - 0.9 μm , and unable to utilize urea as an ammonia source. Its K_s value for ammonia oxidation is $0.133 \pm 0.038 \mu\text{M}$, with a tolerance for ammonium and nitrite

at ten and two mM respectively. Growth requires a salinity of 16 - 96‰, with the optimum of 32 - 37‰ salinity (Konneke, M. et al. 2005, Qin, W., Martens-Habbena, W., Kobelt, J.N. and Stahl, D.A. 2016).

The Group 1.1b AOA cluster belongs to the *Nitrososphaerales* order, with the type genus being *Nitrososphaera*, and *Nitrososphaera viennensis* being the type species (Kerou, M. and Schleper, C. 2016, Kerou, M., Alves, R.J.E. and Schleper, C. 2018). *Nitrososphaera viennensis* is described to form motile irregular cocci with diameters of 0.65 - 0.91 µm and is able to utilize urea as an ammonia source. The optimal ammonia concentration for growth is at 2.6 mM, with inhibition occurring at 15 mM. Growth is inhibited at nitrite concentration of 10 mM. There is no salinity requirement for growth (Tournu, M. et al. 2011, Stieglmeier, M. et al. 2014, Kerou, M. and Schleper, C. 2016). Although the substrate half saturation constant for ammonia oxidation is not available for *Nitrososphaera viennensis*, it should be expected to be similar in range to that of *Nitrososphaera maritimus*.

With a very low substrate half saturation constant for ammonia oxidation, the Group 1.1a and 1.1b AOA are well suited for growth in environments of extreme ammonia limitations. Addition of organic acids like pyruvate and oxaloacetate will greatly improve the growth of both AOA clusters, but recent evidence suggested that scavenging of hydrogen peroxide produced by ammonia oxidation to be the actual mechanism, instead of incorporation of such organic acids into biomass. Hydrogen peroxide produced by ammonia oxidation is toxic, and will inhibit AOA growth, due to

their lack of peroxidase or catalase production (Kerou, M. and Schleper, C. 2016, Kim, J.G. et al. 2016, Qin, W., Martens-Habbena, W., Kobelt, J.N. and Stahl, D.A. 2016).

Like in AOB, the rate limiting step of ammonia oxidation is governed by the AMO gene, but not in an operon. In *Nitrososphaera maritimus*, the gene is arranged as *amoAXCB* whereas in *Nitrososphaera viennensis*, multiple open reading frames (ORF) interspace between *amoC* and *amoB*, between *amoB* and *amoXA*, and between *amoXA* and five copies of *amoC* (Spang, A. et al. 2012, Bayer, B. et al. 2016, Lehtovirta-Morley, L.E. 2018). Similar to AOB, archaeal AMO is a copper-dependent transmembrane enzyme located on the cytoplasmic membrane (Simon, J. and Klotz, M.G. 2013, Kerou, M. et al. 2016). Unlike AOB, ICMs are absent and archaeal AMO are less sensitive to inhibition by copper chelators like allylthiourea (Shen, T. et al. 2013).

Hydroxylamine had been demonstrated to be the intermediate of ammonia oxidation in *Nitrososphaera maritimus*, drawing similarity to the ammonia oxidation pathway of AOB (Vajrala, N. et al. 2013). However, the archaeal counterpart to the bacterial HAO is not detected in the genomes of *Nitrososphaera maritimus* and *Nitrososphaera viennensis*, which made the pathway of hydroxylamine oxidation remained unresolved. To date, there two hypothesized pathways for hydroxylamine oxidation in AOA. The first pathway uses a novel copper-HAO to catalyze the reaction between hydroxylamine, NO and water to form nitrite and liberate five electrons, while the NirK will reduce nitrite to regenerate NO for the hydroxylamine oxidation. The second pathway suggests a three-step hydroxylamine oxidation process similar to that of AOB, with hydroxylamine oxidized to NO to liberate three electrons by an unknown

enzyme, while NO is oxidized to nitrite to liberate one electron by another unknown enzyme. In both proposed pathways, two electrons will be shuttled back to AMO for ammonia oxidation, and two electrons will be shuttled to the respiration chain (Kozłowski, J.A. et al. 2016, Lancaster, K.M. et al. 2018, Lehtovirta-Morley, L.E. 2018).

Like AOB, the oxidation of ammonia to hydroxylamine by AMO is proposed to occur on the outer facing side of the cytoplasmic membrane, while the oxidation of hydroxylamine to nitrite is proposed to occur in the pseudoperiplasmic space between the cytoplasmic membrane and the surface-layer (s-layer) proteins. The electron transport chain for cellular respiration occurs on the cytoplasmic membrane, using quinol to shuttle electrons. Plastocyanin-like electron carrier is used to shuttle electrons from the pseudoperiplasmic space to the electron transport chain (Stieglmeier, M. et al. 2014, Urakawa, H., Martens-Habbena, W. and Stahl, D.A. 2014, Kerou, M. et al. 2016, Kozłowski, J.A. et al. 2016, Qin, W. et al. 2017).

Unlike the gram-negative AOB, the Group 1.1a and 1.1b AOA cells have a s-layer protein array surrounding the cytoplasmic membrane for structural purposes, and as site for surface modifications. In *Nitrososphaera maritimus*, the s-layer proteins are arranged in a hexagonal fashion using six subunits displaying a p6-symmetry, whereas in *Nitrososphaera viennensis*, the s-layer proteins are arranged in a triangular fashion using three subunits displaying a p3-symmetry (Stieglmeier, M. et al. 2014, Qin, W. et al. 2017, Rodrigues-Oliveira, T. et al. 2017). It was recently proposed that the archaeal s-layer protein array architecture may provide greater surface area for capturing ammonium on the cell surface, than that of the AOB gram-negative cell

architecture. It was proposed that the capture of ammonium on the cell surfaces may explain why AOA are adapted to growing in environments of extreme ammonia limitations (Gorman-Lewis, D. et al. 2014).

In contrast to AOB, Group 1.1a and 1.1b AOA are highly suspected to use a modified form of the 3-Hydroxypropionate/4-Hydroxybutyrate (HP/HB) pathway to assimilate carbon dioxide into biomass. The pathway involves the transformation of one molecule of acetyl-CoA and two bicarbonate molecules into succinyl-CoA, followed by the transformation of succinyl-CoA into two molecules of acetyl-CoA. The HP/HB cycle is suggested to be more energetically efficient than the CBB cycle used by AOB, for the assimilation of inorganic carbon (Berg, I.A. et al. 2007, Walker, C.B. et al. 2010, Tourna, M. et al. 2011, Konneke, M. et al. 2014).

2.5 Nitrite-oxidizing Bacteria in drinking water distribution systems

Most of the NOB identified in DWDS, with or without disinfection, belong to the Group II *Nitrospira* cluster, with a minority belonging to the genus *Nitrobacter* (Regan, J.M. et al. 2002, Regan, J.M. et al. 2003, Martiny, A.C. et al. 2005). With *Nitrospira* being the major nitrite oxidizer identified in DWDS, the focus shall remain on the genus. When comparing the oxidation kinetics of the two NOB, *Nitrospira* has a lower half saturation constant K_m of 9 - 27 μM nitrite, compared to 49 - 544 μM nitrite in *Nitrobacter*. It is then argued that *Nitrospira* can be described as a *K* strategist, displaying slower growth and higher affinity for substrates, while *Nitrobacter* can be described as a *R* strategist that display faster growth and lower affinity for substrates. These made

Nitrospira more suitable to persist in an environment that is limiting in nitrite, such as that in the DWDS (Andrews, J.H. and Harris, R.F. 1986, Nowka, B. et al. 2015).

The Group II *Nitrospira* cluster, or *Nitrospira* lineage II, contains the species *Nitrospira moscoviensis*, *Nitrospira japonica* from wastewater treatment, and various uncultured *Nitrospira* from activated sludge (Daims, H. et al. 2001, Ushiki, N. et al. 2013, Daims, H. et al. 2016). *Nitrospira moscoviensis* is a non-motile gram-negative bacteria, possessing a helical to vibroid shape and enlarged periplasmic space, with a size of 0.9 - 2.2 by 0.2 - 0.4 μm that form spirals of up to 3 turns with a width of 0.8 - 1.0 μm , and it lack ICMs and carboxysomes (Ehrich, S. et al. 1995). The *Nitrospira* is capable of mixotrophic growth, when supplied with formate, and is able to hydrolyze urea to liberate ammonia and carbon dioxide, to support its own growth or to support the growth of AOM (Koch, H. et al. 2015). The half saturation constant K_m of *Nitrospira moscoviensis* is at 9 μM nitrite (Nowka, B. et al. 2015).

In contrast to AOM, the oxidation of nitrite to nitrate is a one step process, catalyzed by the enzyme nitrite oxidoreductase (NXR), a membrane bound enzyme complex (Sundermeyer-Klinger, H. et al. 1984). Similar to AOA and AOB, the catalytic face of the enzyme is in the periplasmic space in *Nitrospira*, while in *Nitrobacter* the site of catalysis is in the cytoplasmic space (Spieck, E. et al. 1996, Spieck, E. et al. 1998).

In *Nitrospira*, nitrite is oxidized by NXR to nitrate using water, to release two electrons for respiration, shuttled by Cytochrome c. Part of the electron released will be transferred to a terminal oxidase, where oxygen is reduced to water while releasing proton into the periplasmic space. The protons released to the periplasmic space,

together with the protons generated in nitrite oxidation, will be used by ATPase to produce ATP, and by NADH dehydrogenase to fuel carbon fixation using the reductive tricarboxylic acid (TCA) cycle. It was proposed that *Nitrospira* have an advantage over *Nitrobacter*, by performing nitrite oxidation in the periplasmic space, thus generating more ATP from the increased protons available for protomotive force (Lucker, S. et al. 2010, Nowka, B. et al. 2015). The removed need for nitrite transport into the cell of *Nitrospira* would likely also help in the energy conservation of nitrite oxidation.

The reductive TCA (rTCA) cycle, or the Arnon-Buchanan cycle named after the authors that discovered the pathway (Evans, M.C. et al. 1966), generates two molecules of Acetyl-CoA from two molecules of carbon dioxide, in a reversed TCA cycle (Fuchs, G. 2011). The energy input required for the rTCA cycle to generate one molecule of pyruvate is much lower compared to the CCB cycle and HP/HB cycle, requiring only two molecules of ATP, while CCB cycle requires seven and HP/HB cycle requires nine ATP molecules (Fuchs, G. 2011). Key enzymes of this pathway: 2-oxoglutarate:ferredoxin oxidoreductase and pyruvate:ferredoxin oxidoreductase are generally sensitive to oxygen, with the exception of a unique five-subunit form of the two enzymes present in *Nitrospira* that are more tolerant to oxygen (Lucker, S. et al. 2010, Berg, I.A. 2011).

2.6 Enrichment culture technique for the study of DWDS nitrifiers

The enrichment culture technique has been described as the application of natural selection to obtain cultures that are highly abundant in the organisms of interest while discouraging the growth of organisms outside of interest. This is accomplished by using a selective growth medium if possible and carefully adjusting the physicochemical parameters of the culture, to favor the organism of interest but made unfavorable to its competitors (Asano, Y. and Kaul, P. 2012, Madhuri, R.J. et al. 2019). The technique has been used for multiple unique purposes aside from the isolation of targeted organisms, from the production of specific enzymes (Asano, Y. and Kaul, P. 2012, Madhuri, R.J. et al. 2019) to the selection of biodegradation properties (Sutherland, T.D. et al. 2002) to the biotransformation of selected metabolites (Vanhoutte, I. et al. 2021).

The characterization of the organisms of interest cannot be done properly unless they are in pure culture, as the metabolic activities of the non-target organisms in culture would confound the findings used for the characterization. However, there are also advantages of enrichment cultures compared to pure cultures. For example, “non-culturable” bacteria have been successfully cultured in enrichment cultures (Mu, D.-S. et al. 2018), with the authors concluding that *“enrichment culture methods can be developed and used to construct a model for analyzing mixed cultures and exploring microbial dark matter. This study provides a new train of thought to mining marine microbial dark matter based on mixed cultures.”* In another example, enrichment cultures were found to perform better metabolically, where the degradation of the

compound ioxynil to CO₂ was found to be slower in pure cultures of the isolated ioxynil degraders (Hsu, J.C. and Camper, N.D. 1976).

Lastly, the use of enrichment cultures was argued to enable the detailed study of actual microbial interactions in the environment (Hug, L.A. and Co, R. 2018). The authors speculated that hypothetical proteins, derived from metagenomic analysis of the enriched culture, could be annotated to the characterized microbial consortia in the culture. This was argued to enable the identification of previously unknown metabolic functions, possibly leading to updating our current knowledge of biogeochemical cycles.

3 Enrichment and identification of nitrifiers from drinking water distribution systems

3.1 Introduction

Chloramine disinfection and its relevance to nitrification has been investigated in pure culture using the model AOB organism, *Nitrosomonas europaea*, and at much higher cell concentrations ($\sim 10^5 - 10^6$ AOB/ml) than are usually found in DWDS (Chauret, C. et al. 2008, Wahman, D.G. et al. 2009, Maestre, J.P. et al. 2013, Maestre, J.P. et al. 2016, Keshvardoust, P. et al. 2020). Although such experiments were conducted under drinking water conditions, *Nitrosomonas europaea* is soil-based and allochthonous to drinking water environments, with a different affinity for the substrate and different growth kinetics than the autochthonous ammonia oxidizer, *Nitrosomonas oligotropha* (Regan, J.M. et al. 2002, Regan, J.M. et al. 2003, Nowka, B. et al. 2015).

There is only one study on AOA with monochloramine concentrations typical of DWDS, where nitrifying biofilms containing AOA and AOB were grown from filter effluents of a drinking water treatment plant (Roy, D. et al. 2020). While the taxonomy of the AOA was established based on the archaeal *amoA* gene, the experiment involved a mixed community of AOA and AOB, along with heterotrophic bacteria, cultivated from nitrifying biofilms in the filter effluents. To date, the effect of chloramine disinfection on NOB has not been explored.

Therefore, in the present study specific nitrifier groups allochthonous to the DWDS were enriched from distribution pipe biofilm samples in Singapore (Cruz et al. 2020)

obtained during a PUB project 'Initial survey of biofilms as potential refuge for human pathogens and of biofilm-mediated nitrification and denitrification in drinking water distribution networks' (pub contract no./ref. no. 1150294). Enriching specific nitrifiers from DWDS addresses the first research question:

RQ1: What are the identities of the AOA, AOB and NOB enriched from the local DWDS?

Hypothesis: AOB from the DWDS biofilm are related to *Nitrosomonas oligotropha*, while NOB from the DWDS biofilm are related to *Nitrospira moscoviensis*. Although AOA from DWDS were identified to be related to the *Nitrosopumilus maritimus* cluster in published works, early data suggest that AOA from the DWDS biofilm should be related to *Nitrososphaera viennensis*.

Rationale: 16S rRNA gene amplicon metabarcoding performed in (Cruz, M.C. et al. 2020) reported the AOB to be of the genus *Nitrosomonas*, the NOB to be of the genus *Nitrospira_sublineage II*, and the AOA to be of the phylum of *Thaumarchaeota*. *Nitrosomonas oligotropha* is a commonly found oligotrophic AOB in DWDS, and *Nitrospira moscoviensis*, which belong to *Nitrospira_sublineage II*, is also commonly found in DWDS. *Nitrososphaera viennensis* was identified from an alignment search of the cloned archaeal *amoA* sequences obtained from earlier works done before the start of this dissertation.

Approach: Identify the nitrifiers at the genus level using 16S rRNA gene amplicon metabarcoding on the DWDS biofilm samples, then sequence the *amoA* and *nxB*

genes to identify the nitrifiers at species level by means of phylogenetic relatedness. Identify a common appropriate growth medium based on the identity of the AOA, then enrich the three nitrifiers in separate cultures. Finally, repeat the 16S rRNA gene analysis and sequencing of *amoA* and *nxrB* genes to confirm the identity of the three nitrifier groups.

This chapter covers the taxonomy and community analysis of AOA, AOB and NOB enrichment cultures, the description of novel primers designed for the quantification of nitrifier gene copies using droplet digital PCR (ddPCR), and a novel method for selective enrichment of AOA over AOB using NN'-Dimethylthiourea (DMTU) and sodium pyruvate to stimulate the growth of AOA while inhibiting the growth of AOB.

The nitrifiers found in Singapore's DWDS biofilms were previously reported to belong to the genera *Nitrosomonas* and *Nitrospira* and the phylum *Thaumarchaeota*, obtained through 16S rRNA gene metabarcoding in operational taxonomy units (OTU). This chapter will identify the nitrifiers enriched from the biofilm samples at the species level by sequencing the *amoA* and *nxrB* functional genes, then performing phylogenetic analysis of those sequences. 16S rRNA gene metabarcoding was performed on the nitrifier enrichments, followed by phylogenetic analysis of the amplicon sequence variants (ASV).

The development of novel primers for the quantification of *Nitrososphaera*, *Nitrosomonas* and *Nitrospira* nitrifiers using ddPCR will be explored in this chapter. Established methods for the absolute quantification of AOA, AOB and NOB in literature are limited to quantitative PCR (qPCR) methods, using the primers amplifying the full

sequence of archaeal *amoA* (Francis, C.A. et al. 2005), the full sequence of bacterial *amoA* (Rotthauwe, J.H. et al. 1997) and a partial sequence of *nxrB* (Pester, M. et al. 2014). The amplicon sizes of those methods are close to or greater than 500 base pairs, which while optimized for qPCR operations, are less suitable for ddPCR. The development of absolute quantification with ddPCR was necessary for other work in this thesis to quantify possibly very low copies of nitrifier sequences in samples.

The development of the novel approach to selectively enrich AOA from biofilm samples containing AOA and AOB in this chapter introduces the use of DMTU and sodium pyruvate in the enrichment culture medium. *Nitrososphaera* is a genus of AOA that produces neither catalases nor peroxidases to counteract hydrogen peroxide produced by ammonia oxidation, which will inhibit the growth of AOA if not removed from their environment (Kim, J.G. et al. 2016). Both DMTU and pyruvate are capable of scavenging hydrogen peroxide from the environment, thus removing the growth inhibition of AOA (Jackson, J.H. et al. 1985, Kim, J.G. et al. 2016). Additionally, DMTU is a thiourea with the known property of chelating copper ions important for the proper functioning of ammonia monooxygenase, which would inhibit the growth of AOB (Ginestet, P. et al. 1998), while AOA are reportedly less sensitive to such inhibitors (Shen, T. et al. 2013).

3.2 Materials and methods

3.2.1 Cultivation medium

Nitrifiers were cultivated from drinking water distribution system (DWDS) biofilm samples with the Freshwater Medium (FWM) adopted from and described by (Kerou, M. and Schleper, C. 2016), with the exception of the vitamin solution, which in this study adopted from (Balch, W.E. et al. 1979), with the following composition: 1 g/L NaCl, 0.4 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 g/L KH_2PO_4 , 0.5 g/L KCl, 1 mL/L trace element solution (Per Liter: 100 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 30 mg H_3BO_3 , 36 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 24 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 190 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 144 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), 1 mL/L vitamin solution (Per Liter: 2 mg Biotin, 2 mg Folic acid, 10 mg Pyridoxine HCl, 5 mg Thiamine HCl, 5 mg Riboflavin, 5 mg Nicotinic acid, 5 mg DL-Calcium pantothenate, 0.1 mg Vitamin B12, 5 mg p-Aminobenzoic acid, 5 mg Lipoic acid), and 7.5 μM ferric sodium EDTA. The pH of the FWM was buffered at pH 8.0 with HEPES solution (10 mM HEPES, 6 mM NaOH for AOA and NOB; 20 mM HEPES, 6 mM NaOH for AOB), and 2 mM of NaHCO_3 was supplied as carbon source. 2 mM and 5 mM of NH_4Cl was supplied as energy source for AOA and AOB respectively, while 2 mM of NaNO_2 was supplied as energy source for NOB.

3.2.2 Cultivation of ammonia-oxidizing archaea and ammonia-oxidizing bacteria from DWDS sediments

Enrichment cultures were inoculated with biofilm samples identified to be rich in AOA or AOB (Cruz, M.C. et al. 2020) into 50 mL of FWM in a 100 mL wide-neck Erlenmeyer

flask, with 2 mg/L of $\text{NH}_3\text{-N}$ and 4 mg/L of $\text{NH}_3\text{-N}$ supplied for AOA and AOB respectively, and 24 μM of sodium azide added to inhibit the growth of NOB. The cultures were grown in static condition, in a 30°C incubator. The AOA and AOB cultures were subcultured upon depletion of 90% of the ammonia supplied, by inoculating 10% of the spent culture into fresh media. 24 μM of sodium azide is continuously supplied until accumulation of nitrite was consistently observed, with confirmation that NOB is undetectable with quantitative polymerase chain reaction (qPCR).

To wash out co-cultured AOB from the AOA enrichment culture, 100 μM of DMTU and sodium pyruvate was added to inhibit the growth of AOB, and to stimulate the growth of AOA in the culture. DMTU and sodium pyruvate is constantly supplied, even upon confirmation that AOB is undetectable with qPCR.

3.2.3 Cultivation of nitrite-oxidizing bacteria from DWDS sediments

Nitrite oxidizing bacteria were enriched by inoculating biofilm samples identified to be rich in NOB (Cruz, M.C. et al. 2020) into 50 mL of FWM in a 100 mL wide-neck Erlenmeyer flask, with 4 mg/L of $\text{NO}_2\text{-N}$ supplied, and 86 μM of allylthiourea (ATU) added to inhibit the growth of AOA and AOB. The cultures were grown under static conditions in a 30°C incubator. The NOB cultures were subcultured upon depletion of 90% of the nitrite supplied, by inoculating 10% of the spent culture into fresh media. ATU was continuously supplied at 86 μM until confirmation that AOA or AOB was undetectable by qPCR.

3.2.4 Cultivation of nitrifiers in stirred vessels

The static nitrifier enrichments were diluted 10x into 200 mL of FWM in a sterile 250 mL Erlenmeyer flask with a magnetic stirrer rod. The cultures were supplemented with 4 mg/L NH_4Cl and 4 mg/L NaNO_2 for AOA/AOB and NOB respectively, while mixed on a magnetic stirrer at approximately 100 rpm in room temperature, protected from light.

Upon depletion of the supplied NH_4Cl or NaNO_2 , 150 mL of the spent stirred culture was diluted into 1.5 L of FWM in a stirring culture vessel (Nalgene™ Polycarbonate Magnetic Culture Vessel) (Fig. 2). The cultures were stirred at low speed using a magnetic stirrer, aerated with filtered air at 1 L/min, and incubated at 30°C using a silicon heating jacket with a temperature controller. The cultures were monitored via a sampling port, for substrate utilization and growth of the nitrifiers. When 90% of the supplied ammonia or nitrite was depleted, 500 mL of the spent culture was collected for storage at 4°C, while the remaining spent cultures were wasted. The culture vessels were then scrubbed with Virkon S solution, dishwashing brushes to remove biofilms then rinsed with ultrapure Type 1 water until foaming was no longer observed, after which 15 mL of the spent culture was inoculated into 1.5 L of FWM in the cleaned culture vessels.

For the cultivation of AOA in the stirred culture vessels, the inoculum used for subculturing was filtered through the 0.45 μm filter membrane, once every subculture event.

Fig. 2 Set-up of the stirring culture vessel. A Nalgene™ Polycarbonate Magnetic Culture Vessel wrapped with a temperature-controlled silicon heating jacket sits atop a variable speed magnetic stirrer. The caps are replaced with 3-port caps with tubings, with the thick tubing fitted with a 0.2 μm syringe filter to form a vent, one thin tubing fitted with a 0.2 μm syringe filter and aeration stone, and another fitted with a valve for sampling. The unused lines are closed and secured with cable ties.

3.2.5 Substrate concentration measurement

Ammonia concentrations were determined using the EPA Hach Method 10205 and Ammonia (Nitrogen) Test Strips 0-6.0 mg/L (HACH Co.), while nitrite concentrations were determined using the HACH method 10207 and 10237, and the Nitrate and Nitrite Test Strips (HACH Co.). The HACH methods were performed using the DR3900 Laboratory Spectrophotometer for water analysis (HACH Co.).

3.2.6 Cloning and sequencing of ammonia monooxygenase subunit A (*amoA*), nitrite oxidoreductase beta subunit (*nxrB*) and archaeal 16S rRNA gene

amoA and *nxrB* genes were amplified using the primers listed in Table 1, in a PCR reaction comprised of 12.5 µL of AmpliTaq Gold 360 mastermix (Applied Biosystems™), 200 nM of forward and reverse primer, and 5 µL of template DNA in a 25 µL reaction volume. The PCR reaction was performed with the cycling parameter: 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 60 s at 62 °C then 60 s at 72 °C, followed by 5 min at 72 °C, ending with infinite hold at 4 °C.

The archaeal 16S rRNA gene was amplified using the archaea 16S primers listed in Table 1, in a PCR reaction comprised of 0.2 µL of BioReady Taq DNA polymerase, 2.5 µL of 10x buffer with 15 mM MgCl₂, 1 µL of 25 mM MgCl₂, 1 µL of 10 mM dNTP mix, 0.5 µL of forward and reverse primer, 14.3 µL of nuclease-free water, and 5 µL of template DNA in a 25 µL reaction volume. The PCR reaction was performed with the cycling parameter: 5 min at 94 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at 55 °C then 90 s at 72 °C, followed by 5 min at 72 °C, ending with infinite hold at 4 °C.

The amplicons were immediately cloned into plasmids using the TOPO® TA Cloning® Kit for Sequencing, with One Shot® TOP10 Chemically Competent E. coli (Invitrogen), according to the manufacturer's instructions. 10-20 individual plasmid clones were extracted for plasmids using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen™), according to the manufacturer's instructions.

The plasmids were screened using M13 primers supplied by the cloning kit. Amplicons were generated in 4 replicates from the positive clones, then pooled and purified using the PureLink PCR Purification Kit (Invitrogen). The purified amplicons were sent to 1st BASE for Sanger sequencing using the M13 Forward (-20) primer. The PCR reaction comprised of 12.5 µL of AmpliTaq Gold 360 mastermix (Applied Biosystems™), 200 nM of forward and reverse primer, and 5 µL of template DNA in a 25 µL reaction volume. The PCR reaction was performed with the cycling parameter: 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C then 60 s at 72 °C, followed by 5 min at 72 °C, ending with infinite hold at 4 °C.

3.2.7 Sequence analysis of ammonia monooxygenase subunit A (*amoA*), nitrite oxidoreductase beta subunit (*nxrB*) and archaeal 16S rRNA gene

The sequence data were read with SnapGene Viewer (SnapGene® software from GSL Biotech; available at snapgene.com). A random clone *amoA* or *nxrB* nucleotide sequence was used to translate to the respective protein sequences, using Frame 1 output of the EMBOSS Transeq tool (Madeira, F. et al. 2019). The translated protein sequence was then used in pBLAST (Altschul, S.F. et al. 1990) search, against the non-redundant protein database, to obtain a candidate list of organisms, with in

percentage identity cut-off of 90%. The list of translated *amoA* sequence and the protein sequences of the candidate organisms, were compiled into a FASTA file.

Evolutionary analyses were conducted in MEGA X (Kumar, S. et al. 2018, Stecher, G. et al. 2020). In brief, the multiple sequence alignment was performed on the sequences in the FASTA files, using the ClustalW (1.6) algorithm on the nucleotide sequences, or the MUSCLE algorithm on the peptide sequences, then export the alignment into a .meg file. The *amoA* and *nxB* nucleotide sequences were indicated as “protein coding sequences” during the alignment process, which would account for frameshifting in the sequences. The resultant file was then used to find the best protein/DNA model, using the maximum likelihood method. The best nucleotide or amino acid substitution model was then chosen based on the AIC and BIC scores for the construction of the maximum likelihood tree, using the Bootstrap method for testing the phylogeny with 500 replications. The Nearest-Neighbor-Interchange was chosen for the Heuristic Method, while the default option (NJ/BioNJ) was chosen for making the initial tree.

3.2.8 16S rRNA gene metabarcoding

The PCR reaction was composed of 12.5 µl of 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 200 nM of forward and reverse primer (Table 1), and 12.5 ng of template DNA in a 25 µl reaction volume. The PCR reaction was performed with the cycling parameter: 3 min at 95 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C then 30 s at 72 °C, followed by 5 min at 72 °C, ending with infinite hold at 4 °C. Each

reaction was generated in triplicates, then pooled and purified with SPRISelect magnetic beads (Beckman Coulter) with double size selection ratio of 0.85 - 0.56, according to the manufacturer's instructions. The quality of the amplicons was validated with the Agilent 4200 TapeStation System, using the AgilentD1000 ScreenTape Assay (Agilent Technologies, Inc). Sequencing was performed with MiSeq Illumina 2x300 bp chemistry, and the sequence read was analysed through the DADA2 pipeline (McMurdie, P.J. and Holmes, S. 2013, Callahan, B.J. et al. 2016), performed in R version 3.5.3 (R Core Team 2018).

3.2.9 Designing primers for quantification in droplet digital PCR

Multiple Sequence Alignment (MSA) was performed with the archaeal *amoA*, bacterial *amoA* and *nxB* sequence obtained from Sanger sequencing, against the most closely related BLAST hits using the Clustal Omega program (Sievers, F. et al. 2011). The archaeal *amoA* sequence was aligned against the *amoA* sequence of *Nitrososphaera viennensis*, *Nitrososphaera evergladensis* and *Nitrososphaera gargensis*. The bacterial *amoA* sequence was aligned against the *amoA* sequence of *Nitrosomonas oligotropha* and *Nitrosomonas ureae*. The *nxB* sequence was aligned against the *nxB* sequence of *Nitrospira moscoviensis*, *Nitrospira bockiana* and *Nitrospira cladia*. Primer sites for maximum amplicon size of 200 bp, with the melting temperature of 61 °C to 63 °C, was chosen by manual screening of the alignment outputs, and validated with Primer-BLAST (Ye, J. et al. 2012).

3.2.10 Quantitative PCR and droplet digital PCR

Quantitative PCR (qPCR) reaction was made using the PowerUp Sybr Green MasterMix (Applied Biosystems), 200 nM of primers, and 5 µl of template in a 25 µl reaction volume. The reaction was performed in the StepOnePlus thermocycler (Applied Biosystems) with the following reaction parameters: 50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Plasmids used as standards for absolute quantification was synthesized (Integrated DNA Technologies) containing *Nitrososphaera viennensis amoA*, *Nitrosomonas oligotropha amoA* and *Nitrospira moscoviensis nxrB* for AOA, AOB and NOB respectively.

ddPCR reaction was made using the QX200 ddPCR EvaGreen SuperMix (Bio-Rad), 250 nM of forward primer (100 nM for AOB) and 100 nM of reverse primer, and 5 µL of template in a 25 µl reaction volume. The droplet generation, PCR reaction and droplet reading was performing using the QX200 AutoDG Droplet Digital PCR System (Bio-Rad) with the following reaction parameters: 95 °C for 5 min, 40 cycles of 95 °C for 30 s and 60 °C for 1 min, 4 °C for 5 min, 90 °C for 5 min, and finally hold on 4 °C (All steps with ramp rate of 2 °C.s⁻¹). The primers used in the experiments are listed in Table 1. Concentrations of the plasmid standards and DNA samples were measured with the Qubit 2.0 fluorometer, using the dsDNA HS (high sensitivity) Assay Kit (Invitrogen).

Table 1 Primers and targets used in this study

Primer	Sequence (5'-3')	Target	Reference
Arch21F	TTCCGGTTGATCCYGCCGGA	Archaea 16S	(DeLong, E.F. 1992)
1492R	GGTTACCTTGTTACGACTT	Archaea and Bacteria 16s	(Reysenbach, A.L. et al. 1992)
Arch_amoAF	STAATGGTCTGGCTTAGACG	Archaeal <i>amoA</i>	(Francis, C.A. et al. 2005)
Arch_amoAR	GCGGCCATCCATCTGTATGT		
amoA 332F	GGGGTTTCTACTGGTGGT	Bacterial <i>amoA</i>	(Rotthauwe, J.H. et al. 1997)
amoA 822R	CCCCTCKGSAAAGCCTTCTTC		
nxB 169f	TACATGTGGTGGAACA	<i>nxB</i>	(Pester, M. et al. 2014)
nxB 638r	CGGTTCTGGTCRATCA		
NSS_amoAF	CGCTGCTAACCATCAACGCA	^d Nitrososphaera <i>amoA</i>	This study
	ACACTGCTAACCATCAACGCAG		
NSS_amoAR	GCACCCACAGCGAGCAT		
	GCGCCCACTACGAGCATTG		
NSM_amoAF	TATGTTGCGCTGATTGAGCAAGG	^d Nitrosomonas <i>amoA</i>	This study
	TACGTTGTTTGATTGAACAAGG		

NSM_ amoAR	CCACCATACGCAGAACATCAGCAT		
	CCACCATACACAGAACATCAGCAT		
NSP nxrBF	GTGGAACAACGTGGAGACCAAG	^d Nitrospira <i>nrxB</i>	This study
NSP nxrBR	CATCCCTTCGAACACCCCGTA		
515F	^a GTGYCAGCMGCCGCGGTAA		
		^c 16s rRNA gene	(Parada, A.E. et al. 2016)
926R	^b CCGYCAATTYMTTTRAGTTT		
M13 Forward (- 20)	GTAAAACGACGGCCAG		
		Vector insert	Invitrogen
M13 Reverse	CAGGAAACAGCTATGAC		

^aAdapter: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'

^bAdapter: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'

^cV4-V5 hypervariable region

^dUsed in qPCR and ddPCR

The application of Arch_amoAF/Arch_amoAR primers (Francis, C.A. et al. 2005) for qPCR and ddPCR involved a 25-µl reaction with 12.5 µl of master mix (PowerUp Sybr Green or EvaGreen SuperMix), 400 nM of primers and 5 µl of template. The qPCR reaction was performed in the StepOnePlus thermocycler (Applied Biosystems) with the following reaction parameters: 50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min. The ddPCR reaction was performed with

the following reaction parameters: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min, 4 °C for 5 min, 90 °C for 5 min, and finally hold at 4 °C (All steps with a ramp rate of 2 °C s⁻¹).

The application of amoA332F/amoA822R primers (Rotthauwe, J.H. et al. 1997) for qPCR and ddPCR involved at 25 µl reaction with 12.5 µl of master mix (PowerUp Sybr Green or EvaGreen SuperMix), 1 µM of primers and 5 µl of template. The qPCR reaction was performed in the StepOnePlus thermocycler (Applied Biosystems) with the following reaction parameters: 50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min. The ddPCR reaction was performed with the following reaction parameters: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min, 4 °C for 5 min, 90 °C for 5 min, and finally hold on 4 °C (All steps with ramp rate of 2 °C.s⁻¹).

The application of nxrB169f/nxrB638r primers (Pester, M. et al. 2014) for qPCR and ddPCR involved at 25 µl reaction with 12.5 µl of master mix (PowerUp Sybr Green or EvaGreen SuperMix), 200 nM of primers and 5 µl of template. The qPCR reaction was performed in the StepOnePlus thermocycler (Applied Biosystems) with the following reaction parameters: 50 °C for 2 min, 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min. The ddPCR reaction was performed with the following reaction parameters: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min, 4 °C for 5 min, 90 °C for 5 min, and finally hold on 4 °C (All steps with ramp rate of 2 °C.s⁻¹).

3.2.11 AOB wash-out experiment

After the *amoA* concentrations of the AOA and AOB culture stocks were obtained using ddPCR, the AOA and AOB cultures were diluted and mixed into 10^3 - 10^4 genomic copies/ml concentrations. The AOA+AOB mixed cultures were separated into 3 triplicate groups: With 100 μ M of DMTU and pyruvate; With 100 μ M of pyruvate; Without DMTU or pyruvate. The experiment was conducted with a culture volume of 100 ml in 250 ml Erlenmeyer flasks, shaken at 150 rpm on an orbital shaker covered with black paper, in ambient temperature (average 23 °C). The cultures were supplied with 4 mg/L $\text{NH}_3\text{-N}$ monitored for ammonia consumption with the Ammonia (Nitrogen) Test Strips, 0 - 6 mg/L (HACH). Upon depletion of the supplied ammonia, 60 ml of culture was collected with the SterivaxTM-GP Sterile Vented Filter Unit, 0.22 μ m (EMD Millipore) then stored in -20 °C, while 10 ml of culture was subcultured into a new flask to a culture volume of 100 ml containing 4 mg/L $\text{NH}_3\text{-N}$. The experiment was terminated after 3 collections. The AOA and AOB were quantified via ddPCR for genomic copies of archaeal or bacterial *amoA* (Fig. 3).

Fig. 3 Experimental approach to validate AOB wash out strategy. Each treatment type had 3 replicates, and a sample was collected upon exhaustion of the supplied ammonium during subculturing.

3.3 Results

3.3.1 Taxonomy of enriched Ammonia-oxidizing archaea from DWDS

The cloned archaeal 16S rRNA gene sequences were most similar to those from *Nitrososphaera viennensis* strain EN76 (Fig. A1), and the nucleotide sequences of the cloned archaeal *amoA* were also most related to the *amoA* nucleotide sequences from *Nitrososphaera viennensis* strain EN76 (Fig. A2). The translated peptide sequences of the cloned archaea *amoA* fragments were closely related to *amoA* peptide sequences from the AOA genera of *Nitrososphaera*, including the species *Nitrososphaera viennensis* (Fig. 4). While the AOA enriched from the DWDS shared genetic similarity with the *Nitrososphaera viennensis*, it should be considered functionally unique, based on the translated peptide sequence of the *amoA*. As such, the AOA are best described as *Nitrososphaera viennensis*-like microorganisms.

Fig. 4 Maximum likelihood phylogenetic tree based on translated peptide sequences of cloned archaeal *amoA* nucleotide sequences. The translated peptide sequences of the cloned archaeal *amoA* are closely related to the *amoA* peptide sequences from the AOA genera *Nitrososphaera*, including the species *Nitrososphaera viennensis* (closed circle). The cloned sequences are highlighted by closed triangles. The evolutionary history were inferred using the Maximum Likelihood method (Thomas, R.H. 2001) and Le_Gascuel_2008 model (Le, S.Q. and Gascuel, O. 2008). The unrooted tree with the highest log likelihood (-1321.91) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2277)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 36 amino acid sequences. All positions with less than 90% site coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). The final dataset had a total of 210 positions. The number of bootstrap replications was 500. Evolutionary analyses were conducted in MEGA X (Kumar, S. et al. 2018, Stecher, G. et al. 2020).

3.3.2 Taxonomy of enriched ammonia-oxidizing bacteria from DWDS

The *Nitrosomonas* ASVs generated from 16S rRNA gene metabarcoding of the AOB enrichment are closely related to the 16S rRNA gene sequences from *Nitrosomonas oligotropha* group of AOB (Fig. A3). The nucleotide and translated peptide sequences of the bacterial *amoA* clones are however diversely related to the nucleotide and peptide *amoA* sequences from the AOB genera *Nitrosomonas* and *Nitrosococcus* (Fig. A4 and 5), with most of the clones affiliated with the *Nitrosomonas oligotropha* lineage. Therefore, the AOB enriched from the DWDS should be considered unique *Nitrosomonas oligotropha*-like microorganisms, with the enrichment possibly containing a diverse collection of *Nitrosomonas oligotropha* strains.

3.3.3 Taxonomy of enriched nitrite-oxidizing bacteria from DWDS

The *Nitrospira* ASVs generated from 16S rRNA gene metabarcoding of the NOB enrichment are most related to 16S rRNA gene sequences from the *Nitrospira moscoviensis* group of NOB (Fig. A5). The majority of the nucleotide and translated peptide sequences of the cloned *nxrB* are also most related to the nucleotide and peptide *nxrB* sequences from the *Nitrospira moscoviensis* group of NOB (Fig. A6 and 6). Consequently, the NOB enriched from the DWDS are likely to be local strains of *Nitrospira moscoviensis*.

Fig. 5 Maximum likelihood phylogenetic tree based on translated peptide sequences of cloned bacterial *amoA* nucleotide sequences. The translated peptide sequences of the cloned bacterial *amoA* are closely related to the *amoA* peptide sequences from the AOB genera *Nitrosomonas*, including *Nitrosomonas oligotropha* (closed circle). The cloned sequences are highlighted by closed diamonds. One of the cloned sequences is diversely related to the *amoA* peptide sequences from the AOB genera *Nitrosomonas*, *Nitrospira* and *Nitrosococcus*. The evolutionary history was inferred using the Maximum Likelihood method (Thomas, R.H. 2001) and Le_Gascuel_2008 model (Le, S.Q. and Gascuel, O. 2008). The unrooted tree with the highest log likelihood (-1454.32) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.7441)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 33 amino acid sequences. All positions with less than 90% site coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 144 positions in the final dataset. The number of bootstrap replications was 500. Evolutionary analyses were conducted in MEGA X (Kumar, S. et al. 2018, Stecher, G. et al. 2020).

Fig. 6 Maximum likelihood phylogenetic tree based on translated peptide sequences of cloned *nxB* nucleotide sequences. The translated peptide sequences of the cloned *nxB* appear to be more closely related to the *nxB* peptide sequences from the NOB genera *Nitrospira*, with the majority closely related to the sequences from *Nitrospira moscoviensis* (closed circle). The cloned sequences are highlighted by the closed squares. The evolutionary history was inferred using the Maximum Likelihood method (Thomas, R.H. 2001) and General Reverse Transcriptase model (Dimmic, M.W. et al. 2002). The unrooted tree with the highest log likelihood (-1508.78) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.6257)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 37 amino acid sequences. All positions with less than 90% site coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 162 positions in the final dataset. The number of bootstrap replications was 500. Evolutionary analyses were conducted in MEGA X (Kumar, S. et al. 2018, Stecher, G. et al. 2020).

3.3.4 Designing nitrifier primers for ddPCR and qPCR applications

Two pairs of *Nitrosomonas amoA* primers were designed after multiple alignment of the *amoA* nucleotide sequences of the enriched AOB, *Nitrosomonas oligotropha*, and *Nitrosomonas ureae* (Fig. A16). Additionally, two pairs of *Nitrososphaera amoA* primers were designed after multiple alignment of the *amoA* nucleotide sequences of the enriched AOA, *Nitrososphaera viennensis*, *Nitrososphaera evergladensis*, and *Nitrososphaera gargensis* (Fig. A15). Finally, one pair of *Nitrospira nxrB* primers was designed after multiple alignment of the *nxrB* nucleotide sequences of the enriched NOB, *Nitrospira moscoviensis*, *Nitrospira calida* and *Nitrospira bockiana* (Fig. A17). The specificity of the newly designed primers was checked in silico using PRIMER-BLAST, and no cross reactions with *Nitrosomonas* not from the *Nitrosomonas oligotropha* group, with non-*Nitrososphaera* AOA, or with non-*Nitrospira* NOB were found. For PCR applications involving more than one primer pair, the primer pairs were mixed in equimolar concentrations.

Primers were designed according to guidelines for ddPCR provided by Bio-Rad Laboratories, Inc. Furthermore, the primers were specifically designed to obtain amplicon lengths less than 250 bp. The primer pairs were checked with Primer-BLAST for cross-reactivity with non-target nitrifiers and found to be very specific to the target organisms with cross reactions having multiple mismatches on the primers and producing amplicons longer than the intended size (Table 2).

Table 2 *In-silico* cross reaction of designed primers with non-target nitrifiers

Targets	NSS_amoA (106 bp amplicon)	NSM_amoA (111 bp amplicon)	NSP_nxrB (157 bp amplicon)
<i>Nitrosopumilus</i>	No cross reactions	N.A.	N.A.
<i>Nitrosotenuis</i>	3 – 5 mismatches on primers producing >1.5 kbp amplicons	N.A.	N.A.
<i>Nitrosocosmicus</i>	3 – 4 mismatches on primers producing 106 bp amplicons	N.A.	N.A.
<i>Nitrosocaldus</i>	5 mismatches on primers producing >2.6 kbp amplicons	N.A.	N.A.
<i>Nitrosococcus</i>	N.A.	2 – 5 mismatches on primers producing 111 bp amplicons	N.A.
<i>Nitrosospira</i>	N.A.	1 – 5 mismatches on primers producing 111 bp amplicons	N.A.
<i>Nitrospina</i>	N.A.	N.A.	No cross reactions
<i>Nitrobacter</i>	N.A.	N.A.	No cross reactions
AOA	N.A.	No cross reactions	No cross reactions
AOB	3 – 5 mismatches on primers producing 400 bp – 4 kbp amplicons	N.A.	No cross reactions
NOB	2 – 5 mismatches on primers producing 220 bp – 3.7 kbp amplicons	No cross reactions	N.A.

The design of functional gene primers to quantify nitrifiers using ddPCR was necessary to overcome the limitations of qPCR in terms of a higher detection limit and sensitivity to amplification. In the survey of bulk water and biofilm communities in the local DWDS network with in-pipe sensors, a discrepancy was observed between the quantitation of total Archaea with ddPCR and the detection of archaeal *amoA* with qPCR (Kitajima, M. et al. 2020).

In that study, an archaeal 16S rRNA gene primer was used for ddPCR quantification (Yu, Y. et al. 2005, Kitajima, M. et al. 2020), while the full-length archaeal *amoA* primer was used for qPCR quantification (Francis, C.A. et al. 2005, Kitajima, M. et al. 2020). The amplification product of the archaeal *amoA* primer exceeds 600 bp, which is not ideal for qPCR and ddPCR applications (Fig. 7, 9 and A12.).

Fig. 7 Comparison of standard curves produced by NSS_amoAF/R and Arch_amoAF/R qPCR reactions. The designed NSS_amoAF/R (red) primer pair produced a qPCR reaction with a smaller confidence interval than that of the published Arch_amoAF/R (blue) primer pair (Francis, C.A. et al. 2005).

The designed primer pairs were compared to the published primers pairs in ddPCR and qPCR experiments involving serial 10-fold dilutions of DNA extracts from the nitrifier enrichment and diluted plasmids containing the PCR targets to be used as reaction positive controls. Six serial dilutions of the nitrifier genomic DNA were used in the experiments, together with one dilution of the positive control plasmid and one no template control. The same set of dilutions was used for the ddPCR and qPCR reactions. The genomic copies in each reaction were obtained with ddPCR using the designed primers.

Comparing the designed and published primers for archaeal *amoA* in qPCR, the designed primers were able to achieve more than 97 % reaction efficiency with the limit of quantification appearing close to 10s of copies per reaction (Fig. A12A) producing a smaller confidence interval (Fig. 7), while the published primers (Francis, C.A. et al. 2005) were able to achieve more than 81 % reaction efficiency with the limit of quantification appearing close to tens of copies per reaction (Fig. A12B) but produced a larger confidence interval (Fig. 7). The positive control plasmid quantified with the designed primers was much closer to the amount quantified with ddPCR (3890 copies).

Fig. 8 Visualization of amplicons produced by NSS_amoAF/R and Arch_amoAF/R qPCR reactions. Agarose gel (2 %) electrophoresis of the qPCR reactions using the NSS_amoA primer pairs with the expected amplicon length of 106 bp (A), and reactions using the Arch_amoA primer pair with the expected amplicon length of 635 bp (Francis, C.A. et al. 2005) (B). The lane L represents the Gene ruler 100 bp plus DNA ladder. Lane A represents the no template control. Lanes B – H represent the 1/100 to 1/10⁷ dilution of the AOA enrichment genomic DNA extract.

Agarose gel electrophoresis of the qPCR products from both primer sets revealed single bands of the expected amplicon length, and the band was most visible from the most diluted sample amplified with the designed primers (Fig. 8). Using the designed primers for ddPCR, the signals between the positive and negative droplets were clearly separated (Fig. 9A). When the published primers were used, the signals of the positive droplets were poorly separated from those of the negative droplets (Fig. 9B).

Fig. 9 Comparison of ddPCR reactions using NSS_amoAF/R and Arch_amoAF/R primers. 1D plots from the archaeal *amoA* ddPCR droplet reads showed good signal separation for the positive droplets from the negative droplets of the reactions using the designed primers (A), but the signal separation between the positive and negative droplets of the reactions using the published primers (Francis, C.A. et al. 2005) was poorer (B).

Comparing the designed and published primers for bacterial *amoA* in qPCR, the designed primers were able to achieve more than 91 % reaction efficiency with the limit of quantification appearing close to single copies per reaction (Fig. A13A)

producing a smaller confidence interval (Fig. 10), while the published primers (Rotthauwe, J.H. et al. 1997) were able to achieve more than 84 % reaction efficiency with the limit of quantification appearing close to 10s of copies per reaction (Fig. A13B) but produced a larger confidence interval (Fig. 10). The positive control plasmid quantified with the designed primers deviated slightly from the amount quantified with ddPCR (4790 copies), but the plasmid failed to amplify in qPCR with the published primers.

Fig. 10 Comparison of standard curves produced by NSM_amoAF/R and amoA332F/822R qPCR reactions. The designed NSM_amoAF/R (red) primer pair resulted in a qPCR reaction with a smaller confidence interval than that of the published amoA332F/822R (blue) primer pair (Rotthauwe, J.H. et al. 1997).

Agarose gel electrophoresis of the bacterial *amoA* qPCR products from both primer sets revealed single bands of the expected amplicon length, and the band for the positive control plasmid was only detected from the reaction using the designed primers (Fig. 11), indicating that the plasmid may not be a reliable standard for the *amoA*332F/822R reaction. Using the designed primers for ddPCR, the signals between the positive and negative droplets were clearly separated (Fig. 12A). When the published primers were used, the signals of the positive droplets were poorly separated from those of the negative droplets (Fig. 12B). Similar to the qPCR reactions, the positive control plasmid failed to amplify in the ddPCR reaction.

Fig. 11 Visualization of amplicons produced by NSM_amoAF/R and amoA332F/822R qPCR reactions. Agarose gel (2 %) electrophoresis of the qPCR reactions using the NSM_amoA primer pairs with the expected amplicon length of 111 bp (A), and reactions using the amoA332F/822R primer pair with the expected amplicon length of 491 bp (Rotthauwe, J.H. et al. 1997) (B). The lane L represents the Generuler 100 bp plus DNA ladder. Lane A represents the no template control. Lanes B – H represent the $1/10^3$ to $1/10^8$ dilution of the AOB enrichment genomic DNA extract.

Fig. 12 Comparison of ddPCR reactions using NSM_amoAF/R and amoA332F/822R PCR reactions. 1D plots from the bacterial *amoA* ddPCR droplet reads showed good signal separation for the positive droplets from the negative droplets of the reactions using the designed primers (A), but the signal separation between the positive and negative droplets of the reactions using the published primers (Rotthauwe, J.H. et al. 1997) was poorer and the positive control plasmids failed to amplify (B).

The comparison between designed and published primers for *nxB* in qPCR showed that the designed primers were able to achieve more than 97 % reaction efficiency

with the limit of quantification appearing between 10s to 100 copies per reaction (Fig. A14A) producing a smaller confidence interval (Fig. 13), while the published primers (Pester, M. et al. 2014) were able to achieve more than 76 % reaction efficiency with the limit of quantification appearing close to tens of copies per reaction (Fig. A14B) but produced a larger confidence interval (Fig. 13). The positive control plasmid quantified with the designed primers was much closer to the amount quantified with ddPCR (5128 copies).

Fig. 13 Comparison of standard curves produced by NSP_nxrBF/R and nxrB169f/638r qPCR reactions. The designed NSP_nxrBF/R (red) primer pair resulted in a standard curve with a smaller confidence interval than that of the published nxrB169f/638r (blue) primer pair (Pester, M. et al. 2014).

Agarose gel electrophoresis of the *nxB* qPCR products from both primer sets revealed single bands of the expected amplicon length for the designed primer (Fig. 14A), while the published primers produced bands that are larger than the expected length (Fig. 14B). Using the designed primers for ddPCR, the signals between the positive and negative droplets were clearly separated (Fig. 15A). The ddPCR reactions failed in general when the published primers were used (Fig. 15B).

Fig. 14 Visualization of amplicons produced by NSP_nxrBF/R and nxrB169f/638r qPCR reactions. Agarose gel (2 %) electrophoresis of the qPCR reactions using the NSP_nxrB primer pairs with the expected amplicon length of 157 bp (A), and reactions using the nxrB169f/638r primer pair with bands larger than the expected amplicon length of 485 bp (Pester, M. et al. 2014) (B). The lane L represents the Generuler 100 bp plus DNA ladder. Lane A represents the no template control. Lanes B – H represent the 1/100 to 1/10⁸ dilution of the NOB enrichment genomic DNA extract.

Fig. 15 Comparison of ddPCR reactions using NSP_nxrBF/R and nxrB169f/638r primers. 1D plots from the *nxB* ddPCR droplet reads showed good signal separation for the positive droplets from the negative droplets of the reactions using the designed primers (A), but ddPCR reactions failed with the use of the published primers (Pester, M. et al. 2014) (B).

3.3.5 Enrichment strategy for AOA

The overall strategy for enriching AOA from the DWDS biofilms is outlined in Fig. 16. Freshwater Medium (Kerou, M. and Schleper, C. 2016) was identified to be the most suitable culture medium for the cultivation of the AOA identified to be *Nitrososphaera viennensis*-like.

Initial efforts to enrich AOA from the DWDS biofilm samples resulted in preferential growth of AOB over AOA, with a relative abundance of 7.5% of AOB compared to 0.19% of AOA, based on 16S amplicon metabarcoding. The non-nitrifiers were at a relative abundance of 92.3%. After successive rounds of culturing with DMTU and pyruvate, AOB were successfully reduced to below detection compared to the earlier relative abundance of 7.5%, while the relative abundance of AOA improved greatly to 39.5%. To improve the enrichment of AOA, filtration through 0.45 µm membrane filter was reintroduced while maintaining the use of DMTU and pyruvate, resulting in the increase of AOA relative abundance to 51.6% (Table 3).

Filtration of the enrichment culture through 0.45 µm membrane filters or the addition of antibiotics to the growth medium as suggested by (French, E. et al. 2012, Li, Y.Y. et al. 2016, Chen, H.Y. et al. 2017) successfully suppressed AOB, but did not improve AOA growth (Table 3). The growth of AOA was likely impacted by reduced interactions with bacteria, as suggested by (Santoro, A.E. and Casciotti, K.L. 2011). A likely beneficial interaction would be the production of catalase by the bacteria, to remove hydrogen peroxide in the culture produced by AOA that can inhibit their growth (Zinser, E.R. 2018).

Table 3 Relative enrichment of AOA with and without filtration and addition of DMTU and pyruvate to inhibit AOB

Treatment	Relative abundance (%)		
	<i>Nitrososphaera</i>	<i>Nitrosomonas</i>	Non-nitrifiers
None	0.20	7.50	92.3
DMTU ^A , pyruvate	39.5	ND ^B	60.5
Filtration, DMTU, pyruvate	51.6	ND	46.3

^A NN'-Dimethylthiourea ^B Not detected

Fig. 16 Outline of the AOA enrichment strategy. Biofilm samples were collected from DWDS pipe sections and the archaeal *amoA* sequences from the samples were cloned and sequenced for analysis. The identity of *Nitrososphaera viennensis* was determined from *amoA* sequence analysis and Freshwater Medium was identified to the appropriate culture medium. A starter culture was inoculated with the biofilm

samples using Freshwater Medium and the culture was subsequently scaled up into the stirred reactor. The enrichment of AOA was checked using 16S metabarcoding, which revealed AOB to be outgrowing AOA, prompting the use of DMTU and pyruvate treatment to selectively washout AOB from the culture. The enrichment was checked again with 16S metabarcoding, confirming the washout of AOB. The strategy for selective enrichment of AOA was replicated with the validation experiment.

3.3.6 Validation of enrichment strategy for AOA

To validate the AOA enrichment strategy, AOA and AOB enrichments were diluted to approximately 10^3 genomic copies of *amoA* per ml and mixed in approximately equal proportions before subjection to three treatment types: addition of DMTU and pyruvate, addition of pyruvate only, and absence of DMTU and pyruvate. Each treatment group had three replicates, and the experiment was conducted for a period covering three sampling events. Sampling occurred when the supplied ammonium was exhausted in the culture, which was then followed by a subculture event.

ddPCR was used to quantify AOA and AOB based on the newly designed *Nitrososphaera amoA* primers and *Nitrosomonas amoA* primers, respectively (Table 3). The specificity of the primers was confirmed in qPCR negative control experiments, where the *Nitrososphaera amoA* primers were challenged with 10^7 copies of *Nitrosomonas amoA* plasmids per reaction, and *Nitrosomonas amoA* primers were challenged with 10^7 copies of *Nitrososphaera amoA* plasmids per reaction. While the upper limit of detection for ddPCR is around 10^4 copies per reaction, 10^7 copies of non-target templates were added to test the robustness of the PCR assay in the

presence of such a high number of other sequences. Both negative control experiments showed no evidence of cross reaction of the newly designed primers. One copy of *Nitrososphaera amoA* and one copy of *Nitrosomonas amoA* were assumed to represent one cell each of AOA and AOB, respectively (Spang, A. et al. 2012).

The proportion of AOA in the AOA+AOB mixture increased from 32.4% to 99.9% when cultured with addition of 100 μ M DMTU and pyruvate. In the cultures supplied with only 100 μ M pyruvate, the proportion of AOA increased to 60.7%, while in cultures without DMTU and pyruvate, the proportion of AOA increased to 53.5%, before the first subculture. However, after two subcultures, the AOA were outcompeted by the AOB in cultures not amended with 100 μ M DMTU and pyruvate (Fig. 17).

Fig. 17 Change in proportion of AOA in culture with subsequent sampling events. The proportion of AOA saw an increase to 99.5%, then eventually to 99.9%, from the initial 32.4%. $n = 3$, error bars represent SD. P = pyruvate; D = DMTU. Grey, cultures with

DMTU and pyruvate; orange, cultures with pyruvate only; and blue, cultures without DMTU and pyruvate. Sampling 0 refers to the starting culture, and sampling 1, 2 and 3 occurred sequentially.

3.3.7 Enrichment strategy for AOB

Early efforts to enrich AOB from the DWDS biofilm samples using Freshwater Medium were poor, with the first 16S metabarcoding attempt revealing the relative abundance of *Nitrosomonas* AOB to be 0.74% and most of the microorganisms in the culture to be non-nitrifiers. Subsequent efforts saw the abundance of *Nitrosomonas* greatly increased to 80.03%, with a concurrent increase in abundance of NOB *Nitrospira* at 0.97% and *Nitrobacter* at 0.15%. Later efforts with reducing the period of culturing from one week to three days, together with treatment using 24 μ M of sodium azide (Ginestet, P. et al. 1998), reduced the NOB *Nitrospira* to 0.07% while *Nitrobacter* were not detected, although the abundance of *Nitrosomonas* reduced slightly to 71.16% (Table 4).

Table 4 Improvement of relative enrichment of AOB in culture over time

Metabarcoding event	Relative abundance (%)			
	<i>Nitrosomonas</i>	<i>Nitrospira</i>	<i>Nitrobacter</i>	Non-nitrifiers
First	0.74	ND ^A	ND	99.26
Second	80.03	0.97	0.15	18.85
Third	71.16	0.07	ND	28.77

^A Not detected

3.3.8 Enrichment strategy for NOB

Freshwater Medium was used to enrich NOB from the DWDS nitrifying biofilms, to limited success earlier with the relative abundance of *Nitrospira* NOB reaching 20.95% initially, then increased to 33.32% later in the enrichment effort. Treatment of the culture with 50 mg/L of ampicillin greatly improved with abundance of *Nitrospira* to 73.46%. A slight increase in *Nitrososphaera* AOA was observed with increasing abundance of *Nitrospira* in the culture, from 0.03% to 0.14% relative abundance (Table 5).

Table 5 Improvement of relative enrichment of NOB in culture over time

Metabarcoding event	Relative abundance (%)		
	<i>Nitrospira</i>	<i>Nitrososphaera</i>	Non-nitrifiers
First	20.95	ND ^A	79.05
Second	33.32	0.03	66.65
Third	73.46	0.14	26.4

^A Not detected

3.3.9 Community analysis of nitrifier enrichment cultures

Initial strategies yielded a very poor AOA enrichment, with AOA overgrown by AOB and most other bacteria from the Proteobacteria phylum (Fig. A7). The enrichment improved considerably after the introduction of DMTU and pyruvate into the culture, where AOB were washed out to below detection and AOA reached a relative abundance of close to 40%. The bulk of the bacterial contaminants shifted from the

Proteobacteria phylum to the Bacteroidetes phylum, where most genera were unknown (Fig. A8). With the reintroduction of filtration, the relative abundance of AOA improved further to 51.6%, and flipped the relative abundances of the Proteobacteria and Bacteroidetes phyla (Fig. A9).

The enrichment of AOB was easier, with *Nitrosomonas* reaching close to 71.2% relative sequence abundance in the last round of amplicon sequencing (Fig. A10). Most of the other bacterial contaminants were from the Proteobacteria phylum, and all the contaminants were present at a very low relative abundance. Of note, NOB *Nitrospira* were present in very low abundance. NOB were initially assumed to have been washed out of the enrichment, because nitrite accumulation was observed following successive treatment of the culture with sodium azide, coupled with a high frequency of subculturing (every two days) when the culture was diluted ten-fold. The rapid increase in nitrite concentration was thought to be disadvantageous for NOB to co-culture alongside AOB in the enrichment, but this was not observed in this enrichment effort.

The enrichment of NOB was challenging, because the medium used was less than optimal for their cultivation, with *Nitrospira* initially reaching close to 33% relative sequence abundance, before improving to 73.5% after treatment with 50 µg/ml ampicillin (Fig. A11). Most of the other bacterial contaminants were from the phyla Proteobacteria and Elusimicrobia, while a small amount of AOA (*Candidatus_Nitrososphaera*) was also present.

3.4 Discussion

The enriched AOA, AOB and NOB were closely related to *Nitrososphaera viennensis*, *Nitrosomonas oligotropha* and *Nitrospira moscoviensis*, respectively. Taxonomic identification was done by combining 16S rRNA gene and either *amoA* or *nxrB* sequence analysis. *Nitrosomonas oligotropha* was previously reported in bulk water or biofilms of several DWDS, through sequence analysis of the bacterial *amoA* and 16S rRNA (Regan, J.M. et al. 2002, Regan, J.M. et al. 2003). In other studies, sequence analysis of the bacterial *amoA* gene revealed *Nitrosomonas*-type AOB in the DWDS, without species level identification. *Nitrosomonas oligotropha* is considered well suited to grow in oligotrophic environments like drinking water (Koops, H.P. et al. 1991, Koops, H.-P. and Pommerening-Röser, A. 2015); hence, the discovery of *Nitrosomonas oligotropha*-like AOB in the present enrichment from DWDS biofilms was expected.

Similarly, the selection of *Nitrospira moscoviensis* in the NOB enrichment agreed with previous reports of NOB in bulk water or biofilms of DWDS, through sequence analysis of the 16S rRNA gene (Regan, J.M. et al. 2002, Regan, J.M. et al. 2003, Martiny, A.C. et al. 2005). Most studies identifying NOB from DWDS have pointed to the Group II *Nitrospira* cluster, which includes *Nitrospira moscoviensis*. Its adaption fits the niche environment of drinking water (Nowka, B. et al. 2015).

In contrast, while the AOA member enriched here was related to *Nitrososphaera viennensis*, previous work reported *Nitrosopumilus* in the bulk water of DWDS through

direct sequence analysis of the archaeal *amoA* gene. Although biofilm samples were collected from one pipe section (Roy, D. et al. 2020), it is unclear if those samples were used in the sequence analysis of the archaeal *amoA* gene. This difference between bulk water and biofilm AOA was also observed in freshwater environments (Li, M. et al. 2018), where the Group 1.1a *Nitrosopumilus* cluster of marine-related AOA was more abundant in the water column and *Nitrososphaera* were mostly found in sediments. It is, therefore, plausible that *Nitrososphaera viennensis*-like AOA were enriched from DWDS sediments in this study.

Our nitrifier enrichments built on earlier work of (Cruz, M.C. et al. 2020) who used 16S rRNA gene sequence analysis of the same biofilm samples obtained from pipe sections of the present DWDS in Singapore, but without enrichment step. Like the earlier studies, *Nitrosomonas* and *Nitrospira* were identified in those samples, while Marine Group I Thaumarchaeota was the AOA in the biofilm. Due to the limitation of 16S rRNA sequence analysis using the V4-V5 hypervariable region (515F and 926R in Table 3) alone, no species information was obtained. It is significant that our cultivation method for AOA led to the enrichment of *Nitrososphaera viennensis*-like AOA rather than Group I Thaumarchaeota. This may be due to different physiologically relevant conditions in the growth medium versus in-situ biofilms in the DWDS or indicate that the Group I Thaumarchaeota cells were either not viable or outcompeted by *Nitrososphaera viennensis* during enrichment.

The AOB and NOB enriched from the DWDS nitrifying biofilms were similar to those identified in DWDS bulk water in published works, but the enriched AOA members

differed from those known to occur in DWDS bulk water. This is significant because AOA in nitrifying biofilms have a longer residence time in DWDS compared to AOA in bulk water, whose presence in the DWDS would be transient under constant flow conditions. Therefore, the impact of AOA in nitrifying biofilms on monochloramine in DWDS is higher than that of AOA in the bulk water.

The biofilm samples used for AOA enrichment contained both AOA and AOB, which complicated the AOA enrichment. The concentration of ammonium supplied was not sufficiently low for AOA to outgrow AOB, and direct removal of AOB cells with filtration and antibiotic treatment (French, E. et al. 2012, Li, Y.Y. et al. 2016, Chen, H.Y. et al. 2017) severely impacted the overall growth of AOA. This can be attributed to the removal of catalase producing heterotrophic bacteria, which remove hydrogen peroxide produced during ammonia oxidation by AOA (Santoro, A.E. and Casciotti, K.L. 2011, Zinser, E.R. 2018).

Hydrogen peroxide and other reactive oxygen species (ROS) are natural by-products of biological aerobic metabolism, and they are generally toxic to cells at high concentrations, introducing oxidative damage to DNA and cellular components (Fridovich, I. 1983). Thiourea, in addition to being a hydroxyl radical scavenger (Anbar, M. and Neta, P. 1967), is also a metal chelator, allowing it to act as a non-specific hydroxyl radical scavenger. The ability to chelate copper ions makes allylthiourea a potent inhibitor of ammonia oxidation in AOB (Ginestet, P. et al. 1998), but less so in AOA (Shen, T. et al. 2013). DMTU, in addition to being a hydroxyl radical scavenger,

also acts as a hydrogen peroxide scavenger (Jackson, J.H. et al. 1985), rendering the compound a suitable agent for removing hydrogen peroxide from the culture.

α -keto acids like pyruvate are known to aid the growth of the AOA *Nitrososphaera*, perhaps by enabling mixotrophy (Kerou, M. and Schleper, C. 2016). In a study to determine how α -keto acids aid in the growth of AOA, (Kim, J.G. et al. 2016) determined that the organic acids functioned as hydrogen peroxide scavengers, as the effects were replicated when the α -keto acids were substituted with DMTU.

In the present study, it was decided to supplement the enrichment culture with DMTU and pyruvate to enhance AOA growth and inhibit AOB, based on their increased sensitivity to thiourea inhibition (Ginestet, P. et al. 1998).

Both DMTU and pyruvate are hydrogen peroxide scavengers, capable of replacing catalase producing heterotrophic bacteria to support the growth of AOA (Kim, J.G. et al. 2016), while AOB are more sensitive than AOA to inhibition by DMTU (Ginestet, P. et al. 1998, Shen, T. et al. 2013). Adding both agents to the growth medium proved successful in selectively enriching AOA over AOB from DWDS biofilms, and the method was further validated experimentally.

In the experiment to validate the use of DMTU and pyruvate to selectively enrich AOA over AOB from the nitrifying DWDS biofilms, 3 different treatments (With DMTU and pyruvate; With pyruvate only; Without DMTU and pyruvate) were compared. DMTU and pyruvate shared the same property of scavenging hydrogen peroxide, with DMTU carrying the additional property of inhibiting AOB growth. Therefore, the treatment

“With DMTU and pyruvate” served to promote AOA growth while inhibiting AOB growth, the treatment “With pyruvate only” served to promote AOA growth without inhibiting AOB growth, and the treatment “Without DMTU and pyruvate” was a baseline control. Pyruvate was used together with DMTU to maximize the removal of hydrogen peroxide from the culture.

As the experiment started with a low density of nitrifier cells in the media, ddPCR was chosen to quantify the nitrifiers confidently and accurately in the experiment, which required new primers to be designed. While published primers were available for the quantification of the nitrifiers with PCR methods (Rotthauwe, J.H. et al. 1997, Francis, C.A. et al. 2005, Pester, M. et al. 2014), the amplicons produced were between 485 and 635 bp, a size range that is less suitable for quantitative PCR methods. This is due to the less efficient PCR reactions and the presence of degenerate sequences in the primers that often require higher concentrations to be used. In combination, these sequences do not make for a clear differentiation of the signals from positive reactions and negative background signal, which is important for ddPCR applications. The experiments comparing designed and published primers showed that the designed primers produced PCR reactions with greater efficiencies and led to a clear separation of signals between the positive and negative reactions.

Although filtration of the AOA enrichment culture through 0.45 µm syringe filters, accompanied with consistent treatment with DMTU and pyruvate, improved the enrichment of *Nitrososphaera* AOA, close to half of the culture community were non-

nitrifiers with the majority coming from the phylum Proteobacteria. In particular, the genus *Hyphomicrobium* was the most abundant Proteobacteria after filtration.

Ultramicrobacteria, defined as bacteria possessing a cellular volume $< 0.1 \mu\text{m}^3$, are ubiquitous in the natural environment. They include some with small genomes adapted to small cell sizes, some capable of morphological changes like budding to produce cells with reduced sizes, and some adapting to stressed conditions like nutrient starvation by shrinking, while others have very slender morphologies like filamentous bacteria (Nakai, R. 2020). Indeed, filterable bacteria from phyla that included Proteobacteria and Bacteroidetes, originating from Lake Sanaru in Hamamatsu, Japan, were capable of passing through 0.22- μm filters (Maejima, Y. et al. 2018).

The genus *Hyphomicrobium* consists of Gram-negative, non-spore forming bacteria that reproduce by producing budding hyphae 0.2 – 0.3 μm in diameter (Gliesche, C. et al. 2015). Since the hyphae buds have diameters smaller than the 0.45 μm pore size of the filters used, young *Hyphomicrobium* buds can join *Nitrososphaera* in the filtrate for regrowth to occur using the soluble microbial products produced by the AOA.

The observation of a small relative abundance of AOA in the NOB enrichment was not surprising, as the NOB was originally enriched from the same biofilm sample that produced the AOA enrichment. Additionally, *Nitrospira* are capable of producing urease that will break down urea in the culture to produce ammonia (Koch, H. et al. 2015), which would support the growth of *Nitrososphaera*.

With the separate enrichment of individual nitrifiers (AOA, AOB, NOB), the effect of chloramination can be tested on these nitrifiers without the confounding effects of interactions between each nitrifier group. Although the micro-diversity among the individual nitrifiers was not investigated their existence was evident from the analysis of the cloned *amoA* and *nxB* sequences and the ASVs from 16S rRNA gene metabarcoding experiments. While interactions between nitrifier strains cannot be discounted, the interactions between the individual strains of the same nitrifier would be more similar than the interactions between the different nitrifiers.

The cultivation of nitrifying biofilms would be easier using highly enriched nitrifier starting cultures, as compared to bulk waters from the DWDS or water treatment plant filter backwashes requiring many months to cultivate a stable nitrifying biofilm. Even more relevant, the nitrifying biofilms cultivated from highly enriched nitrifier cultures would be dominated by the nitrifiers, unlike the nitrifying biofilms from previous works that included mostly heterotrophic bacterial communities (Lee, W.H. et al. 2011, Pressman, J.G. et al. 2012, Aggarwal, S. et al. 2018, Roy, D. et al. 2020).

By having highly enriched cultures of nitrifiers autochthonous to drinking water environments, studies in the context of drinking water conditions can be freed from reliance on model nitrifiers that are usually allochthonous to drinking water environments, which should produce results that are more relevant to DWDS. Additionally, these highly enriched nitrifier cultures will allow us to ask more detailed questions about the roles of specific drinking water nitrifiers.

In conclusion, the identities of the nitrifiers from Singapore DWDS matched those in previous works apart from the *Nitrososphaera viennensis*-like AOA, which have not been reported before in DWDS. These nitrifiers can be easily enriched separately using biofilm samples obtained from the DWDS, and the approach can be used for other DWDS studies. The availability of enriched autochthonous nitrifier cultures should allow for more accurate studies involving conditions of the DWDS and remove the reliance on commercial model nitrifier cultures. A study limitation is that the enriched nitrifiers came from single biofilm samples and may not fully represent the entire nitrifying community in DWDS biofilms.

4 Decay and regrowth of enriched nitrifiers in planktonic conditions after challenge from monochloramine

4.1 Introduction

In the DWDS, chloramine disinfection occurs both in the bulk water and in biofilms on the inner pipe surfaces. For nitrifying biofilms to form, enough planktonic cells must survive chloramine disinfection and colonize the surfaces in the DWDS.

Prior to this work, the effect of monochloramine on AOB in planktonic conditions had been studied on pure cultures of *Nitrosomonas europaea*, with cell concentrations that were not representative of actual DWDS conditions (Chauret, C. et al. 2008, Wahman, D.G. et al. 2009). In addition to being allochthonous to the drinking water environment, *Nitrosomonas europaea* is also physiologically distinct from the commonly found AOB, *Nitrosomonas oligotropha*; it possesses a much higher substrate half saturation constant, can tolerate much higher concentrations of ammonia, and is unable to break down urea to release ammonia. While the AOB enriched from the DWDS were classified as *Nitrosomonas oligotropha*-like, it is unknown how similar their physiology is to that of known *Nitrosomonas oligotropha* strains; likewise, the variability among the strains in the enrichments is unknown. However, the physiological traits of the characterized AOB can still be used to estimate those of the enriched AOB.

The effects of monochloramine on AOA and NOB have been largely overlooked, and the only available studies on AOB (Chauret, C. et al. 2008, Wahman, D.G. et al. 2009) should not be considered a close approximation to real world drinking water

environments. Having enriched three types of DWDS nitrifiers (see Chapter 4), the effect of monochloramine on these autochthonous organisms can now be investigated.

In addition, the previously mentioned studies focused on the inactivation of AOB by monochloramine and overlooked the possibility of inactivated AOB recovering after the removal of monochloramine. The lower reactivity of monochloramine compared to free chlorine was demonstrated by monochloramine's preferential reaction with sulfuryl groups in proteins (Ingols, R.S. et al. 1953). In contrast, free chlorine not only oxidizes sulfuryl groups, but also affects other important functional groups on proteins like the iron-bearing centers of heme enzymes. Both permanent structural alterations to proteins and increased permeability of the cell membrane have been observed (Ingols, R.S. et al. 1953, Venkobachar, C. et al. 1977).

As such, nitrifiers inactivated by monochloramine should have greater potential for regrowth than those inactivated by free chlorine. Therefore, to compare the nitrifiers' survivability and regrowth potential, the enriched nitrifiers were first diluted to 10^2 - 10^3 cells (genomic copies of *amoA* or *nxrB*) per ml of water before subjecting them to 24 h of chloramination using two concentrations of monochloramine: high at 3 mg/L and low at 1 mg/L. The small landmass of Singapore means that the distances between the endpoint consumer and the water treatment plants are small, and 24 h was assumed to be the maximum water age. Hence it was chosen as the period of disinfectant exposure. To assess the regrowth potential of the nitrifiers post

disinfection, they were cultivated in Fresh Water Medium (FWM) for 14 d, after quenching the residual monochloramine with Na_2SO_3 .

This chapter will address the following research question:

RQ2a: Do planktonic AOB have a higher tolerance for monochloramine than AOA and NOB?

Hypothesis: AOB have a higher tolerance for monochloramine disinfection than AOA and NOB.

Approach: Introduce monochloramine into diluted nitrifier cultures and sample for nitrifier and monochloramine quantification, then split into five small-volume reactor flasks for 24 h of incubation. After 24 h, sample from each flask for nitrifier and monochloramine quantification, then quench the remaining monochloramine. Incubate reactor flasks for 14 d to study recovery of inactivated nitrifiers. After 14 d, sample from each flask for nitrifier quantification.

4.2 Materials and methods

4.2.1 Fixation of AOA and AOB enrichment cultures

Forty milliliters of the nitrifier cultures were collected 50 ml centrifuge tubes, then centrifuged at 16,000 g for 10 min to pellet the nitrifier cells. The cell pellets were resuspended in 40 ml of 1x PBS buffer then centrifuged again at 16,000 g to pellet the washed cells. The washed pellets were resuspended with 1 ml of ice-cold 2% formaldehyde in 1x PBS buffer then incubated at 4 °C for 3 h. After incubation, the fixed samples were washed with ice-cold 1x PBS buffer, followed by resuspension in 1 ml of ice-cold 1:1 1x PBS and 96% ethanol to be stored at -20 °C.

4.2.2 Fluorescence In-Situ Hybridization of AOA and AOB

Before performing in-situ hybridization, the fixed samples stored in -20 °C were vortexed briefly and 10 µl of suspension were applied onto the cells of a Teflon coated microscopy slides with Adcell (Thermo Fischer), to be dried in a 46 °C oven for approximately 15 min. After the samples were dried, the slides were dipped sequentially into 50%, 80% then 96% ethanol for 3 min each, followed by drying the slides in a 46 °C oven until the samples were completely dehydrated.

The probes used for labelling AOA and AOB for confocal laser scanning microscopy (CLSM) are listed in Table 6, and they were diluted to 30 ng/µl working concentration before performing in-situ hybridization and kept on ice. Two microliter of each probe was added into 20 µl of Hybridization Buffer with 35% formamide (Per ml: 180 µl 5 M NaCl, 20 µl 1 M Tris-HCl, 350 µl formamide, 1 µl 10% SDS, 449 µl ddH₂O) then applied

to each sample cell on the slides. Each slide was then placed in a 50 ml centrifuge tube atop a paper tower soaked with the remaining volume of Hybridization Buffer, then the tube was capped tightly and placed horizontally on a rack placed in a 46 °C oven and incubated for 2 h. After incubation the slides were wash with Washing Buffer (Per 50 ml: 0.7 ml 5 M NaCl, 1 mL 1 M Tris-HCl, 0.5 ml 0.5 M EDTA, 47.8 ml ddH₂O) prewarmed to 48 °C, then transferred into the remaining Washing Buffer and incubated in a 48 °C water bath for 10 min. The slides were quickly dipped in ice-cold ddH₂O after incubation, then air dried under the flow of nitrogen gas to quickly dry the samples. A small drop of Prolong Diamond Antifade mountant with DAPI (Invitrogen) was applied per four cells in each slide, then a microscopy cover slip was carefully applied onto the samples spreading the mountant evenly across each cell. The samples were left to cure for 24 h before microscopic observation.

Table 6 Oligonucleotide probes targeting ribosomal RNA used in this study

Probe	Fluorophore ^a	Sequence (5'-3')	Competitor sequence ^b (5'-3')	FA ^c (%)	Target	Reference
S-D-Arch-0915-a-A-20	Alexa Fluor 448	GTGCTCCCCCGCCAATTCCT		35	Archaea	(Amann, R.I. et al. 1990, DeLong, E.F. 1992)
Nm_OL_703	Cy5	GCCATCGATGTTCTTCCATATCTC	GCCATCGGTGTTCTCCATATCTC GCCATCGGTGTTCTCCACATCTC	35	N. oligotropha, N. ureae, N. aestuarii, N. marina lineage (cluster 6a+6b)	(Reysenbach, A.L. et al. 1992, Lukumbuzya, M. et al. 2020)

^a Fluorophore(s) used were labelled on the 5' and 3' ends of the probes. ^b Competitors were unlabeled oligonucleotides and added in equimolar concentrations as the labeled probes. ^c Formamide, with the concentration in the hybridization buffer (v/v) indicated.

4.2.3 Confocal Laser Scanning Microscopy

The samples were observed with the Carl Zeiss LSM 780, utilizing the 100x objective with oil immersion, and the software ZEN 2.3 SP1 FP3 Black (Carl Zeiss), using an Airy Unit of 1 for all scans. Image acquisition was performed with z-stack at maximum scan speed, using a frame size of 512 x 512 pixels and an averaging of 2 with Line mode, Mean method and 8 Bit depth, to obtain an image size of 85.0 μm x 85.0 μm .

Image processing of the CLSM images was done with the Imaris ver. 9.0.0 (Bitplane AG). In brief, the image was loaded into the “Surpass” function with “3D View” and “Smoothing” was performed with “Median Filter” on all channels with the filter size of 3x3x1, followed by “Background Subtraction” on all channels with the default “Filter Width”. “Volume” and “Ortho Slicer” was checked under “Scene”, with the “XY Plane” option was checked under “Slice Orientation”, the maximum thickness chosen for “Extended Section” and “Slice Position”, and the “Show Frame” option unchecked. The scale bar was adjusted accordingly before the image was exported as a TIFF image with the “Snapshot” function.

4.2.4 Formation of monochloramine in solution

One molecule of monochloramine is formed when one molecule of hypochlorite reacts with one molecule of ammonia (Eq. 1). Stoichiometrically, one molecule of chlorine (Cl) reacts with one molecule of nitrogen (N), which gives rise to the Cl:N mass ratio of 5:1. To improve the stability of monochloramine in solution, the Cl:N mass ratio of 4:1 was chosen, introducing a slight abundance of ammonia (1 molecule of chlorine

to 1.25 molecule of nitrogen) to discourage the formation of dichloramine (Eq. 2). 1 M NH_4Cl was used as the ammonia source, with a calculated equivalent concentration of 14 g/L N- NH_3 , and 5% sodium hypochlorite (Sigma Aldrich) was used as the chlorine source, with a calculated equivalent concentration of 50 g/L Cl.

There are two colorimetric methods for the quantification of monochloramine, the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method and the indophenol method (Hach MonochlorF, HACH Method 1017). Both methods accurately measure monochloramine in nanopure water, but the MonochlorF method is able to specifically quantify monochloramine over other chloramines like dichloramine and organic chloramines (Lee, W. et al. 2007).

According to Hach Company, the HACH method 10172 Indophenol Method¹ has a detection range of 0.1 – 10 mg/L for monochloramine, and the chemical compounds present in the Incomplete FWM are listed as non-interfering substances. Under ideal conditions tested by the company, the method had a 95% confidence interval of 5.6–6.2 mg/L monochloramine, with a sensitivity of 0.1 mg/L monochloramine, when tested with a monochloramine standard of 5.9 mg/L concentration.

4.2.5 Decay and regrowth of planktonic nitrifiers after 24 hours monochloramine exposure

The overall outline of the experiment is described in Figure 18. All surfaces to be in contact with monochloramine were pretreated with 5000 mg/L hypochlorite solution

for 24 h (Wahman, D.G. et al. 2009), then rinsed thoroughly with ultrapure Type 1 water. The rinsed surfaces were then dried in a 70 °C oven until used.

Monochloramine was formed in Incomplete FWM (1 g/L NaCl, 0.4 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 g/L KH_2PO_4 , 0.5 g/L KCl). To achieve the concentrations of 1 mg/L and 3 mg/L monochloramine, 1 mg/L or 3 mg/L of chlorine was added to the Incomplete FWM with 5% active chlorine hypochlorite (Sigma Aldrich), using HACH Method 10231 to measure free chlorine concentration. Then, 0.25 mg/L or 0.75 mg/L of $\text{NH}_3\text{-N}$ was added to the chlorinated Incomplete FWM with 1M NH_4Cl , to form 1 mg/L or 3 mg/L monochloramine. The monochloramine concentration was measured with HACH Method 10172.

The nitrifiers were diluted into a bulk volume of chloraminated Incomplete FWM to achieve 10^2 - 10^3 *amoA* genomic copies/ml of AOB, and 10^3 *amoA* and *nxB* genomic copies/ml of AOA and NOB. The bulk chloraminated nitrifier cultures were then separated into 100 ml Schott bottles with a liquid volume of 130 ml to minimize headspace. For each nitrifier, and for each level of monochloramine (0, 1, 3 mg/L), there are 5 replications placed in an unlit 30 °C incubator for 24 h. A 50-ml sample was drawn from each leftover nitrifier bulk cultures, then filtered through a Sterivax™ filter unit to collect the nitrifier biomass, while the filtrate is used to measure the starting monochloramine concentration.

At the end of the 24 h incubation, 10 ml of sample was drawn and filtered through a 0.2 μm syringe filter, to measure the end monochloramine concentration. The

monochloramine in the remaining 120 ml culture was quenched by adjusting 7.35 mg/L Na_2SO_3 into the culture using 1 g/L Na_2SO_3 solution. The FWM was completed by adding the remaining trace element solution, B vitamin solution, Ferric-iron EDTA solution, bicarbonate solution, HEPES buffer, and adjusting the culture to pH 8.0, followed by the addition of NH_4Cl or NaNO_2 . 50 ml of sample was drawn from each replicate and filtered through the SterivaxTM filter unit to collect the nitrifier biomass, while 60 ml of the culture from each replicate was transferred into a 100 ml Erlenmeyer flask covered with black paper.

The 60 ml recovering cultures were incubated in a 30 °C shaker incubator for 2 weeks for nitrifier regrowth to occur. At the end of the incubation, 50 ml of sample was drawn from each replicate and filtered through the SterivaxTM filter unit to collect the nitrifier biomass.

The nitrifier biomasses were quantified via ddPCR, using the NSS_amoA, NSM_amoA and NSP_nxrB primer sets (Table 3) for AOA, AOB and NOB respectively. Decay of biomass were determined by the difference in gene copy numbers before and after 24 h of chloramination. Regrowth of biomass was based on the difference in gene copy numbers before and after 14 d of recovery of the remaining biomass post-chloramination, after the leftover monochloramine had been quenched.

Fig. 18 Experimental approach to determine decay and regrowth of chloraminated nitrifiers in planktonic conditions

4.2.6 Statistical analysis of decay and regrowth of planktonic nitrifiers data

One-way ANOVA was performed using the R package Rstatix version 0.6.0, with R version 3.6.2 (R Core Team 2018). Outliers were identified using the “identify_outliers” function, and outliers were removed only when assumption of homogeneity of variance was violated in the Levene’s test. Assumption of normality was tested using Shapiro-Wilk’s normality test, before proceeding to performing ANOVA. When both assumptions were not violated, ANOVA was performed using the “anova_test” function, while Welch’s ANOVA was performed using the “welch_anova_test” function,

when the assumption of homogeneity of variance was violated after the removal of outliers. Tukey's honest significance test was performed on the 3 factors to determine significant difference between the factors. Type III sum of squares was chosen for the ANOVA tests, to account of the unbalanced observations due to removal of outliers, or observations lost to sampling error.

4.3 Results

4.3.1 Microscopic observation of enriched AOA and AOB

AOB cells were not detected in the AOA enrichment observed under CLSM, and the AOA cells were observed to have irregular spherical morphologies with diameters of $< 0.5 \mu\text{m}$ (Fig. 19 and A18). AOA cells were not detected in the AOB enrichment observed under CLSM, AOB cells were observed to have short rod morphologies with lengths of $0.5 - 1 \mu\text{m}$ (Fig. 19 and A19).

Fig. 19 Fluorescent in-situ hybridization of the AOA and AOB enrichment observed with confocal laser scanning microscopy under 100x objective with oil immersion. The scale bar represents 1 μm . In the AOA enrichment AOB cells were not detected, and total cells were stained with DAPI. The AOA cells have a diameter of approximately < 0.5 μm . In the AOB enrichment AOA cells were not detected although faint Archaea 16S rRNA signals were generated, and total cells were stained with DAPI. The AOB cells have a length of approximately 0.5 – 1 μm .

4.3.2 Stability of monochloramine in Incomplete Freshwater Medium

To estimate the stability of monochloramine in the chosen growth medium, a simple experiment was performed with five replicates, using the same materials as in the study for planktonic nitrifier decay and regrowth, involving high (3.0 mg/L) and low (1.0 mg/L) concentrations of monochloramine. Incomplete Freshwater Medium (FWM) (1 g/L NaCl, 0.4 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 g/L KH_2PO_4 , 0.5 g/L KCl) was used instead of the complete FWM (refer to Chapter 3.13), to remove the chlorine demand from the medium, to enable maximum reaction between monochloramine and the nitrifier biomass. The incomplete FWM is the Freshwater Medium without the addition of trace elements, HEPES buffer, soluble iron, sodium bicarbonate and B vitamins.

After a 24 h incubation of monochloramine in FWM under abiotic conditions at 30°C, solutions with the low monochloramine concentration had decreased by 0.3 (± 0) mg/L MC, while solutions with a high monochloramine concentration had decreased by 0.5 (± 0) mg/L monochloramine.

4.3.3 Decay and regrowth of AOA after monochloramine exposure

After 24 h, the enriched *Nitrososphaera viennensis* exposed to either the high or the low concentration of monochloramine experienced significantly higher decay compared to the control group without added monochloramine; there was no significant difference between high and low concentrations of monochloramine (Fig. 20A). After 14 d of recovery, the percentage recovery of AOA was not significantly different among the control group and treatment groups of high and low monochloramine concentration (Fig. 20B).

In the control group where monochloramine and ammonia were absent, some decay was observed but was significantly higher at both concentrations of monochloramine. Before AOA were diluted to the experimental concentration, the cells were washed with Incomplete FWM via centrifugation, and since *Nitrososphaera viennensis* do not have an outer membrane covering the S-layer cell wall, the AOA are likely to be more permeable to leak substrates during the washing step. The potential lack of residual substrates in the washed cells may explain the decay of the AOA in the control group.

The recovery studies suggested that the potential for regrowth of *Nitrososphaera viennensis* was not affected by inactivation by monochloramine, indicating that the AOA are resilient but not resistant to inactivation by monochloramine.

Fig. 20 Comparing decay and regrowth of AOA between treated and non-treated groups. The decay of AOA after 24 h of chloramination was similar with either concentration of monochloramine and was significantly higher than that of non-chloraminated AOA, (**A**) but chloraminated AOA shared similar regrowth to non-chloraminated AOA (**B**). $N = 5$. “****” $p < 0.0001$. Non-significant interactions are not

shown in the plots. In (B) the y-axis depicts percentage regrowth from leftover biomass after 24 h of incubation with or without monochloramine.

4.3.4 Decay and regrowth of AOB after monochloramine exposure

After 24 h, AOB exposed to either high or low concentrations of monochloramine had a significantly higher decay compared to the control group without added monochloramine, while the difference in decay was not significantly different between treatments of high and low concentration of monochloramine (Fig. 21A). After 14 d of recovery, the recovery of AOB was significantly lower than that of the control group. Like the decay of AOB, there was no significant difference in the recovery of AOB between the high monochloramine group and the low monochloramine group, but the difference between the control, which saw no decay, and treatment groups was approximately 3 – 4 orders of magnitude (Fig. 21B and C).

In the absence of monochloramine and a source of ammonia, AOB were able to achieve a small amount of growth within 24 h in the control group. This could be attributed to residual growth of late-log phase AOB used early in the experiment. As such, the recovery of AOB in the control group was a continuation of the previous growth.

Similar to AOA, *Nitrosomonas oligotropha* was sensitive to inactivation by monochloramine, leading to the decay of biomass over a 24 h period. However, the regrowth was very poor compared to the non-chloraminated control, suggesting that the AOB were neither resistant nor resilient to inactivation by monochloramine.

Monochloramine ▢ Control ▢ Low ▢ High
pwc: **Tukey HSD**; p.adjust: **Tukey**

Monochloramine ▢ Control ▢ Low ▢ High
pwc: **Games Howell**; p.adjust: **Tukey**

Monochloramine ▢ Low ▢ High
pwc: **Tukey HSD**; p.adjust: **Tukey**

Fig. 21 Comparing decay and regrowth of AOB between treated and non-treated groups. The decay of AOB after 24 h of chloramination was similar with either concentration of monochloramine but non-chloraminated AOB did not experience decay, (**A**) and the regrowth of the chloraminated AOB was also similar for either concentration of monochloramine (**B** and **C**). N = 5. “****” $p < 0.0001$. “ns” Not Significant Non-significant interactions are not shown in the plots **A** and **B**. In (**B**) the y-axis represents percentage regrowth from leftover biomass after 24 h chloramination, and the regrowth of non-treated AOB was the continuation of growth after 24 h of incubation without monochloramine.

4.3.5 Decay and regrowth of NOB after monochloramine exposure

After 24 h, NOB exposed to a high concentration (3 mg/L) of monochloramine had a significantly higher decay compared to the control group without added monochloramine, while the difference in decay was not significantly different between the control and the treatment with a low concentration (1 mg/L) of monochloramine (Fig. 22A). After 14 d of recovery, NOB failed to recover and continued to decay (Table S1). The extent of decay after the 14 d recovery period was not significantly different between the two levels of monochloramine (Fig. 22B).

Based on these results, NOB appear to be resistant to cellular decay at 1 mg/L monochloramine within a 24 h period, but less resilient than AOA and AOB to cellular injury by monochloramine, by failing to regrow after 14 d of recovery.

Fig. 22 Comparing decay and regrowth of NOB between treated and non-treated groups. There was significantly less decay of NOB after 24 h of chloramination with a low concentration of monochloramine than with a high concentration, while non-chloraminated NOB did not experience any decay (**A**). The chloraminated NOB failed to re-grow after exposure to either concentration of monochloramine (**B**). N = 5. “*” p < 0.05, “***” p < 0.001, “****” p < 0.0001. Non-significant interactions are not shown in the plots. In (**B**) the y-axis is for percentage regrowth from leftover biomass after 24 h chloramination, and the regrowth of non-treated NOB was the continuation of growth after 24 h incubation without monochloramine.

4.3.6 Comparing nitrifier decay and regrowth after chloramination

When exposed to low concentrations of monochloramine, NOB decayed more slowly than AOA and AOB, which experienced similar levels of decay. At the high monochloramine level NOB also decayed less than the AOM, but AOA experienced significantly greater decay than AOB (Fig. 23A). The non-treated AOB and NOB did not decay, but displayed residual growth over the treatment period, and the amount of growth was not significantly different between the two nitrifier types (Fig. 23A). The regrowth of the two AOM was similar post exposure to the high monochloramine concentration, but AOA showed significantly higher levels of regrowth than AOB post exposure to the low concentration of monochloramine. Although the regrowth of NOB did not occur, the regrowth of treated AOB was low enough to not be significantly different from NOB (Fig. 23B). Continued growth of non-treated AOB was significantly higher than that of non-treated NOB and regrowth of non-treated AOA, but the

regrowth of non-treated AOA was not significantly different from the continued growth of non-treated NOB (Fig. 23A).

Fig. 23 Comparing decay and regrowth among AOA, AOB and NOB, within the treated and non-treated groups. The decay of non-treated AOA was significantly higher than that of non-treated AOB and NOB, which did not suffer decay. The decay of NOB treated with a high concentration of monochloramine was significantly lower than that of AOA and NOB, and the decay of AOA treated with a low concentration of monochloramine was significantly higher than that of treated AOB and NOB, of which AOB had significantly higher decay than NOB (**A**). The continued growth of non-treated AOB was significantly higher than that of non-treated NOB and of regrowth of non-treated AOA, while the regrowth of AOA treated with low concentration of

monochloramine was significantly higher than that of AOB and NOB, and the regrowth of AOA treated with high concentration of monochloramine was significantly higher than that of NOB but not of AOB (**B**). $N = 5$. “*” $p < 0.05$, “**” $p < 0.01$, “***” $p < 0.001$, “****” $p < 0.0001$. Non-significant interactions are not shown in the plots. In (**B**) the y-axis is for percentage regrowth from leftover biomass after 24 h chloramination, and the regrowth of non-treated AOB and NOB was the continuation of growth after 24 h incubation without monochloramine.

4.4 Discussion

Decay after 24 h of monochloramine exposure in the absence of ammonia or nitrite was only observed in AOA, whereas AOB and NOB cell numbers increased slightly. Both AOB and NOB are gram-negative bacteria that possess an enclosed periplasmic space, while AOA contain a pseudo-periplasmic space enclosed by a much more permeable S-layer protein array. The experimental setup involved washing of the cultured nitrifier cells prior to inoculation, which was meant to remove traces of ammonium or nitrite from the culture. This may have led to the leakage of residual ammonia and hydroxylamine from the more permeable AOA cells, while their less permeable outer cell membrane would allow AOB and NOB cells to better retain the substrates in their periplasmic space during the washing step. It is possible that low substrate levels in the periplasmic spaces of AOB and NOB allowed for residual growth in the control group of the experiment, even in the absence of externally supplied substrates.

It has been experimentally demonstrated that AOB resistance to monochloramine is the result of the reaction between monochloramine and hydroxylamine produced by ammonia oxidation, likely depleting monochloramine from the local environment of the cells (Wahman, D.G. et al. 2014, Wahman, D.G. and Speitel, G.E. 2015). If AOB were able to retain hydroxylamine in their periplasmic space during the washing step, they would be better protected from cell damage due to monochloramine, compared to AOA that are less likely to retain hydroxylamine. Since monochloramine reacts with nitrite, residual nitrite in the periplasmic space of NOB may explain their apparent resistance to the low monochloramine concentration. The present study is the first to investigate resistance of NOB to monochloramine and no other mechanism of monochloramine resistance in NOB has been proposed to date. I posit that actively growing NOB may resist at low concentrations of monochloramine by depleting monochloramine intracellularly using residual levels of nitrite present.

In future experiments using more enriched nitrifier cultures with a higher biomass density, the need to wash the cells before chloramination can be avoided by extensively diluting the nitrifier cultures. Here, the nitrifier enrichments resulted in a cell density in late-log cultures of approximately 10^6 to 10^7 cells/ml, which would then result in a 1000 to 10,000-fold dilution of the culture in the experiment to achieve the starting density of 10^3 /ml of nitrifier cells. This may have effectively diluted out the nitrite in the culture. Therefore, residual growth of AOA may also be observed after 24 h in the control group without monochloramine exposure.

Alternatively, 0.05 mg/L of ammonium or nitrite can be introduced into the washed and diluted nitrifier cells 24 h prior to chloramination, to allow the build-up of ammonium and hydroxylamine in the AOM periplasmic space, and the accumulation of nitrite in the NOB periplasmic space. This would allow the nitrifiers to better prepare for the stress of chloramination, and this effect could be demonstrated by comparing the treatment with washed and diluted nitrifier cells in the absence of augmentation by ammonium or nitrite.

The data from the recovery experiments suggest that the nitrifiers were not completely resistant to monochloramine, and the surviving nitrifier cells were likely injured by monochloramine during 24 h of exposure. In all three nitrifiers, the enzymes responsible for ammonia/nitrite oxidation are in the periplasmic space, which exposes them to reactions with monochloramine. Both hydroxylamine oxidoreductase (HAO) (Cedervall, P. et al. 2013) and nitrite oxidoreductase (NXR) (Yamanaka, T. and Fukumori, Y. 1988, Cabello, P. et al. 2009) are heme containing enzymes, where the heme molecules have important structural roles in the catalytic sites. The irreversible alteration of heme groups, through oxidation of sulfuryl groups by monochloramine, has been well documented and is considered the main mode of inactivation of microbial cells by monochloramine (Ingols, R.S. et al. 1953). Therefore, when hydroxylamine or nitrite is depleted from the nitrifier cells, the absence of those substrates within the vicinity of HAO or NXR will leave those key enzymes vulnerable to inactivation by monochloramine.

The *Nitrososphaera viennensis* cells are the smallest amongst the nitrifiers of the DWDS, and the maintenance energy budget of this AOA differs from that of AOB and NOB. The AOA were microscopically confirmed to be smaller than the AOB using fluorescent in situ hybridization (FISH). When placed in an energy-scarce environment, the smaller AOA cells must devote more maintenance energy to protein repair than the larger cells of AOB and NOB. This handicap likely exerts a selective pressure on AOA to evolve more resilient metabolic pathways, and/or evolve more efficient protein turnover machinery (Kempes, C.P. et al. 2017). The ammonium monooxygenase (AMO) subunit AmoC possesses chaperon-like functions that can stabilize the function of the AMO protein complex (Qin, W. et al. 2018), and *Nitrososphaera viennensis* possesses multiple copies of *amoC* in its genome (Spang, A. et al. 2012, Bayer, B. et al. 2016, Lehtovirta-Morley, L.E. 2018), giving the AOA the advantage of forming a more stable AMO than AOB. AOA and AOB share a similar pathway for ammonia oxidation (Vajjala, N. et al. 2013); yet the archaeal homolog to HAO remains elusive, which opens up the possibility that the archaeal HAO of *Nitrososphaera viennensis* is structurally more resilient to monochloramine damage than the bacterial HAO of AOB.

In conclusion, the hypothesis of AOB being more resistant to monochloramine than AOA is true only for concentrations lower than 3 mg/L, and non-dormant NOB appear to be more resistant than AOB. In addition, AOA are the most resilient to chloramination of the three nitrifiers, given that prior chloramination appeared to have no significant impact on their growth potential. This may indicate a strain-level variation

among the enriched AOA, where a subpopulation of AOA is more resistant to chloramination, which would suggest a future experiment to sequence the archaeal *amoA* genes selected by chloramination.

5 Decay or growth of enriched nitrifiers in mixed biofilms continuously exposed to monochloramine

5.1 Introduction

Secondary disinfection in DWDS primarily serves to maintain biological stability of the distributed water (Prest, E.I. et al. 2016), to provide a level of protection against potential microbial contamination events and serve as indicator of such events when disinfectant demand increases unexpectedly (Olivieri, V.P. et al. 1986). The residence time of microorganisms in the bulk water is dependent on the water age in the network, whereas microorganisms in DWDS biofilms have much longer residence times that are related to the age of the drinking water pipe instead. Therefore, contact times with the residual disinfectant of nitrifiers will be longer in biofilms than in the bulk water.

Microbial regrowth in the DWDS is monitored through sampling of bulk water to perform heterotrophic plate counts, or in advanced facilities to perform flow cytometry or quantitative PCR. However, the build-up of biofilms in the DWDS would elude the surveillance of microbial regrowth in the bulk water, thus would be undetected with regular monitoring.

According to the United States Environmental Protection Agency's guideline for nitrification (Water, O.o.G.W.a.D. 2002), monitoring for nitrification can be done indirectly based on one or more of the following DWDS properties: loss of residual chloramine; increase in water temperature; decrease in dissolved oxygen; decrease in pH and alkalinity; and increase in heterotrophic plate count. Additionally, nitrite

levels of 0.05 mg/L can be used as a trigger for breakpoint chlorination in DWDS (Wolfe, R.L. et al. 1988, Odell, L.H. et al. 1996, Wilczak, A. et al. 1996). Nitrite in the bulk water of DWDS is likely released from actively nitrifying biofilms on pipes, since the slow growth rates of nitrifiers would not result in appreciable nitrite production by ammonia oxidizers in the bulk water itself, which has a limited residence time before reaching consumers.

Access by researchers to the biofilms in the DWDS is usually only possible when pipe sections require maintenance or replacement. With regular monitoring of biofilms in the DWDS being unfeasible, understanding the response of nitrifying biofilms to chloramination can potentially allow for the prediction of nitrification episodes in the DWDS.

In the previous chapter, the decay of nitrifier in planktonic cultures after 24 h of chloramination and the regrowth of the remaining nitrifiers for 14 d were explored, to determine the resiliency of the nitrifiers insulted by chloramination. To relate those observations to nitrifiers growing in biofilms, nitrifying biofilms grown from either AOA or AOB were continuously challenged by chloramination for 14 d, to compare post-insult regrowth of free living AOM with growth or decay of sessile AOM under constant insult. NOB were not studied in this experiment because the addition of nitrite to allow NOB growth would have created a high chloramine demand.

Hence, this chapter seeks to expand our knowledge on the effects of chloramination on planktonic single enriched nitrifier cultures and biofilms, and addresses the following research question:

RQ2b: Do AOB in biofilms have a higher tolerance for monochloramine than AOA?

Hypothesis: AOB have a higher tolerance for monochloramine disinfection than AOA.

Approach: Cultivate AOA or AOB biofilms on cement coupons in a CDC biofilm reactor (see Chapter 3.15), then transfer coupons with AOA or AOB biofilms to flow cells. Sample two coupons from the biofilm reactor for each flow cell. Introduce monochloramine into filtered and dechloraminated tap water, then continuously supply the rechloraminated tap water to the flow cells and operate as a flow-through system for 14 d. After 14 d, sample the coupons from the flow cells to quantify the AOM and image cells using FISH.

5.2 Materials and methods

5.2.1 Decay or growth of Ammonia-oxidizing microorganisms in biofilm with constant monochloramine exposure

The 2-L amber Schott glass bottles used as reservoirs were pretreated with 5000 mg/L hypochlorite solution for 24 h (Wahman, D.G. et al. 2009), then rinsed thoroughly with ultrapure Type 1 water. The rinsed bottles were then dried in a 70 °C oven until used. The tubing and bubble traps used in the flow-cell set up were pretreated with 5000 mg/L hypochlorite solution for 24 h at a flowrate of 0.4 ml/min, then rinse with 2 L of ultrapure Type 1 water using gravity flow.

Fifteen liters of DWDS water was obtained from a high pressure tap in the laboratory after wasting approximately 15 L of tap water, to rinse the premise plumbing of

stagnant building water. The 15 L of tap water (approximating DWDS water) was filtered using a 0.2 μm filtration unit into 3 separate 5-L bottles. The filtered tap waters were autoclaved at 121°C for 15 mins to accelerate the decay of monochloramine in the water. After cooling, the autoclaved waters were tested with HACH Method 10172 to confirm the removal of monochloramine in the waters, which were then deemed to be dechloraminated DWDS waters.

For the cultivation of AOA, 40 ml of late-log AOA enrichment culture were inoculated into 400 ml of FWM in the reactor, with 2 mM of NH_4Cl , 100 μM of DMTU and 100 μM of pyruvate supplied. When approximately 90% of the NH_4Cl were exhausted, the sample coupons together with the coupon rods were temporally stored in 400 ml of dechloraminated DWDS water, while the reactor vessel and the stir bar/vane assembly scrubbed cleaned with Virkon S Solution and rinsed thoroughly with ultrapure Type 1 water. Excess biofilms were removed from the back of the coupon rods with paper towels, before the coupon rods were returned to the cleaned reactor with 400 ml of dechloraminated DWDS water supplemented with 7.5 μM ferric sodium EDTA, 10 mM HEPES, 6 mM NaOH, 2 mM NaHCO_3 , 200 μl of vitamin solution, 200 μl of trace element solution and 2 mM of NH_4Cl .

On the same day that the AOA biofilms were transferred to dechloraminated DWDS water, the cultivation of AOB biofilms were initiated by inoculating 4 ml of late-log AOB enrichment culture into another reactor with 400 ml of FWM with 5 mM of NH_4Cl . Upon exhaustion of approximately 90% of the substrate, the steps described above were mirrored for the AOB biofilms, with the exception of 5 mM of NH_4Cl supplied.

When approximately 80% of the NH_4Cl were consumed in the dechloraminated DWDS water, 2 AOA biofilm coupons and 2 AOB biofilm coupons were transferred into each of the 3 FC 274-AL anodized aluminum Dual Channel Flat Plate Coupon Evaluation Flow Cells (BioSurface Technologies Corporation) that were previously brush cleaned with Virkon S Solution. Additionally, 3 AOA biofilm coupons and 3 AOB biofilm coupons were collected from the reactor, for DNA extraction using the DNeasy PowerBiofilm Kit (Qiagen), using micro foam head Critical Swabs (VWR) to collect the biofilms from the coupons. Three more AOA biofilm coupons and AOB biofilm coupons were collected from the reactor for Fluorescence In-Situ Hybridization (FISH) of AOA and AOB cells, to be visualized under co-focal laser scanning microscopy (CLSM).

One coupon flow cell (1 channel with AOA biofilms and 1 channel with AOB biofilms) was subjected to 0.4 ml/min of dechloraminated DWDS water containing 3 mg/L of monochloramine, while another coupon flow cell was subjected to 0.4 ml/min of dechloraminated DWDS water containing 1 mg/L of monochloramine, and the last coupon flow cell was subjected to 0.4 ml/min of dechloraminated DWDS water as control (Fig. 24). The flow cells were under constant flow for a period of 14 d, with 2 L of DWDS water replenished every 2 d between Mondays and Fridays, and every 3 d between Fridays and Mondays. Reservoir waters were tested with HACH Method 10172 to measure the monochloramine concentrations at the start and finish of each replacement. At the end of 14 d, one coupon from each channel was collected for DNA extraction, while the remaining coupon from each channel was collected for future FISH and visualization under CLSM.

Fig. 24 Scheme of flow cell set up. Starting from the water reservoirs (a) & (b) in 2 L amber bottles containing 2.2 L of filtered dechloraminated water with or without monochloramine, to the peristaltic pumps (c), to the dual-channel coupon evaluation flow cells (d), to the 20 L waste reservoir (e). Each water reservoir had two fluid lines; one connected to the pump via a 3-way port for the purpose of priming the fluid line, another reserved for sampling from the reservoir. Each reservoir also had one filtered air valve. In each dual-channel flow cell, one channel occupied two coupons with nitrifying biofilms containing AOA, and another two coupons with nitrifying biofilms containing AOB. Between the flow cells (d) and the pumps (c), the fluid lines were connected by a bubble trap.

5.3 Results

5.3.1 Surface pH of cement coupons

The surface pH of the cement coupons was measured by pressing the test face of the wetted non-bleeding pH indicator strips (Merck Millipore) onto the wetted cement surfaces and then rubbing the back of the strips for approximately 10 s. The process was repeated after the top cement surfaces had been filed away with steel wool to measure the pH of the cement underneath the material with biofilm growth.

Both AOA and AOB lowered the pH of the cement surface from 9 – 10 to 6 – 7, and lower layers of the cement coupon changed to a pH of 8 – 9. It was observed that the cement had hardened after the growth of AOM biofilms, and it was noticeably harder to file away after the surface had been neutralized due to the growth of AOM biofilms, especially so in the case of AOA (Fig. 25).

In addition, the cement coupons used for AOB biofilm growth showed greater surface erosion than the coupons used for AOA biofilm growth; this was also observed during biofilm collection from the coupons, when more cement materials were collected on the swabs. The biodeterioration of cement materials caused by nitric acid produced from biological ammonia oxidation has been previously documented (Sand, W. and Bock, E. 1991). Greater deterioration of the cement coupons colonized by AOB compared to coupons containing AOA was expected since more AOB biofilm mass was produced.

Fig. 25 Conceptual model of pH measurement on cement coupons. The pH of the cement surface decreased from pH 9 – 10 to pH 6 – 7 after AOM biofilm growth. After the surface layer of cement was filled away, the pH of the cement layer underneath was measured to be between pH 8 – 9. The cement coupons were noticeably softer after AOB biofilm growth, which resulted in more cement materials being removed from the surface.

5.3.2 AOM in the nitrifying biofilms were of the same order of magnitude on every coupon

The cement coupons used in the experiments were made by BioSurface Technologies Corporation by curing cement filled on polycarbonate cups and were made to order. As such, it is very likely that variations exist between batches of coupons and between individual coupons of each batch, varying in surface topology and surface chemistry.

To determine if such variations could affect biofilm growth of the AOM on such coupons, test biofilm cultures with either AOA or AOB enrichments were made in the CDC biofilm reactors, and four coupon rods each holding three coupons were collected from each reactor to enumerate the AOM in the biofilms growing on the coupon surfaces.

The AOA in the nitrifying biofilms were found to range from 4×10^6 to 6×10^6 cells per coupon on 12 coupons (Fig. 26A), while the AOB in the nitrifying biofilms ranged from 2×10^7 to 4×10^7 cells (Fig. 26B). Since the respective nitrifiers were of the same order of magnitude on replicate coupons, the use of the cement coupons for experimenting with nitrifying biofilms was deemed suitable.

Fig. 26 Variability of AOM grown on replicate cement coupons. The amount of AOA (*amoA* genomic copies) per area of cement coupon was on the same order of magnitude varying between 4×10^6 and 6×10^6 cells (A) as was the amount of AOB (*amoA* genomic copies) varying between 2×10^7 – 4×10^7 cells (B)

5.3.3 Stability of monochloramine in rechloraminated tap water

Monochloramine was formed in dechloraminated tap water, obtained by autoclaving filtered tap water for 15 min at 121 °C, and stored in 2 L amber bottles before being fed to the flow cell channels. The residence time of the water in the flow cell channels was approximately 1.1 s and, therefore, it was assumed that the monochloramine in each flow cell channel would not degrade significantly during the flow cell run. The 2.2 L of reservoir water was sufficient to sustain continuous flow conditions for a maximum

of 3 d flowing at a rate of 0.5 ml/min, to be replenished every 2 – 3 d over the experimental period of 14 d. The concentration of monochloramine in the water reservoir was monitored throughout, and concentrations decreased by 0.1 – 0.3 mg/L every 2 – 3 d. The high-concentration monochloramine reservoir had slightly higher fluctuations, owing to fluctuating residual monochloramine concentrations in the dechloraminated waters obtained periodically (Fig. 27).

As tap water was used and dechloramination was achieved through acceleration of chloramine decay through autoclaving the filtered tap water at 121 °C, a low concentration (0.1 – 0.6 mg/L) of monochloramine persisted in the control waters. Chemical decay of monochloramine was not chosen because the residual levels of the chemical compounds after dechloramination would interfere with the reintroduction of monochloramine into the waters and present a higher chloramine demand during the experiments.

Fig. 27 Concentration of monochloramine in all six reservoirs during the 14 d experimental period. High concentration: 2.8 – 3.3 mg/L monochloramine; low concentration: 0.9 – 1.1 mg/L monochloramine; control: 0.2 – 0.6 mg/L monochloramine

5.3.4 AOA and AOB share similar susceptibility to decay after continuous chloramination

After 14 d of constant exposure to monochloramine, the decay of the AOM biomass was neither significantly different between the low and high concentrations of monochloramine, nor was it significantly different between the control and treated samples (Fig. 28). Although the control samples in AOA skewed towards a much lower percentage of decay, a single outlier (Table S4) pushed the difference in decay, between the control and treated samples, to be not significant (Fig. 28A). Meanwhile, the control samples in AOB skewed towards a higher percentage of decay, which saw the difference in decay between the control and treated samples to be not significant (Fig. 28B), although a single outlier had a much lower decay in the control (Table. S2). Due to the single outliers in the control samples of AOA and AOB, the decay of non-treated AOA and AOB biomass from the nitrifying biofilms was not significant (Fig. 28C).

In the treated biofilm samples, the decay of AOA biomass from the biofilms was not significantly different between treatment with low and high concentrations of monochloramine (Fig. 28A), and the same was observed for the treated AOB biomasses (Fig. 28B). When the AOM are compared, the differences in decay of biomass from the nitrifying biofilms were not significant between AOA and AOB, after treatment with low or high concentrations of monochloramine (Fig. 28C).

Fig. 28 Comparing decay of AOA and AOB biomass between treated and non-treated groups, and among AOA and AOB within the treated and non-treated groups. The decay of AOA after 14 d of constant chloramination was not significantly different between low and high concentrations of monochloramine and there was no difference between control and treatment (A), like the decay of AOB from 14 d of constant chloramination that was neither significantly different between Low and High concentrations of monochloramine, nor between control and treated samples (B). The levels of decay were not significantly different between AOA and AOB (C). N = 3. “ns” Not Significant.

5.3.5 AOA and AOB both persist in nitrifying biofilms after continuous chloramination

After 14 d of continuous chloramination, the amount of persistent AOA biomass in biofilm was not significantly different between treatment with low and high concentrations of monochloramine, nor was it significantly different between the treated and non-treated biofilms (Fig. 29A). The same was observed for the AOB biomass in biofilms (Fig. 29B). A single outlier likely resulted in the difference in AOA biomass persistence to be not significant between the treated and non-treated biofilms (Table. S2). When the persistence of biomass after continuous chloramination was compared between the AOM, no significant differences were observed between AOA and AOB for treatment with either low or high concentrations of monochloramine, nor was there a significant difference between non-treated AOA and AOB (Fig. 29C).

Fig. 29 Comparing persistence of AOA and AOB biomass between treated and non-treated groups, and among AOA and AOB within the treated and non-treated groups. The persistence of AOA biomass in biofilms was not significantly different between treated and non-treated biofilms (A), the persistence of AOB biomass in biofilms was not significantly different between treated and non-treated biofilms (B), and the persistence of AOA and AOB biomass in biofilms was not significantly different (C). $N = 3$. “*” $p < 0.05$. “ns” Not Significant.

5.4 Discussion

The ability of AOA and AOB to resist monochloramine in biofilms was tested with ammonia-oxidizing biofilms cultivated from enriched AOA and AOB cultures, resulting in biofilms that were highly abundant in AOA or AOB biomass. Resistance to monochloramine was expected through continued growth of AOA or AOB in the biofilms, but decay was observed instead. Therefore, the difference in levels of decay was used to assess the difference in monochloramine resistance of the AOM.

Although there was extensive loss of biomass from the biofilms for both nitrifier groups, there was no significant difference between the control and treatment groups due to the high variability in the control group. Both high and low monochloramine treatment groups showed much lower variability. Importantly, both AOM were equally susceptible to chloramination, thereby rejecting the hypothesis that AOB are more tolerant than AOA to monochloramine in biofilms. It can be observed that the variability of AOM biomass decay and persistence in replicate biofilms was reduced for treatment with monochloramine as a function of concentration. This would suggest that monochloramine has a dose-dependent effect on the decay and survivability of AOM in nitrifying biofilms, where higher concentrations of monochloramine are more reliable in controlling AOM growth in biofilms.

An important note is that a single outlier in the control AOA biomass (Table. S4) in biofilm led to the conclusion that no significant difference was observed between the treated and non-treated biofilms, as the starting AOA biomass was one order of

magnitude higher. This is because the decay of non-treated AOA from biofilm skewed to a much lower 28 – 30 % (Table. S2 – 3), which may suggest that treated water from the water treatment plants cannot control AOA growth in biofilm without chloramination. This is unsurprising as AOA are adapted to growth at very limiting concentrations of ammonium.

Both AOA and AOB cells persisted at reduced levels after 14 d of chloramination and this persistence may be the result of monochloramine cometabolism, where the breakdown of monochloramine by ammonia monooxygenase (AMO) occurs in parallel with ammonia oxidation by AMO as demonstrated by (Maestre, J.P. et al. 2013), with the mechanism originally proposed by (Woolschlager, J.E., III 2000). According to the latter, ammonia oxidation by AMO provided the energy needed to maintain AOM biomass, while monochloramine cometabolism contributed to its decay thus reducing the extent of damage to the cells.

Monochloramine formed in this study followed the 4:1Cl₂:NH₃-N mass ratio, with approximately 0.16 mg/L of free ammonia available at 3 mg/L of monochloramine. According to Equation 1 in Chapter 2.1, one molecule of HOCl reacts with one molecule of NH₃ to produce one molecule of NH₂Cl (monochloramine) and one molecule of water. 3 mg/L of Cl₂ is equivalent to 42.31 µM of Cl₂, which should react with 42.31 µM of NH₃-N to produce 3 mg/L of monochloramine. At the 4:1 ratio, the molar concentration of NH₃-N would be 53.55 µM, which results in an excess NH₃-N concentration of 11.24 µM that is equivalent to 0.16 mg/L. Nitrifying activity in nitrifying biofilms under long-term exposure to monochloramine in a previous study was only

possible if a large excess of free ammonia was supplied (Pressman, J.G. et al. 2012), even when monochloramine fully penetrated the nitrifying biofilms within 24 h and beyond (Pressman, J.G. et al. 2012, Lee, W.H. et al. 2018). Decay of the nitrifier biomass was not demonstrated, and the nitrifiers present in the biofilms were uncharacterized. The previously demonstrated cases of monochloramine cometabolism involved excess ammonia concentrations and a $\text{Cl}_2\text{-N}$ mass ratio of 1 or below (Maestre, J.P. et al. 2013, Maestre, J.P. et al. 2016), similar to the observations in (Pressman, J.G. et al. 2012).

In both previous studies, when the $\text{Cl}_2\text{:NH}_3\text{-N}$ mass ratio was controlled at 4:1, the complete penetration of monochloramine into the nitrifying biofilms arrested nitrifying activity in all layers of the biofilms, where dissolved oxygen was able to completely penetrate the biofilm (Pressman, J.G. et al. 2012, Lee, W.H. et al. 2018). Because free chlorine is more reactive than monochloramine, monochloramine can penetrate approximately 2000 μm of biofilm within two days, whereas free chlorine could only penetrate approximately 1500 μm of biofilm in the same time period, failing to arrest oxygen consumption in all layers of the biofilms (Pressman, J.G. et al. 2012, Lee, W.H. et al. 2018).

Previous studies involving cometabolism of monochloramine were performed either on pure cultures of allochthonous AOB (Maestre, J.P. et al. 2013), or on mixed but undefined nitrifying biofilms (Maestre, J.P. et al. 2016). Although monochloramine cometabolism was not mentioned in the study involving chloramination of mixed but undefined nitrifying biofilms with excess ammonia, where both high ($>3\text{ mg/L}$) and low

(roughly 1 mg/L) concentrations of monochloramine were tested, the excess ammonia involved were very exaggerated, involving a $\text{Cl}_2\text{-N}$ mass ratio of 1 or below (Pressman, J.G. et al. 2012), monochloramine cometabolism could explain the continued nitrification observed in the presence of monochloramine.

This study considered the possibility of monochloramine cometabolism by AOM at a low concentration of available ammonia, instead of excess ammonia levels addressed in previous works. Both *Nitrosomonas oligotropha* and *Nitrososphaera viennensis* have evolved to grow in oligotrophic environments like drinking water with limiting substrate concentrations (Koops, H.P. et al. 1991, Koops, H.-P. and Pommerening-Röser, A. 2015, Kerou, M. and Schleper, C. 2016, Kerou, M., Alves, R.J.E. and Schleper, C. 2018), and AOM enriched from the DWDS should not require a large excess of ammonia for monochloramine cometabolism to occur. While AOA are better suited to growing with limiting ammonia concentrations than AOB, both AOM were shown here to persist in nitrifying biofilms under continuous chloramination at both concentrations of monochloramine. As was observed in the decay data, the variability of the persistent AOM biomass after 14 d continuous chloramination decreased with increasing concentrations of monochloramine. However, the variability of the persistent AOA biomass in the low concentration monochloramine group appeared to be higher than that of AOB. This could indicate better long-term persistence (on a time scale beyond the experimental period of 14 d) of AOA than of AOB in nitrifying biofilms under continuous assault by low concentrations of monochloramine.

A recent study concluded that AOA in biofilms were less susceptible to both free chlorine and monochloramine than AOB (Roy, D. et al. 2020), in contrast to experimental results in this study. There are two important differences in the study design that would likely explain the discrepancy. Firstly, in the published study, mixed AOM nitrifying biofilms were cultivated from the filter backwash of the ammonia oxidizing biological filters of a water treatment plant. The AOM from such sources were not acclimated to the presence of monochloramine, as disinfection was introduced after the filtration step. While the feed water was ozonated prior to the filtration step, the short half-life of ozone (~30 min) in water would mean that the AOM in the filter biofilms were unlikely to be under constant oxidative stress. As such, the observed behavior of AOA and AOB would not reflect conditions in operational DWDS. Secondly, the AOM in the earlier study were exposed to monochloramine for 96 h compared to 14 d in this experiment, as the intent of the authors was to examine the response of AOM to different levels of chlorine/monochloramine at different temperatures exclusively (Roy, D. et al. 2020).

Previous research on the effect of chloramine disinfection on nitrifying biofilms involved mixed nitrifier biofilms cultivated either from water in the DWDS or from water treatment plant filter backwashes (Aggarwal, S. et al. 2018, Roy, D. et al. 2020). Those nitrifying biofilms required a long cultivation period of 25 – 30 d (Roy, D. et al. 2020) and 28 months (Aggarwal, S. et al. 2018) and were dominated by heterotrophic bacteria. In contrast, this study used AOA and AOB nitrifying biofilms derived from highly enriched cultures of AOA and AOB allochthonous to chloraminated drinking

water environments. Additionally, the biofilms in this chapter were cultivated to contain either AOA or AOB, at greater relative abundance in the microbial community than the earlier studies.

A conceptual model has been proposed regarding the formation and activity of nitrifying biofilms in chloraminated DWDS based on unplanned surveillance of pipes in the same operational DWDS as yielded the enriched cultures in the present work (Cruz et al. 2020). Sections of the DWDS with higher residual concentrations of monochloramine would select for lower diversity nitrifying biofilms featuring AOB, while sections with reduced residual levels would select for higher diversity nitrifying biofilms featuring AOA and NOB (Cruz, M.C. et al. 2020). It was argued that in those low-diversity nitrifying biofilms, ammonia oxidation would be performed by monochloramine-resistant AOB, thus releasing nitrite into the bulk water. Due to the high concentration of monochloramine and the rapid reaction between monochloramine and nitrite (Vikesland, P.J. et al. 2001), insufficient nitrite would be available to support the growth of NOB, in addition to NOB being hypothesized to be less resistant to monochloramine than AOB. Subsequently, it was argued that the reduced monochloramine concentration in the high-diversity nitrifying biofilms with AOA and NOB allowed for their biological activity and that very low concentrations of ammonia enabled AOA to outgrow AOB, owing to their reduced substrate threshold and half-saturation constant (K_m) (Martens-Habbena, W. et al. 2009). NOB would then support the growth of AOA by removing nitrite from the local environment (Cruz et al. 2020).

Overall, I have demonstrated that both concentrations (~1 mg/L and ~3 mg/L) of residual monochloramine in the bulk water can similarly inactivate the bulk of the AOM in established nitrifying biofilms, with higher concentrations of monochloramine appearing more reliable (less variability in biomass decay). Lower concentrations (~1 mg/L) of residual monochloramine in the bulk water might favor the survivability of AOA over AOB in the biofilms, as seen with the greater variability of persistent AOA biomass. NOB were not addressed in this chapter, as the continual growth of NOB in the biofilm requires a supply of nitrite, which would readily react with monochloramine in the water. The model proposed in (Cruz et al. 2020) was based on observations made in a survey study, in contrast to the controlled experiments reported in this chapter.

In conclusion, the hypothesis that AOB in biofilms are more resistant to monochloramine than AOA is rejected. It is likely that in drinking water distribution pipes, AOA and AOB in biofilms are equally resistant to monochloramine, but higher concentrations of monochloramine are more reliable in controlling AOM populations in DWDS biofilms. The persistence of AOM at both concentrations of monochloramine suggests that monochloramine cometabolism is possible at very low concentrations of free ammonia in chloraminated waters. There is evidence that lower concentrations of monochloramine may favor the persistence of AOA biomass in DWDS biofilms.

The limitations of this chapter include the variability of nitrifying biofilm formed on the cement coupon, owing to the inherent inconsistency of those commercially made coupons, and the inability to control the amount of nascent AOM biomass when

forming the nitrifying biofilms. Further, the constant supply of chloraminated water at a given level may not reflect actual DWDS conditions by excluding stagnation periods and the design of the flow cells limited the number of coupons that could be withdrawn per experimental period. The 14-d period chosen to mimic long-term chloramination may not be sufficient to represent actual DWDS operations, but the duration was necessary to allow comparison with the planktonic study in this dissertation. Extending the period of this study may stabilize the biomass population in the nitrifying biofilms, potentially reducing the variability in the data produced.

I performed image analysis combined with fluorescence in-situ hybridization (FISH) to detect the AOA and AOB cell clusters in the biofilms. This was meant to be an independent method to confirm sequencing and ddPCR analyses. Unfortunately, the method did not yield reproducible results and hence they were excluded from this chapter. The FISH methodology for the labelling of planktonic nitrifier cells was adopted for the biofilm samples on the cement coupon surfaces, with the appropriate increase in volume of probe solutions to adequately cover the coupon surfaces. Furthermore, the staining time was increased to 24 h to increase the sensitivity of the procedure. This proved to be inadequate as the cement material was more porous than the microscopic glass slides and absorbed the bulk of the probe solution during the 24-h period, rendering the staining ineffective. Further improvement of the FISH procedure would include modifying the application of probe solution onto the biofilm samples, by immersing the biofilm face down in a bulk probe solution instead of applying a smaller volume of probe solution to the biofilm samples.

6 Study highlights and future directions

Three nitrifiers (AOA, AOB and NOB) were successfully enriched from biofilm samples obtained from DWDS receiving monochloramine as secondary disinfectant in Singapore. The AOB and NOB communities in the long-term enrichment cultures were closely related to *Nitrosomonas oligotropha* and *Nitrospira moscoviensis* respectively, as has been observed in other DWDS, identified through direct sequence analysis of the 16S rRNA and *amoA* genes for AOB, and of the 16S rRNA gene only for NOB (Regan, J.M. et al. 2002, Regan, J.M. et al. 2003, Martiny, A.C. et al. 2005). In contrast, the AOA communities in a long-term enrichment culture were closely related to *Nitrososphaera viennensis*, a soil-related AOA first isolated from a garden soil (Stieglmeier, M. et al. 2014, Kerou, M. and Schleper, C. 2016). In previous studies, AOA communities in DWDS were reported to be closely related to the Group 1.1a *Nitrosopumilus* cluster, a group of marine-associated AOA, based on 16S and *amoA* sequences obtained from bulk water alone (de Vet, W.W. et al. 2009, van der Wielen, P.W.J.J. et al. 2009, Niu, J. et al. 2016, Roy, D. et al. 2020).

The AOA communities in this study were cultured and identified from biofilms in the DWDS, whereas the previously described communities were identified from the bulk water. Hence the biofilm phase is relevant as was also reported in a survey of a river system that revealed an unequal distribution of AOA in the freshwater environment, with *Nitrososphaera* predominantly found in sediments whereas *Nitrosopumilus* and *Nitrosotalea* were predominantly found in the water column based on sequence

analysis alone without enrichment (Li, M. et al. 2018). In conclusion, this is the first report of AOA enrichment from DWDS biofilms.

Selective enrichment of AOA from nitrifying biofilms containing mixed communities of AOA, AOB and NOB was successfully demonstrated with the use of NN'-Dimethylthiourea (DMTU) and pyruvate, to selectively inhibit the growth of AOB (Ginestet, P. et al. 1998) while relieving the growth inhibition of AOA by hydrogen peroxide produced during ammonia oxidation (Shen, T. et al. 2013). NOB were easily washed out of the culture with the use of sodium azide (Ginestet, P. et al. 1998). Although early attempts to selectively enrich AOA by filtration through 0.45 µm pore size filters (French, E. et al. 2012, Li, Y.Y. et al. 2016, Chen, H.Y. et al. 2017) did not yield favorable results, the reintroduction of filtration into the enrichment in the present study further improved the level of enrichment of AOA.

In the planktonic nitrifier experiments, AOB were observed to be more resistant than AOA to decay after 24 h of exposure to a low concentration of monochloramine (1 mg/L), and NOB were more resistant than both AOA, which may be attributed to the presence of residual nitrite inside active NOB cells. The opposite was observed in regrowth experiments of chloraminated AOA, which recovered more than both AOB and NOB. The implication is that while AOB and NOB may better survive chloramination in DWDS, AOA may be more resilient towards inactivation by monochloramine, and therefore more apt at repopulating the nitrifying biofilms when the chloramine pressure recedes.

In the biofilm nitrifier experiments, low (~1 mg/L) and high (~3 mg/L) concentrations of monochloramine were equally capable of inactivating and reducing AOA and AOB populations in nitrifying biofilms, with increasing concentrations of monochloramine appearing to have a dose response effect. This was similarly observed for the persistent AOA and AOB populations in the biofilms, where increasing concentrations of monochloramine appeared to have greater effects on suppressing both AOM populations in biofilm. When continuously challenged with a low (~1 mg/L) concentration of monochloramine, the range of persistent AOA biomass in biofilms was larger than that of AOB, indicating that such concentrations of monochloramine may favor AOA over AOB in DWDS.

It is known that complete penetration of monochloramine in biofilms resulted in the loss of observable nitrifying activity, thorough the observed loss of oxygen consumption, and the lack of nitrite accumulation in all layers of biofilm penetrated by monochloramine (Pressman, J.G. et al. 2012). The presence of a large excess of ammonia was shown to partially negate the action of monochloramine in the biofilms and subsequently led to improved regrowth of the nitrifiers, which could be due to the phenomenon of monochloramine cometabolism by the AOM. This work suggests that cometabolism of lower concentrations (~1 mg/L) of monochloramine can occur at limiting concentrations of excess ammonia in DWDS, since the AOM present in the biofilms of chloraminated DWDS evolved to grow with limiting ammonia concentrations.

The phenomenon of monochloramine cometabolism by AOB had been described as the oxidation of monochloramine concurrently with the oxidation of ammonia by AMO (Maestre, J.P. et al. 2013), with ammonia oxidation supplying the cells with energy and monochloramine cometabolism depleting the intracellular concentration of monochloramine. That work was done in *Nitrosomonas europaea*, monochloramine cometabolism was observable with a $\text{Cl}_2\text{-N}$ mass ratio of 1 or below, while this work is suggesting that monochloramine cometabolism is viable for AOM in biofilms, with a $\text{Cl}_2\text{-N}$ mass ratio of 4 or above.

Monochloramine has been characterized as a weaker disinfectant because it is less reactive than free chlorine (Ingols, R.S. et al. 1953), but the ease of penetration of biofilms by monochloramine and its ability to arrest biological activity in those areas shows that monochloramine is still an effective disinfectant, especially for biofilms. The reduced reactivity of monochloramine is due to the selective reactivity of monochloramine to proteins, particularly the accessible thiol groups present in proteins (Ingols, R.S. et al. 1953). Free chlorine reacts more indiscriminately with organic molecules, which allows the disinfectant to physically weaken the biofilm structure, rendering biofilms susceptible to erosion in DWDS (Lee, W.H. et al. 2018). In contrast, monochloramine is more reactive in protein-rich biofilms, resulting only in swelling and loosening the biofilm (Lee, W.H. et al. 2018). This may be insufficient to remove microorganisms capable of recolonizing the pipe surface. However, the swelling of the biofilm may increase its susceptibility to sloughing during routine

flushing events in the DWDS, although this was not observed in the experiments of the previous study (Lee, W.H. et al. 2018).

In addition, because monochloramine is selectively reactive to proteins with accessible thiol groups, which are prominently heme-containing enzymes, its action may be bacteriostatic, compared to free chlorine, which is bactericidal and can irreversibly alter the structure and function of proteins in the cell (Ingols, R.S. et al. 1953). By inactivating the heme-containing enzymes, important metabolic pathways like the respiratory chain will be compromised, thereby arresting the metabolic activities while the cell remains physically intact.

If the nitrifiers inactivated by monochloramine remain physically intact but metabolically inactivated, the potential for regrowth of nitrifiers would be greater than if the nitrifiers were inactivated by free chlorine. It was reported that exogenous hydroxylamine failed to resume ammonia oxidation after the activity was arrested from chloramination, but the oxidation of the exogenous hydroxylamine to nitrite was unaffected (Wahman, D.G. and Speitel, G.E. 2015). Since heme proteins are an essential component of the electron transport chain, they will be susceptible to inactivation by monochloramine, especially when ammonia oxidation ceases and no longer provides a hydroxylamine screen against monochloramine. Without a functioning electron transport chain, the electrons liberated by hydroxylamine oxidation cannot be relayed to AMO to restart activity. While HAO is a heme-enzyme, the protein possess 24 heme cofactors, which may allow the enzyme to be extra resilient against inactivation by monochloramine (Cedervall, P. et al. 2013).

Very small microorganisms spend most of their maintenance energy on protein turnover, which suggests that AOA have evolved to produce proteins that are more resilient to damage and/or possess a more efficient protein turnover machinery, to cope with the immense energy cost (Kempes, C.P. et al. 2017). Therefore, the observed resiliency of AOA to injury by monochloramine could be explained by its additional gene copies of AMO chaperon proteins (Spang, A. et al. 2012, Bayer, B. et al. 2016, Lehtovirta-Morley, L.E. 2018, Qin, W. et al. 2018), and by having HAOs that are structurally less reactive to monochloramine, which may explain why the archaeal counterpart to AOB's HAO remains to be discovered.

In light of the findings of this study, the following scenarios for the development of AOB-AOA-NOB nitrifying biofilms in chloraminated DWDS are proposed to update the conceptual model by (Cruz, M.C. et al. 2020), as illustrated in Fig. 30:

1. High concentrations of monochloramine (3 mg/L) in DWDS proximal to the service reservoirs, with an ammonia-monochloramine ratio of 0.25 or below, are effective in inactivating the majority of AOM cells in the bulk water, but less so in the case of active NOB cells. The bulk of the AOM biomass in biofilms in the DWDS would also be inactivated, but a small number of cells will persist, supported by monochloramine cometabolism of limiting concentrations of excess ammonia. Since the regrowth of chloraminated NOB is very poor compared to AOM, persistence of NOB biomass in biofilms is not likely to be supported at high concentrations of monochloramine.

2. While planktonic AOA and AOB cells have a similar tolerance to high concentration of monochloramine in the bulk water, AOB have a higher growth rate than AOA. As a result, when the surviving AOM cells begin to attach to pipe surfaces, AOB will outgrow AOA at high concentrations (3 mg/L) of monochloramine, leading to nitrifying biofilms dominated by AOB.
3. Due to the persistence of AOB in the nitrifying biofilms, the decomposition of monochloramine will be accelerated by monochloramine cometabolism, and by the reaction between monochloramine and nitrite produced by ammonia oxidation. These will deplete both ammonia and monochloramine in the bulk water to be distributed downstream of the DWDS. Due to the rapid rate of reaction (Equation 8 in Chapter 1) between monochloramine and nitrite (Hao, O.J. et al. 1994, Vikesland, P.J. et al. 2001), the regrowth of NOB in the biofilm would not be supported.
4. Although AOA appear to survive less during chloramination, their greater regrowth potential enables them to colonize parts of the DWDS more distal from the service reservoir, where the decomposition of monochloramine would be more extensive. This scenario is favoured by higher water age and lower flow rates, which is expected at pipe locations further away from the service reservoirs and closer to the consumer.
5. Monochloramine and excess ammonia in the bulk water will be depleted by the nitrifying biofilms dominated by AOB in the DWDS proximal to the service reservoirs, relieving the nitrifying biofilms of chloramine stress in the distal parts of the DWDS. The better regrowth potential of AOA after exposure to lower

concentrations (1 mg/L) of monochloramine, together with a depleted concentration of excess ammonia, favours the persistence of AOA in the biofilm at lower concentrations of monochloramine, as these conditions are more advantageous to ammonia oxidation and monochloramine cometabolism by AOA.

6. Nitrification by AOA provides a niche environment suitable for the regrowth of NOB, as the nitrite produced by AOA shields NOB cells from monochloramine by rapidly reacting with monochloramine and provides them with an energy source.
7. Less monochloramine in the bulk water means that more nitrite is available for NOB to derive energy from.
8. Unlike free chlorine, monochloramine does not lead to the physical destruction of the biofilm EPS, but instead results in the loosening of the biofilm structure. Therefore, nitrifier biomass would be better retained in chloraminated biofilms than in chlorinated biofilms.

Future work can uncover sequence and functional variations among strains present among the nitrifier populations in the long-term enrichment cultures. Improvements to the study of free living nitrifier decay and regrowth in response to chloramination could be made by performing the experiments in actual drinking water instead of Freshwater Medium, for more accurate simulation of drinking water conditions. The hypothesis of chloramine resistance by AOM and NOB can be tested by preincubating the test cultures before chloramination, with trace concentrations of ammonium or nitrite, to

build up ammonia, hydroxylamine, and nitrite in their periplasmic spaces. The nitrifier populations before and after chloramination can be studied to explore the possibility of chloramination selecting for resistant strains. The study of sessile nitrifiers' decay and persistence in response to chloramination can be improved by extending the experimental period to allow the populations in biofilm to stabilize, to reduce the variations observed in this study. The regrowth of chloraminated nitrifiers in nitrifying biofilms can also be explored, to complement what was observed in the free living nitrifiers. The application of fluorescence in-situ hybridization directly to biofilms on cement coupon can be further improved, by modifying the approach of applying the fluorescent-labelled probes onto the biofilms.

The updated conceptual model of nitrifier succession calls for additional hypotheses about the impact of different levels of residual monochloramine on distinct nitrifier populations and their activities in DWDS or DWDS-like environments. Highly enriched and distinct autochthonous nitrifier populations obtained in this study may enhance the regrowth potential of heterotrophic communities in DWDS differently, regardless of monochloramine levels, and can be used for mechanistic studies on the co-metabolism of monochloramine.

A

B

Fig. 30 Conceptual model for the succession of AOB-dominated nitrifying biofilms to AOA-dominated nitrifying biofilms in DWDS. In the presence of higher concentrations (3 mg/L) of monochloramine (MCA), the biofilm community is populated dominantly by *Nitrosomonas* AOB (blue rods) with a few *Nitrososphaera* AOA (green spheres), while *Nitrospira* NOB (red spirals) are absent. Ammonia oxidation by AOB and AOA in the biofilm leads to the co-metabolism of MCA, and the nitrite produced by ammonia oxidation rapidly reacts with MCA. This accelerates the decomposition of MCA (**A**). In the presence of lower concentrations (< 1 mg/L) of MCA, the biofilm community is populated predominantly by *Nitrososphaera* AOA (green spheres) with a few *Nitrosomonas* AOB (blue rods) and *Nitrospira* NOB (red spirals). Ammonia oxidation by AOA and AOB in the biofilm leads to the cometabolism of MCA, and the nitrite produced by ammonia oxidation rapidly reacts with MCA, which provides a niche for NOB to grow. As the residual concentration of MCA is reduced, more nitrite is available for NOB to be oxidized for energy (**B**).

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APPENDIX

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Fig. A1 The cloned archaeal 16S rRNA gene sequences are most closely related to the 16s rRNA gene sequence from *Nitrososphaera viennensis* strain EN76 (closed circle). The cloned sequences are highlighted by closed triangles.

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura, R.H. 2001). The unrooted tree with the highest log likelihood (-8752.35) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.0804)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 34 nucleotide sequences. All positions with less than 90% site coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1397 positions in the final dataset. The number of bootstrap replications was 500. Evolutionary analyses were conducted in MEGA X (Kumar, S. et al. 2018, Stecher, G. et al. 2020).

Fig. A2 The cloned archaeal *amoA* nucleotide sequences are most closely related to the *amoA* nucleotide sequence from *Nitrososphaera viennensis* strain EN76 (closed circle). The cloned sequences are highlighted by closed triangles.

The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model (Thomas, R.H. 2001). The unrooted tree with the highest log likelihood (-4008.34) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.0704)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 37.69% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 30 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 90% site coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 600 positions in the final dataset. The number of bootstrap replications was 500. Evolutionary analyses were conducted in MEGA X (Kumar, S. et al. 2018, Stecher, G. et al. 2020).

Fig. A3 The 16S amplicon sequence variant (ASV) nucleotide sequences are closely related to the 16S sequences from the *Nitrosomonas oligotropha* group (closed circles) of AOB. The ASV sequences are highlighted by the filled diamonds.

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Thomas, R.H. 2001). The unrooted tree with the highest log likelihood (-883.41) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.7320)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 43.93% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 27 nucleotide sequences. All positions with less than 90% site coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 321 positions in the final dataset. The number of bootstrap replications was 500. Evolutionary analyses were conducted in MEGA X (Kumar, S. et al. 2018, Stecher, G. et al. 2020).

Fig. A4 The cloned bacterial *amoA* nucleotide sequences are diversely related to *amoA* nucleotide sequences from the AOB genera *Nitrosomonas* and *Nitrosococcus*, including the species *Nitrosomonas oligotropha* (closed circle). The cloned sequences are highlighted by the filled diamonds.

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Thomas, R.H. 2001). The unrooted tree with the highest log likelihood (-4809.00) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.0460)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 25.57% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 28 nucleotide sequences. All positions with less than 90% site coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 479 positions in the final dataset. The number of bootstrap replications was 500. Evolutionary analyses were conducted in MEGA X (Kumar, S. et al. 2018, Stecher, G. et al. 2020).

Fig. A5 16S amplicon sequence variant (ASV) nucleotide sequences are closely related to the 16S sequences from *Nitrospira moscoviensis* group (closed circles). The ASV sequences are highlighted by the filled squares.

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Thomas, R.H. 2001). The unrooted tree with the highest log likelihood (-1919.43) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4127)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 56 nucleotide sequences. All positions with less than 90% site coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 317 positions in the final dataset. The number of bootstrap replications was 500. Evolutionary analyses were conducted in MEGA X (Kumar, S. et al. 2018, Stecher, G. et al. 2020).

Fig. A6 The cloned *nxB* nucleotide sequences are closely related to *nxB* nucleotide sequences from the NOB genera *Nitrospira*, with the majority most related to *nxB* nucleotide sequences from *Nitrospira moscoviensis* (closed circles). The cloned sequences are highlighted by the filled squares.

The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model (Thomas, R.H. 2001). The unrooted tree with the highest log likelihood (-2987.74) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2785)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 31 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 90% site coverage were eliminated, i.e., fewer than 10% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 485 positions in the final dataset. The number of bootstrap replications was 500. Evolutionary analyses were conducted in MEGA X (Kumar, S. et al. 2018, Stecher, G. et al. 2020).

Fig. A7 Relative abundance of the 16S amplicon sequence reads from the AOA enrichment culture before the introduction of DMTU and pyruvate.

The amplicon sequence variants (ASV) are plotted against the relative abundance in a bar plot, grouped together and faceted by genus. The color coding represent the phylum each genus belongs to. AOA are represented by *Candidatus_Nitrososphaera* at 0.19% relative abundance while AOB are represented by *Nitrosomonas* at 7.5% relative abundance. The NA genus refer to sequences not identifiable to the genus level. The bulk of the bacterial contaminants are from the Proteobacteria phylum, with the *Hyphomicrobium* genus prominently at 33.1% relative abundance.

Fig. A8 Relative abundance of the 16S amplicon sequence reads from the AOA enrichment culture after the introduction of DMTU and pyruvate.

The amplicon sequence variants (ASV) are plotted against the relative abundance in a bar plot, grouped together and faceted by genus. The color coding represent the phylum each genus belongs to. AOA are represented by *Candidatus_Nitrososphaera* at 39.5% relative abundance. The second and third most abundant phyla are Bacteroidetes at 32.7% and Proteobacteria at 24.4%.

Fig. A9 Relative abundance of the 16S amplicon sequence reads from the AOA enrichment culture filtration through 0.45 μm pore size membrane.

The amplicon sequence variants (ASV) are plotted against the relative abundance in a bar plot, grouped together and faceted by genus. The color coding represent the phylum each genus belongs to. AOA are represented by *Candidatus_Nitrososphaera* at 51.6% relative abundance. The second and third most abundant phyla are Proteobacteria at 36.7% and Bacteroidetes at 5.1%.

Fig. A10 Relative abundance of the 16S amplicon sequence reads from the AOB enrichment culture in the same period of the first improvement in the AOA enrichment culture.

The amplicon sequence variants (ASV) are plotted against the relative abundance in a bar plot, grouped together and faceted by genus. The color coding represent the phylum each genus belongs to. AOB are represented by *Nitrosomonas* at 71.2% relative abundance. The second and third most abundant genera are from the Proteobacteria phylum, with the *Pseudomonas* at 9.4% and *Phenyllobacterium* at 3.9%.

Fig. A11 Relative abundance of the 16S amplicon sequence reads from the NOB enrichment culture in the same period of the first improvement in the AOA enrichment culture.

The amplicon sequence variants (ASV) are plotted against the relative abundance in a bar plot, grouped together and faceted by genus. The color coding represent the phylum each genus belongs to. NOB are represented by *Nitrospira* at 73.5% relative abundance. The second and third most abundant phylum are Proteobacteria at 16.7% and Bacteroidetes at 7.8%.

Fig. A12 The designed NSS_amoAF/R primers produced more efficient qPCR reaction of >97 % compared to that of the published Arch_amoAF/R primers at >81 %. A standard curve was generated with a dilution series of DNA extraction from AOA enrichment culture and the genomic copies of archaeal *amoA* were obtained from ddPCR reactions using the NSS_amoA primer pairs. The standard curve output for the NSS_amoA primer pair quantified the plasmids to 4451 copies (A), while the output for the Arch_amoA primer pair (Francis, C.A. et al. 2005) quantified the plasmids to 636 copies (B). The unknown points in blue belong to the diluted archaeal *amoA* plasmids used as a positive control.

Fig. A13 The designed NSM_amoAF/R primers produced more efficient qPCR reaction of >91 % compared to that of the published amoA332F/822R primers at >84 %. A standard curve was generated with a dilution series of DNA extraction from AOB enrichment culture and the genomic copies of bacterial *amoA* were obtained from ddPCR reactions using the NSM_amoA primer pairs. The standard curve output for the NSM_amoA primer pair quantified the plasmids to 2566 copies (A), while the output for the amoA332F/822R primer pair (Rotthauwe, J.H. et al. 1997) failed to quantify the plasmids (B). The unknown points in blue belong to the diluted archaeal *amoA* plasmids used as a positive control.

Fig. A14 The designed NSP_nxrBF/R primers produced more efficient qPCR reaction of >97 % compared to that of the published nxrB169f/638r primers at >76 %. A standard curve was generated with a dilution series of DNA extraction from NOB enrichment culture and the genomic copies of *nxrB* were obtained from ddPCR reactions using the NSP_nxrB primer pairs. The standard curve output for the NSP_nxrB primer pair quantified the plasmids to 4523 copies (A), while the output for the nxrB169f/638r primer pair (Pester, M. et al. 2014) (B) quantified the plasmids to 1901 copies (B). The unknown points in blue belong to the diluted archaeal *amoA* plasmids used as a positive control.

Primer Map results

Results for linear 200 residue sequence "Singapore_DWDS_AOA_amoA_Clone_2" starting "CTAATGGTCT"

```

1 CTAATGGTCTGGCTTAGACGTACAACGCACTACCTATTCTAGTAGTGGTGTCAAC
1 10 20 30 40 50
1 GATTACGACGCGAATCTGCATGTTGGTGATGGATAAGTATCATCAGCAACGACAGTTG
    >>>NSS_amoAF1>>> 65 to 84
61 AGCAAGCTGCTAACCATCAACGAGGAGACTACATCTTCTACAGACTGGGGTGGACG
61 70 80 90 100 110
61 TCGTGGGACGATTGGTAGTTGGTCTCTCTGATGTAGAAGATGTGTCTGACCCGACCTGTC
    <<<NSS_amoAR1<<< 154 to 170
121 TCCTTTGTAGTATTCTCAATCTCGCAGTCGACTATGCTGCTGGTGGCAATATACTAC
121 130 140 150 160 170
121 AGGAAACATCATAGAGTTAGAGCGTCAGCTGATACGAGCGACACCCAGCTTATATGATG
181 ATGTTTTCACGGGTGTTCC
181 190
181 TACAACAAGTGCCCAAGG

```

Primer:	Sequence:
NSS_amoAF1	5'-CGCTGCTAACCATCAACGCA-3'
NSS_amoAF2	5'-ACACTGCTAACCATCAACGCGAG-3'
NSS_amoAR1	5'-GCACCCACAGCGAGCAT-3'
NSS_amoAR2	5'-GCGCCCACTACGAGCATTG-3'

Results for linear 210 residue sequence "F8773159.1:1-632 Nitrososphaera viennensis EN76 partial amoA gene for ammonia-monooxygenase subunit A, type strain EN76T" starting "ATGGTCTGGC"

```

1 ATGGTCTGGCTTAGACGTACAACGCACTACCTATTCTAGTAGTGGTGTCAACAGC
1 10 20 30 40 50
1 TACGACGACGATCTGCATGTTGGTGATGGATAAGTATCATCAGCAACGACAGTTGTG
    >>>NSS_amoAF1>>> 62 to 81
61 ACGCTGCTAACCATCAACGAGGAGACTACATCTTCTACAGACTGGATGGACATCC
61 70 80 90 100 110
61 TCGGACGATTGGTAGTTGGTCTCTCTGATGTAGAAGATGTGTCTGACCTACACCTGAGG
    <<<NSS_amoAR1<<< 151 to 167
121 TTGCTAGTATTCTCCGTCACAGTCGACGATGCTGCTGGGTGGGTATACTACATG
121 130 140 150 160 170
121 AGCATCATTAAGAGGCACAGTGTACGCTGTACGAGCGACACCCAGCCATATGATGAC
181 TTGTTACAGCGCTTCGGGTACAGCCACA
181 190 200
181 AACCAAGTGTCCGCAAGGCCATGTGCGGTG

```

Primer:	Sequence:
NSS_amoAF1	5'-CGCTGCTAACCATCAACGCA-3'
NSS_amoAF2	5'-ACACTGCTAACCATCAACGCGAG-3'
NSS_amoAR1	5'-GCACCCACAGCGAGCAT-3'
NSS_amoAR2	5'-GCGCCCACTACGAGCATTG-3'

Results for linear 210 residue sequence "CP007174.1:c821032-820400 Candidatus Nitrososphaera evergladensis SR1, complete genome" starting "AATGGTCTGG"

```

1 AATGGTCTGGCTTAGACGTACAACGCACTACCTATTCTAGTAGTGGTGTCAACAG
1 10 20 30 40 50
1 TTACGACGACGATCTGCATGTTGGTGATGGATAAGTATCATCAGCAACGACAGTTGTC
    >>>NSS_amoAF1>>> 63 to 82
61 CACGCTGCTAACCATCAACGAGGAGACTACATCTTCTACAGACTGGATGGACATC
61 70 80 90 100 110
61 GTGGACGATTGGTAGTTGGTCTCTGATGTAGAAGATGTGTCTGACCTACACCTGTAG
    <<<NSS_amoAR1<<< 152 to 168
121 CTTCGTAGTATTCTCCGTGTACAGTCGACGATGCTCGCTGGGTGGGTATACTACAT
121 130 140 150 160 170
121 GAAGCATCATTAAGAGGCACAGTGTACGCTGTACGAGCGACACCCAGCCATATGATGTA
181 GTTGTTCACAGGCGTTCCGGGACAGGCCAC
181 190 200
181 CAACAAGTGTCCGCAAGGCCGCTGCGGTG

```

Primer:	Sequence:
NSS_amoAF1	5'-CGCTGCTAACCATCAACGCA-3'
NSS_amoAF2	5'-ACACTGCTAACCATCAACGCGAG-3'
NSS_amoAR1	5'-GCACCCACAGCGAGCAT-3'
NSS_amoAR2	5'-GCGCCCACTACGAGCATTG-3'

Results for linear 210 residue sequence "CP002408.1:c1974905-1974272 Candidatus Nitrososphaera gargensis Ga9.2, complete genome" starting "TAATGGTCTG"

```

1 TAATGGTCTGGCTTAGACGTACAACGCACTACTTCTCATAGTAGTGGTGTCAACA
1 10 20 30 40 50
1 ATTACGACGACGATCTGCATGTTGGTGATGAACAAGTATCATCACAACGACAGTTGT
    >>>NSS_amoAF2>>> 63 to 84
61 GCACACTGCTAACCATCAACGAGGAGTACATCTTCTACAGGACTGGATGGACCT
61 70 80 90 100 110
61 TCGTGTGACGATTGGTAGTTGGTCTCACTGATGTAGAAGATGTGCTGACCTACACCTGGA
    <<<NSS_amoAR2<<< 151 to 169
121 CCTTGTAGTATTCTCGATCTCCGCTGTACAGTCTGCTGATGGGGTGGATATACTACA
121 130 140 150 160 170
121 GGAAGCATCATTAAGAGCTAGAGGGTCAAGTGTACGAGCATCACCCGCGCTATATGATGT
181 TGCTCTTCACAGGAGTACAGGAGGAGGCCA
181 190 200
181 ACGAGAAGTGTCTCATGTCTCTTGCCGT

```

Primer:	Sequence:
NSS_amoAF1	5'-CGCTGCTAACCATCAACGCA-3'
NSS_amoAF2	5'-ACACTGCTAACCATCAACGCGAG-3'
NSS_amoAR1	5'-GCACCCACAGCGAGCAT-3'
NSS_amoAR2	5'-GCGCCCACTACGAGCATTG-3'

Fig. A15 Mapping of the designed NSS_amoAF/R primers to the *amoA* nucleotide sequence of the targeted AOA. The mapping was performed using the Primer Map tool of the Sequence Manipulation Suite in bioinformatics.org (Stothard, P. 2000).

Primer Map results

Results for linear 173 residue sequence "Singapore_DWDS_NOB_nxrB_Clone_1" starting "TACATGTGGT"

```
>>>NSP_nxrBF>>> 9 to 30
1 TACATGTGGTGGGAACACCTGGAGACCAAGCCCTACGGCGGGTATCCCCAGTTCTACGAC
1 10 20 30 40 50
1 ATGTACACCACCTTGTTCACCTCTGGTTCGGGATGCCGCCATAGGGGTCAAGATGCTG
61 GTGAAGATCAACCACTTGATCGAGCAGGTGAACCCGGCGGGCAGGTGTGGAACGTGGG
61 70 80 90 100 110
61 CACTTCTAGTGGTCAACTAGCTCTGCCACTTGGCCCCCGCTCCACACCTTGCACGCC
<<<NSP_nxrBF<<< 145 to 165
121 GTGGCGCCGAAGCACCATGCGCCCTACGGGGTGTTCGAAGGATGACCATTTT
121 130 140 150 160 170
121 CAGCGCGCGTTCTGGTACGCGGGATGCCCCACAAGCTTCCTACTGGTAAAA
```

Primer:	Sequence:
NSP_nxrBF	5'-GTGGAACAACGTGGAGACCAAG-3'
NSP_nxrBF	5'-CATCCCTTCGAACACCCCGTA-3'

Results for linear 208 residue sequence "KC884858.1 Nitrospira moscoviensis strain M-1 clone 20 nitrite oxidoreductase beta subunit (nxrB) gene, partial cds" starting "GCAGGAGTAC"

```
>>>NSP_nxrBF>>> 16 to 37
1 GCAGGAGTACATGTGGTGAACAACCTGGAGACCAAGCCCTACGGCGGGTATCCCCAGTT
1 10 20 30 40 50
1 CGTCTCATGTACACCACCTTGTTCACCTCTGGTTCGGGATGCCGCCATAGGGGTCAA
61 CTACGACGTGAAGATCACGCACTGATCGAGCAGGTGAACCCGGCGGGCAGGTGTGGAA
61 70 80 90 100 110
61 GATGCTGCACCTTCTAGTGCCTGACTAGCTCTTCACTTGGGCCCGCGTCCACACCTT
<<<NSP_nxrBF<<< 152 to 172
121 CGTGGCGGTGGCGCGCAAGCACCATGCGCCCTACGGGGTGTTCGAAGGATGACCATTTT
121 130 140 150 160 170
121 GCAGCCCAACCCGGCTTCTGGTACGCGGGATGCCCCACAAGCTTCCTACTGGTAAAA
181 CGACGCGGGGCAAGTGGGCGAGGCG
181 190 200
181 GCTGGCGCCCGGTTCACCCGCTCCGC
```

Primer:	Sequence:
NSP_nxrBF	5'-GTGGAACAACGTGGAGACCAAG-3'
NSP_nxrBF	5'-CATCCCTTCGAACACCCCGTA-3'

Results for linear 209 residue sequence "KC884905.1 Candidatus Nitrospira bockiana clone 17 nitrite oxidoreductase beta subunit (nxrB) gene, partial cds" starting "GCAGGAGTAC"

```
>>>NSP_nxrBF>>> 16 to 37
1 GCAGGAGTACATGTGGTGAACAACCTGGAGACCAAGCCCTACGGCGGGTATCCCCAGTT
1 10 20 30 40 50
1 CGTCTCATGTACACCACCTTGTTCACCTCTGGTTCGGGATGCCGCCATAGGGGTCAA
61 CTACGACGTGAAGATCACGCACTGATCGAGCAGGTGAACCCGGCGGGCAGGTGTGGAA
61 70 80 90 100 110
61 GATGCTGCACCTTCTAGTGCCTGACTAGCTCTGCCACTTGGGCCCGCGTCCACACCTT
<<<NSP_nxrBF<<< 152 to 172
121 CGTGGCGGTGGCGCGCAAGCACCATGCGCCCTACGGGGTGTTCGAAGGATGACGATCTT
121 130 140 150 160 170
121 GCAGCCCAACCCGGCTTCTGGTACGCGGGATGCCCCACAAGCTTCCTACTGCTAGAA
181 CGACGCGGGGCAAGATCGGCGAGGCGG
181 190 200
181 GCTGGCGCCCGGTTCAGCCGCTCCGC
```

Primer:	Sequence:
NSP_nxrBF	5'-GTGGAACAACGTGGAGACCAAG-3'
NSP_nxrBF	5'-CATCCCTTCGAACACCCCGTA-3'

Results for linear 210 residue sequence "KC884905.1 Nitrospira calida strain Ns10 clone 1 nitrite oxidoreductase beta subunit (nxrB) gene, partial cds" starting "GAGTACATGT"

```
>>>NSP_nxrBF>>> 12 to 33
1 GAGTACATGTGGTGAACAACCTGGAGACCAAGCCCTACGGCGGGTATCCCCAGTTCTAC
1 10 20 30 40 50
1 CTCATGTACACCACTTGTTCACCTCTGGTTCGGGATGCCGCCATAGGGGTCAAGATG
61 GACGTGAAGATCACGCACTGATCGAGCAGGTGAACCCGGCGGGCAGGTGTGGAACGTA
61 70 80 90 100 110
61 CTGCACTTCTAGTGGTGTGACTAGCTCTGCCAGTTGGGCCCGCGTCCACACCTTGCAT
<<<NSP_nxrBF<<< 148 to 168
121 CGGGTGGGCGCGCAAGCACCATGCGCCCTACGGGGTGTTCGAAGGATGACGATCTTCGAC
121 130 140 150 160 170
121 GCCCACCGCGGTTCTGTGTACGCGGGATGCCCCACAAGCTTCCTACTGTAGAAGCTG
181 GCCGGGCGAAGTGGGCGAGGCGGCATC
181 190 200
181 CGGCCCCGGTTCCACCGGTCCCGCGGTAG
```

Primer:	Sequence:
NSP_nxrBF	5'-GTGGAACAACGTGGAGACCAAG-3'
NSP_nxrBF	5'-CATCCCTTCGAACACCCCGTA-3'

Fig. A17 Mapping of the designed NSP_nxrBF/R primers to the *nxrB* nucleotide sequence of the targeted NOB. The mapping was performed using the Primer Map tool of the Sequence Manipulation Suite in bioinformatics.org (Stothard, P. 2000).

Fig. A18 Fluorescent in-situ hybridization of the AOA enrichment observed with confocal laser scanning microscopy under 100x objective with oil immersion. The scale bar is at 1 μm . AOB cells were not detected, and total cells were stained with DAPI. The AOA cells have a diameter of approximately $< 0.5 \mu\text{m}$.

Fig. A19 Fluorescent in-situ hybridization of the AOB enrichment observed with confocal laser scanning microscopy under 100x objective with oil immersion. The scale bar is at 1 μm . AOA cells were not detected although faint Archaea 16S signals were generated, and total cells were stained with DAPI. The AOB cells have a length of approximately 0.5 – 1 μm .

Table S1 Percent decay and regrowth of planktonic nitrifiers

	Percent Decay	Percent Regrowth
AOA-H	81.2% ± 1.8%	285.0% ± 193.4%
AOA-L	83.7% ± 2.5%	474.7% ± 187.9%
AOA-C	28.9% ± 3.2%	401.7% ± 139.7%
AOB-H	70.6% ± 7.1%	115.9% ± 98.4%
AOB-L	58.6% ± 13.5%	71.1% ± 67.3%
AOB-C	*36.6% ± 8.4%	77002.5% ± 9081.7%
NOB-H	26.0% ± 14.2%	#39.6% ± 12.8%
NOB-L	*7.1% ± 23.0%	#22.2% ± 18.5%
NOB-C	*33.8% ± 15.5%	368.4% ± 90.6%

H: High concentration monochloramine; L: Low concentration monochloramine; C: Control

*Growth observed instead of decay

#Decay observed instead of regrowth

Table S2 Decay of AOA and AOB in the presence of monochloramine over the first 14 d experiment

Nitrifier enrichment	Treatment (mg monochloramine^α/L)	Day 0 <i>amoA</i> (gc^β/cm²)	Day 14 <i>amoA</i> (gc/cm²)	Biomass loss (%)
AOA	High (2.8 – 3.3)	2,072,202	155,119	92.51%
AOA	Low (0.9 – 1.1)	2,983,970	148,014	95.04%
AOA	Control (0.2 – 0.6)	496,934	346,156	30.34%
AOB	High (2.8 – 3.3)	9,749,215	754,676	92.26%
AOB	Low (0.9 – 1.1)	6,188,975	1,205,824	80.52%
AOB	Control (0.2 – 0.6)	6,177,134	4,870,660	21.15%

^α Monochloramine

^β Gene copies

Table S3 Decay of AOA and AOB in the presence of monochloramine over the second 14 d experiment

Nitrifier enrichment	Treatment (mg monochloramine ^α /L)	Day 0 <i>amoA</i> (gc ^β /cm ²)	Day 14 <i>amoA</i> (gc/cm ²)	Biomass loss (%)
AOA	High (2.8 – 3.1)	6,808,662	97,097	98.57%
AOA	Low (0.9 – 1.1)	1,732,755	509,564	70.59%
AOA	Control (0.1 – 0.4)	4,815,402	3,449,722	28.36%
AOB	High (2.8 – 3.1)	7,207,314	290,108	95.97%
AOB	Low (0.9 – 1.1)	4,065,462	259,321	93.62%
AOB	Control (0.1 – 0.4)	8,604,570	1,626,185	81.10%

^α Monochloramine

^β Gene copies

Table S4 Decay of AOA and AOB in the presence of monochloramine over the third 14 d experiment

Nitrifier enrichment	Treatment (mg monochloramine ^α /L)	Day 0 <i>amoA</i> (gc ^β /cm ²)	Day 14 <i>amoA</i> (gc/cm ²)	Biomass loss (%)
AOA	High (2.8 – 3.1)	36,391,806	505,038	98.61%
AOA	Low (0.9 – 1.1)	32,207,932	2,612,947	91.89%
AOA	Control (0.1 – 0.4)	30,194,936	4,183,874	86.14%
AOB	High (2.8 – 3.1)	21,235,132	131,042	99.38%
AOB	Low (0.9 – 1.1)	7,657,278	419,572	94.52%
AOB	Control (0.1 – 0.4)	7,815,160	1,172,274	85.00%

^α Monochloramine

^β Gene copies