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ABSTRACT

AMMONIA AS THE DRIVING FACTOR FOR AEROBIC AMMONIA OXIDIZERS

by

Sabita Ghimire

Ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA) and complete ammonia oxidizers (comammox) comprise aerobic ammonia oxidizers (AO). Here, we present the ammonium concentration as a determinant of the distribution of ammonia-oxidizers.

The ecophysiology of comammox enrichment Cmx-BO4 was characterized by growing it in different ammonium concentrations, pH, light and nitrite concentrations. Cmx-BO4 oxidized up to 3 mM ammonium, with the highest growth rate at 10 μM ammonium. The comammox enrichment oxidized ammonium through pH 6 to 8 and was inhibited by blue and white light. Cmx-BO4 oxidized nitrite in presence of ammonium but did not oxidize nitrite only. The genomic characterization of comammox strain *Nitrospira* sp. BO4 in the comammox enrichment Cmx-BO4 was performed through metagenomic sequencing. The metagenome assembled genome (MAG) of *Nitrospira* sp. BO4 possessed genes for ammonia and nitrite oxidation. The MAG, however, lacked genes for assimilatory nitrite reduction and nitrite transporters, corresponding to the inability of the culture to consume nitrite as the nitrogen source.

Comammox *Nitrospira* sp. BO4 was competed for ammonium against AOA *Nitrosoarchaeum* sp. BO1 to investigate if the two oligotrophic AO had niche differentiation or if they co-existed in nature. Cmx-BO4 and AOA-BO1 have *Nitrosopira* sp. BO4 and *Nitrosoarchaeum* sp. BO1 as the AO respectively. *Nitrospira* sp. BO4 outcompeted *Nitrospira* sp. BO1 at all three tested ammonium concentrations (<10-, 50- and 500 μM). Comammox may have an advantage over AOA at ammonium limited environments and the AOA may occupy microniches devoid of comammox to be able to coexist in oligotrophic environments.

The effect of elevated ammonium availability was investigated to characterize the toxic effects of high ammonium concentration on *Nitrosomonas* sp. Is79 and *N. eutropha*. We cultivated both cultures at low, intermediate and high ammonium concentrations to investigate the effect of ammonium concentrations on gene expression.

Overall, the activity and growth of AO depends on the ammonium concentration. The concentration of ammonium is a factor determining the distribution of AO in nature.

AMMONIA AS THE DRIVING FACTOR FOR AEROBIC AMMONIA OXIDIZERS

A DISSERTATION

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DEDICATION

I am dedicating this dissertation to all the strong women who dare to speak their opinions, make their own decisions, and live their lives on their own terms.

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INTRODUCTION

I. The Nitrogen cycle

The nitrogen cycle is the biogeochemical cycle where nitrogen flows through atmospheric, terrestrial, oceanic and biotic components in nature (1). Nitrogen cycle is comprised of multiple steps and interactions among various organisms in different environments (Figure 1). The nitrogen cycle can be divided into the following key steps:

a. Nitrogen fixation and anthropogenic nitrogen deposition

Approximately 78% of atmosphere is nitrogen gas which is biologically unavailable to most organisms because of its very stable nature (2). Nitrogen fixation is the process of converting atmospheric nitrogen to biologically available reactive forms of nitrogen such as ammonia (3, 4). Nitrogen fixation by bacteria in root nodules of leguminous plants is the biological method of nitrogen fixation, while lightning process is the physical method of nitrogen fixation. Human activities, such as the addition of nitrogenous fertilizers for agricultural practices can cause excess nitrogen deposition in the form of nitrogen oxides and ammonium (5). Nitrogen oxides and ammonium are either assimilated by living organisms or used for energy generation through respiration.

b. Nitrification

The stepwise oxidation of ammonia to nitrite and then to nitrate is commonly termed as nitrification (6). In aerobic environments, nitrification is caused by ammonia-oxidizing bacteria (AOB) (7), ammonia-oxidizing archaea (AOA) (8), complete ammonia oxidizer (comammox) (9, 10) and nitrite-oxidizing bacteria (NOB) (7, 11). In the nitrogen cycle, the nitrification process connects the most reduced component, ammonium with the most oxidized component, nitrate (6). Nitrification is important for converting ammonium to nitrate, the preferred N substrate for assimilation by some plants and algae (12), decreasing pH as ammonium oxidation yields protons, production of greenhouse gas (N_2O) (13), producing substrate for denitrification (14) and consumption of oxygen thereby creating oxygen-limited condition (15).

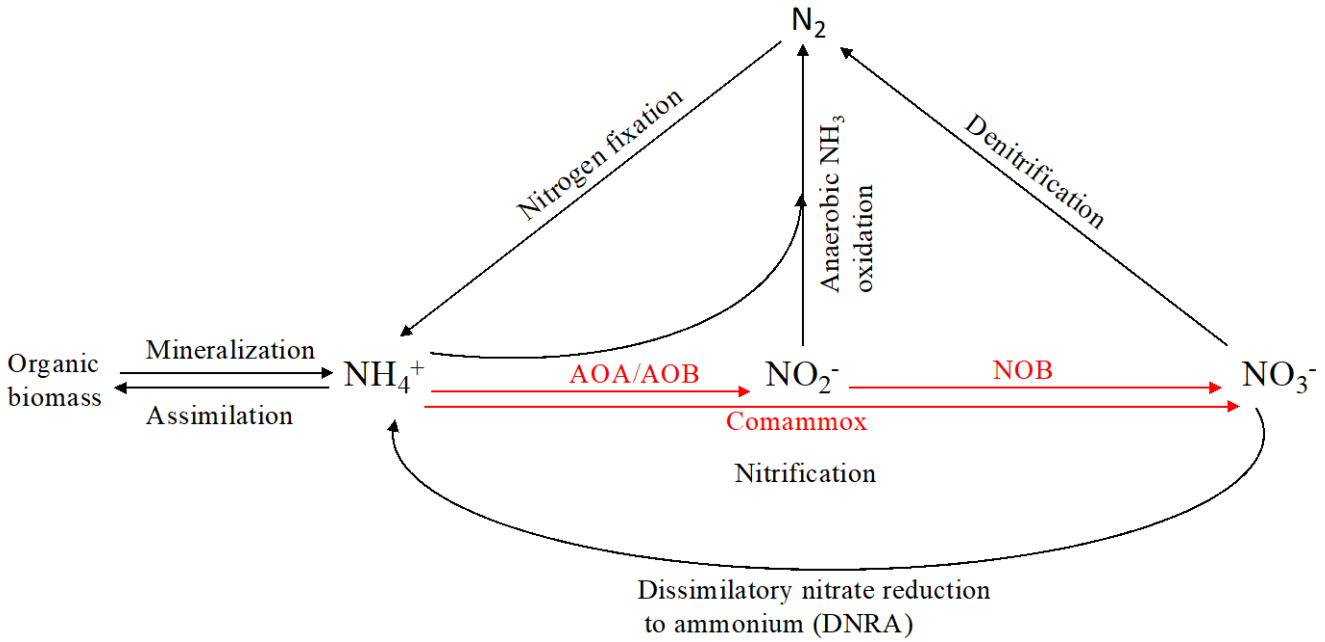

Figure 1. Simplified model of Nitrogen cycle highlighting the nitrification process in red.

a. Assimilation

Photosynthetic organisms like plants, algae, cyanobacteria, as well as some heterotrophic microbes assimilate ammonium, nitrite, nitrate or urea into specific tissue compounds in the form of organic molecules such as amino acids and nucleotides (16, 17). Heterotrophic organisms including animals, fungi and bacteria that cannot assimilate inorganic nitrogen acquire assimilated organic forms of nitrogen such as amino acids and nucleotides (18–20).

b. Denitrification and dissimilatory nitrate reduction

Denitrification is the process of the conversion of nitrate to nitrogen gas depleting the bioavailable form while returning it to the atmosphere (14). Nitrate (NO_3^-) is reduced to nitrogen gas (N_2) through a series of intermediate gaseous nitrogen oxides namely nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) by denitrifying heterotrophic bacteria such as *Pseudomonas* sp., *Comamonas* (21) and *Paracoccus denitrificans* (22). Denitrification is significant for i) loss of fertilizer from agriculture system and ii) production of greenhouse gas, N_2O (23). In addition, dissimilatory nitrate reduction to ammonium (DNRA) is the anaerobic respiration using nitrate as the terminal electron acceptor producing ammonium (24). DNRA process helps recycle reactive forms of nitrogen preventing their loss from the ecosystem.

c. Anaerobic ammonia oxidation

Anaerobic ammonia oxidation is the conversion of ammonium to nitrogen gas in anaerobic environments using nitrate as the electron acceptor (25, 26). Anaerobic ammonia oxidizers (anammox) include a broad range of microbes in terms of biochemistry and taxonomy: from heterotrophs to autotrophs growing on hydrogen and carbon dioxide to photosynthetic microbes (27). Some examples of anaerobic ammonia oxidizers include *Candidatus* Kuenenia stuttgartiensis (28), *Candidatus* Brocadia anammoxidans (29), *Candidatus* Jettenia asiatica (30).

d. Mineralization

Nitrogen mineralization is the conversion of complex organic nitrogen compounds such as proteins, nucleic acids and amino acids to the inorganic form, ammonium (31). Nitrogen mineralization is caused by microbial and animal activity and extracellular enzymes such as protease, deaminase and urease (32). Mineralized nitrogen becomes available for assimilation by organisms capable of assimilating.

I. Aerobic ammonia oxidizers (AO)

Aerobic ammonia oxidizers (AO) comprise of ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB) and complete ammonia oxidizer (comammox). Together these microbes play a role in the nitrification step of nitrogen cycle (7–10, 33, 34) (Fig. 1). The three groups of AO are phylogenetically and physiologically unique.

Ammonia-oxidizing bacteria (AOB) are chemolithotrophs that generate energy by oxidizing ammonium to nitrite for growth and metabolic activity (34). Based on 16S rRNA gene sequence similarity, AOB are divided into two monophyletic lineages: a) betaproteobacteria (beta-AOB) comprising *Nitrosomonas* (including *Nitrosococcus mobilis*) and *Nitrosospira* (including *Nitrosolobus* and *Nitrosovibrio*) and b) gammaproteobacteria (gamma-AOB) comprising *Nitrosococcus oceani* and *Nitrosococcus halophilus* (35). AOB possess an ammonia monooxygenase gene cluster, *amoCAB*, in one to three copies coding for the enzyme ammonia monooxygenase (34). AOB also possess an *amoC* singleton of unknown function (36). AOB acquire their organic carbon by assimilating inorganic carbon dioxide through the Calvin-Benson-Bassham (CBB) cycle in which carboxylation is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) enzyme (37). AOA also derive their energy by oxidizing ammonia to nitrite (38) and fix CO_2 by the 3-hydroxypropionate/4-hydroxybutyrate cycle (39). AOA belong to subphylum Thaumarchaeota including genera like *Nitrosopumilus* sp., *Nitrosocaldus* sp., *Nitrosocosmicus* sp. (40, 41). Complete ammonia-oxidizer (comammox) perform two-step oxidation of ammonium to nitrate through nitrite (9, 10). Comammox belong to the phylum Nitrospirae (42) and they fix their CO_2 by the reductive tricarboxylic acid (rTCA) cycle (9).

AO are distributed widely in all terrestrial and aquatic environments (43–46) with the exception of Comammox which have not been reported in open ocean water. Several studies have attempted to understand the niche differentiation of the AO. Here, I discuss what is known about the effect of environmental factors on AO which helps us better understand the nature of the microhabitat they occupy in nature.

II. Effect of environmental factors on ammonia oxidizer distribution and activity

a. Effect of ammonium concentration

The ammonium concentration is one of the most studied environmental factors affecting the distribution and microbial function of ammonia oxidizers. Two physiological phenomena

determine the ammonium concentration range favorable for an ammonia oxidizer: i) the affinity of ammonia monooxygenase (AMO) enzyme of the AO to ammonia and ii) the critical concentration of ammonium above which ammonium is toxic/inhibitory to the AO (47). The two characteristics typically covary within a specific AO taxa or group. For example, AOB have comparatively low affinities for ammonia, and they grow well at higher ammonium concentrations compared to AOA and comammox (48). AOB *Nitrosomonas eutropha* is eutrophic with an optimum growth rate at 100 mM ammonium (Ghimire et al., unpublished). AOA and comammox have high affinity to ammonia and generally prefer to grow at low ammonium concentrations (48). Comammox enrichment Cmx-BO4 from freshwater Lake Burr Oak grows optimally through 10-25 μM ammonium while its growth is inhibited above 50 μM ammonium concentration (49). *Candidatus* Nitrososphaera gargensis, a moderately thermophilic AOA, has a higher ammonium requirement, as it is highly active at 0.14- and 0.79 mM ammonium and partially inhibited at 3.08 mM ammonium (50). Marine AOA *Nitrosopumilus maritimus* strain SCM1 has a very high affinity to ammonium concentration and can even oxidize ammonium ≤10 nM with an apparent (km (app)) for NH_3 of nearly 3 nM (38). Of all the analyzed ammonia oxidizers, AOA strains *N. maritimus* SCM1 and *Candidatus* Nitrosoarchaeum koreensis MY' (soil archaeon) have the highest affinity for ammonia followed by comammox *Nitrospira inopinata* and AOA *Ca.* N. koreensis AR' (marine archaeon) (48). *N. inopinata* has Km (app) for NH_3 of 49 nM (48). Interestingly, marine environments with ammonium concentrations from below detection limit (100 nmol/L) to 195 nmol/L ammonium concentrations (51) have never been reported to have comammox in ocean water (9) while they are abundant in coastal waters and sediments (43, 52, 53). A simulating competition between *N. inopinata* and non-marine AOAs *Nitrososphaera gargensis* and *Nitrosospira* sp. at low ammonium concentration predicts the comammox to outcompete the AOAs (48, 54), however, an actual competition experiment to investigate the which of the two oligotrphic groups of AO perform better in oligotrophic environments has not been published yet.

Ammonium is toxic in nature causing programmed cell death in plants and yeasts (55, 56), as well as stunting and chlorosis in plants (57). Hyperammonemia, a state of high ammonium in human serum causes irreversible brain damage (58). A correlation between high ammonium and oxidative and nitrosative stress is observed in mammalian and plant cells (59–61). High ammonium concentration is also inhibitory to the growth and activity of AO (47, 62).

AOB *Nitrosomonas eutropha* can tolerate up to 600 mM ammonium (47) compared to a lower ammonium tolerance of 10 mM ammonium in *Nitrosomonas* sp. Is79 (62). There is a gap of knowledge in why ammonium/ammonia is toxic to ammonia oxidizers at high concentrations.

b. Effect of pH

Ammonia and ammonium exist in a pH-dependent equilibrium (63). The fractions of unionized ammonia to ionized ammonium can be calculated by using equation: unionized ammonia (%) = 100 $(1+ 10^{(pKa-pH)})$, in which pka=0.09108+2729.92(273.2+Temperature) (64, 65). Hence, pH determines the availability of free ammonia and ammonium molecules. Ammonia can enter cells through diffusion without energy expenditure because of its uncharged nature while ammonium must be actively transported through cellular membranes with the use of energy (66). Uncharged ammonia form is the substrate for ammonia oxidation in *Nitrosomonas europaea* (67), which is a big disadvantage in acidic pH and ammonium environments since almost 99% of substrate is in the form of ammonium in most environmental conditions (68). Ammonia oxidation is reported in acidic, neutral as well as in alkaline environments (69) although cultivating acidophilic AO has remained a challenge because acidophilic AO live in aggregates with heterotrophs as a means of adaptation to low ammonium concentrations (70). AOA are the most dominant AO in acidic and alkaline environments (71–73). De Boer et al (1991) enriched AOA from soil with pH 4 (70) and Zhang et al (2012) detected AOA *amoA* but not AOB *amoA* in acidic agricultural soil (74). Cultivation of obligately acidophilic AOA *Nitrosotalea* sp. Nd2 and *Nitrosotalea devanaterra* Nd1 from acidic agricultural soil confirmed the existence of AO adapted to acidic environments (75). Neutrophilic AO are easier to cultivate and have been enriched and isolated widely. A comparative study of the effect of pH on freshwater AOA, AOB and comammox enrichments showed that the non-acidophilic enrichments perform ammonia oxidation through pH 6-8.5/9 (45, 49). However, the cultures described were enriched at pH 7.5 and this could have selected for neutrophilic AOA and comammox(45, 49) . Ammonia oxidation activity also occurs at alkaline pH. For example, comammox *Nitrospira* sp. are present at extremely alkaline (pH 11) condition (76).

c. Effect of temperature

Temperature is one of the major environmental factors affecting the growth of microorganisms (77). Temperature optima depends on the two factors: (i) increase in biochemical reaction rates with increase in temperature and (ii) denaturation of proteins

including energy deriving membrane proteins at high temperature (78). Understanding the effect of temperature on the activity of AO is important in sewage treatment as maintaining temperature can be energy demanding. Ammonia oxidation occurs in psychrophilic, mesophilic and thermophilic environments. Nakagawa et al (2007) observed ammonia oxidation at 4°C (79). Thermophilic AO from hot springs with temperature optima of 50°C and 46°C were observed by Lebedeva et al (80) and Hatzenpichler et al (50). Optimum temperatures for ammonia oxidation by *N. inopinata* and *Nitrososphaera gargensis* are 37°C and 46°C respectively (48), 42 °C for *N. viennensis* (81), and 46 °C for *Ca*. Nitrosotenuis uzonensis (80).

Most of the characterized AO are mesophilic in nature potentially because of the bias of temperature selection in the AO enrichment and/or isolation. A study on the effect of temperature on nitrification rates using a mixed culture of nitrifiers and heterotrophs from a livestock wastewater treatment plant in Yongin city, Korea showed that the optimum temperature for nitrification is 35°C (82). *Nitrosomonas* sp. from trickling filter effluent in Illinois had the optimum growth at 32°C (83). A study on the effect of temperature on AOA shows that at pH 4.5 and below, 20°C is the optimum temperature while at pH 5 and above, 30°C is usually the optimum temperature (84) showing a correlation between temperature and pH.

d. Effect of light

Light sensitivity in ammonia oxidizers is of interest because it helps predict the depth at which AO habitat in terrestrial and aquatic environments, as light intensity and quality vary through the water column. Photoinhibition in AOB is the inhibition of activity of AOB in the presence of light. Photoinhibition in AOB *Nitrosomonas* sp. is targeted in the initial step of ammonia oxidation catalyzed by the enzyme ammonia monooxygenase. The oxygenated state of enzyme ammonia monooxygenase is sensitive to UV and is inactivated by the light of wavelength 400-430 nm (85). Photoinhibition is also observed in AOA and comammox (45, 49, 86, 87). The inhibitory effects of light on growth have been observed in many AO. The specific growth rates of AOB *Nitrosomonas europaea* and *Nitrosospira multiformis*, and AOA *Nitrosopumilus maritimus* and *Nitrosotalea devanaterra* were significantly reduced at both 15 $\mu E\ m^{-2}\ s^{-1}$ and 60 $\mu E\ m^{-2}\ s^{-1}$ of 400-680 nm wavelength spectrum (87). The growth rate of comammox enrichment Cmx-BO4 was also significantly inhibited by white light compared to its growth in the dark incubation and completely inhibited by blue light (470 ± 5 nm) (49). AOA enrichments AOA-AC2, AOA-AC5 and AOA-DW were also completely inhibited by blue light

(470 ± 5 nm) and they did not recover even after transferring them to dark incubation (45). Based on the studies yet, it is observed that AO are inhibited by light, particularly blue light. Of the nitrifiers, nitrite oxidizers are more sensitive than AO giving rise to a primary nitrite maximum (PMN) at the base of the euphotic zone in the ocean (86, 88). The effect of light on the activity of comammox is not well studied, except the photoinhibition observed in a comammox strain *Nitrospira inopinata* (86).

e. Effect of oxygen concentration

Ammonia oxidation is an oxidative process; hence AO require dissolved oxygen (DO) for their activity. However, like all aerobic microbes, AO should contend with intracellular oxygen potentially causing oxidative stress (Imlay, 2013). AOA *amoA* have been detected in very low DO environments such as activated sludge bioreactors (DO < 6.3 μM) operated for simultaneous nitrification-denitrification (Park et al., 2006). AOA have also been detected in water columns of Pacific Ocean and Black Sea with DO <3.1 μM and1 μM respectively (44, 89). A comprehensive study comparing abundance of AO in activated sludge at different DO saturation indicates comammox is more abundant than AOA at low DO conditions (90). Comammox are more dominant than AOB in low DO conditions which can be explained by the higher oxygen sensitivity, slower metabolism of comammox and higher DO demand of AOB for rapid ammonia oxidation (90). In another study, increasing oxygen concentration increased growth rate of AOB while the growth of AOA was unaffected which can be explained by high DO tolerance of both AOB and AOA and higher metabolism of AOB than AOA (45).

CONCLUSION

Abiotic factors determine the distribution and activity of AO. Aerobic AO are found everywhere except for comammox which have not been reported yet in open ocean waters. AOA and comammox are generally oligotrophic and are the dominant AO in oligotrophic environments. Although all three groups of AO are found in alkaline, neutral and acidic environments, AOA are the dominant AO in acidic environments. AO are inhibited by light, particularly blue light and are likely distributed at depths where light cannot penetrate. AOB are favored by high DO while AOA and comammox prefer low DO conditions. All three groups of AO are found through psychrophilic to thermophilic environments. AO are versatile and can perform their ecological function of ammonia oxidation at a wide variation of environmental conditions.

DISSERTATION OVERVIEW

In this dissertation, I describe the impact of environmental factors, particularly ammonium concentration on the activity and distribution of AO.

In **Chapter 1**, I characterized a freshwater comammox enrichment from Lake Burr Oak. Prior, comammox cultures were only characterized from bioengineered systems but not from natural environments. Comammox cultures from natural environments need to be characterized to elucidate the physiological potential of comammox. I investigated effect of environmental factors on the growth of comammox enrichment Cmx-BO4 as well as characterized metagenome assembled genome (MAG) of comammox *Nitrospira* sp. BO4 for its metabolic potential. The enrichment Cmx-BO4 is the first freshwater comammox to be characterized and fifth of all the characterized comammox enrichment cultures.

In **chapter 2**, I investigated the competition between comammox enrichment Cmx-BO4 and AOA enrichment BO1 in limited ammonium environments. Comammox and AOA are generally oligotrophic environments and it is not understood what happens to their abundance when the two are competing for ammonium. Competition for ammonium in chemostat and batch culture followed by quantification by qPCR was used as the method to investigate the competitive interaction between the two oligotrophic enrichments.

In **chapter 3**, I explored ammonium toxicity to AOB *Nitrosomonas* at high concentrations. High ammonium is toxic to AOB *Nitrosomonas* sp., however, the mechanism of high ammonium toxicity is not understood yet. I used growth experiments as the method to investigate the effect of ammonium concentration on the growth of *Nitrosomonas* sp. and RNA-seq to evaluate the effect of different ammonium concentrations on gene regulation of *Nitrosomonas* sp.

CHAPTER 1

Ecophysiological and genomic characterization of the freshwater complete ammonia oxidizer *Nitrospira* sp. BO4

This chapter was reproduced from the following research publication:

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SG contributed to lab work, data evaluation and writing manuscript. MEW contributed to lab work and data evaluation. AB contributed to data evaluation, figure representation and writing manuscript. AB and SG edited the manuscript.

ABSTRACT

Complete ammonia oxidizers (comammox) are a group of ubiquitous chemolithoautotrophic bacteria capable of deriving energy from the oxidation of ammonia to nitrate via nitrite. Here, we present a study characterizing the comammox strain *Nitrospira* sp. BO4 using a combination of cultivation-dependent and molecular methods. The enrichment culture Cmx-BO4 was obtained from the sediment of Lake Burr Oak – a mesotrophic lake in eastern Ohio. The metagenome of the enrichment culture was sequenced, and a metagenome-assembled genome (MAG) was constructed for *Nitrospira* sp. BO4. The closest characterized relative of *Nitrospira* sp. BO4 was *Candidatus* Nitrospira kreftii. All genes for ammonia and nitrite oxidation, reductive TCA cycle and other pathways of the central metabolism were detected. *Nitrospira* sp. BO4 used ammonia and oxidized it to nitrate with nitrite as the intermediate. The culture grew on initial ammonium concentrations between 0.01 and 3 mM with the highest rates observed at the lowest ammonium concentrations. Blue light completely inhibited the growth of *Nitrospira* sp. BO4, while white light reduced the growth and red light had no effect on the growth. *Nitrospira* sp. BO4 did not grow on nitrite as its sole substrate. When supplied with ammonium and nitrite, the culture utilized nitrite after most of the ammonium was consumed. In summary, the genomic information of *Nitrospira* sp. BO4 coupled with the growth experiments shows that *Nitrospira* sp. BO4 is a freshwater comammox. Future research will focus on further characterization of the niches of comammox in freshwater environments.

SIGNIFICANCE

Nitrification is a key process in the global nitrogen cycle. Complete ammonia oxidizers (Comammox) were discovered recently and only three enrichment cultures and one pure culture have been characterized with respect to activity and growth under different conditions. The cultivated comammox strains were obtained from engineered systems such as a recirculating aquaculture system and hot water pipes. Here, we present the first study characterizing a comammox strain obtained from a mesotrophic freshwater lake. In freshwater environments, comammox co-exists with ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). Our results will help elucidate physiological characteristics of comammox, and the distribution and niche differentiation of different ammonia oxidizers in freshwater environments.

INTRODUCTION

Nitrification is one of the key processes in the global nitrogen cycle (6, 41). During nitrification, microorganisms oxidize ammonia completely to nitrate. Until 2015 nitrification was assumed to be a two-step process. Ammonia is first oxidized to nitrite by ammonia-oxidizing archaea (AOA) or ammonia-oxidizing bacteria (AOB) (7, 91). In the second step, nitrite is further oxidized to nitrate by nitrite-oxidizing bacteria (NOB) (7, 11). The complete oxidation of ammonia to nitrate by one microorganism was theoretically proposed in 2006 and discovered several years later (9, 10, 92) refuting the long-held assumption that nitrification is a process carried out by two different microorganisms. The newly discovered complete ammonia oxidizers (comammox) are chemolithoautotrophic bacteria generating energy through the two-step oxidation of ammonia to nitrate via nitrite (9, 10).

Phylogenetically, all identified comammox bacteria belong to phylum *Nitrospirae,* while most AOB belong to the order *Betaproteobacteriales* and AOA belong to the *Thaumarchaeota* (7–10, 38, 93–95).

Comammox bacteria encode the ammonia monooxygenase (*amoCAB*), hydroxylamine dehydrogenase (*haoAB*) and nitrite oxidoreductase (*nxrAB*) genes required to oxidize ammonia completely to nitrate (9, 10, 93). The *amoCAB* gene cluster in comammox is unique but closely related to the *amoCAB* gene cluster in AOB, while *nxrAB* genes are homologous to the *nxrAB* genes in NOB *Nitrospira* (9, 10).

AOA, AOB and comammox in the environment can be detected using the ammonia monooxygenase subunit A (*amoA*) gene (43, 44, 96, 97). Comammox have been widely found in a variety of different environments such as a drinking water treatment plant (52), wastewater treatment plants (98, 99), aquaponics (100), soils (101–105), sediments and water column of freshwater lakes (102, 106, 107) and groundwater fed filters (108, 109). Interestingly, comammox have not been detected in open-ocean samples (43).

Controlling factors of nitrification and ammonia oxidation include but are not limited to ammonium availability, pH, oxygen availability and light (45, 110–112). Ammonium concentration impacts niche differentiation between AOA and AOB with most AOA growing at lower ammonium concentrations and having a higher affinity for ammonia, while AOB have a lower affinity for ammonia and grow better at higher ammonium availability (38, 45, 48). Direct competition for ammonium between AOA and AOB from freshwater resulted in AOA being

successful at limiting ammonium and AOB being successful at higher ammonium availability (113).

Four different comammox strains have been characterized: *Nitrospira inopinata*, *Candidati* Nitrospira nitrosa, Nitrospira nitrificans and Nitrospira kreftii (9, 10, 93). Activity measurements showed that *Nitrospira inopinata* and *Ca.* Nitrospira kreftii have a high affinity to ammonia, which is in the same order of magnitude as the affinity of AOA, and a much higher than the affinity of most AOB to ammonia (48, 93). All characterized strains have been obtained from engineered/man-made systems: *Nitrospira inopinata* from a hot water pipe (9) and the other three strains from a recirculating aquaculture system (10, 93).

Here, we present a study combining cultivation-based and cultivation-independent methods to characterize the newly enriched comammox strain *Nitrospira* sp. BO4. This culture was enriched from the mesophilic Lake Burr Oak in Ohio. We determined the effect of different environmental conditions, such as ammonium, nitrite, pH and light on the growth of *Nitrospira* sp. BO4. In addition, we analyzed the MAG (metagenome-assembled genome) of *Nitrospira* sp. BO4 to characterize the physiological capacity of newly enriched comammox culture.

MATERIALS AND METHODS

Materials

Sediment samples were taken from the near-shore region of Lake Burr Oak in October 2009 (BO; 39°54'N, 82°06'W) and transported at 4°C to the laboratory. Lake Burr Oak is a man-made reservoir in the east of Ohio. The lake is mesotrophic and its watershed is 81% forest, 14% agricultural land and 5% other uses (114). Enrichment cultures were started within 1-2 days after sediment sampling.

Medium

Mineral salts (MS) medium with ammonium was used to enrich and cultivate the comammox strain (115, 116). MS medium contained 10 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$ * $2H_2O$, 0.2 mM $MgSO_4$ * $7H_2O$, and 1 ml $liter^{-1}$ trace elements solution (115, 116). The medium was buffered with HEPES in 4:1 molar ratio to the ammonium concentration except when stated otherwise. The pH of the media was adjusted before autoclaving to 7.5 - 7.8. Sterile KH_2PO_4 solution was supplemented to autoclaved MS media to obtain a final concentration of 0.4 mM. If not differently stated, all cultures were incubated in 50 mL media in 125-mL Erlenmeyer flasks with cotton stoppers.

Enrichment and cultivation of *Nitrospira* sp. BO4

Sediment samples (0.5 g) from Burr Oak Lake were inoculated in MS media supplemented with 0.25 mM ammonium and incubated at 27°C in dark. The ammonium concentration in the enrichment culture was regularly monitored. After the initial added ammonium was depleted, the culture was filtered through a 0.45-µm membrane filter. The filtrate (5 ml) was transferred to a fresh MS medium (50 ml) with 0.25 mM ammonium. Filtering and transfer were repeated 5-6 times. After the initial enrichment, the culture was transferred to MS medium with 0.5 mM ammonium. Subsequent transfers to fresh medium were done every 4-6 weeks by inoculating 5 ml of the outgrown culture into 50 ml of fresh medium. Biomass for molecular analysis was collected by filtering the enrichment culture *Nitrospira* sp. BO4 through 0.1-µm-pore-size polycarbonate filters. The filters were stored at -80°C.

Growth experiments

Growth experiments were performed to test the influence of ammonium, nitrite, pH and light on the growth of *Nitrospira* sp. BO4 (45, 115). For all growth experiments, 50 mL of MS medium was inoculated with 10 % (v/v) (5 mL) late-log-phase culture and incubated in the dark at 27°C. Samples were taken at regular intervals and ammonium, nitrite and nitrate concentrations were measured. The influence of different ammonium concentrations was tested by incubating *Nitrospira* sp. BO4 in MS media with initial ammonium concentrations between 0.01 to 3 mM and the corresponding HEPES concentrations. The influence of the initial pH was determined in two different experiments A. MS medium with 0.5 mM ammonium and 2 mM HEPES was used, and the pH values were adjusted to pH 6 – 8.5. B. MS medium with 0.1 mM ammonium and 2 mM HEPES was used, and the initial pH values were adjusted to values between pH 3 and pH 9. The influence of light was tested by incubating the cultures above light panels which emitted 30 µmol photons m^{-2} s^{-1}. Three different wavelengths of light were tested: white (5000-7000K), red (623 ± 3nm) and blue light (470 ± 5nm). The influence of nitrite was tested in the presence and absence of 0.5 mM ammonium with initial nitrite concentrations between 0.1- and 2 mM nitrite.

To calculate the growth rates, the nitrate concentrations were natural log (ln) transformed (log to the basis 2) and plotted against time. The growth rates were calculated from the slope (linear increase) of the ln-transformed nitrate concentrations over time. These calculations are based on the assumption that the nitrite and/or nitrate production of ammonia-oxidizing cultures

is correlated to their growth (45, 115, 117). The increase in the ln transformed nitrate concentrations was linear for several days (except for very low ammonium concentrations) and the correlation coefficients were always ≥ 0.97 and in most cases ≥ 0.99.

Cultivation for metagenomic analysis

Nitrospira sp. BO4 was cultivated in 2 L Erlenmeyer flasks in 1 L MS medium with 0.5 mM ammonium. When the ammonium was consumed, the biomass was collected by centrifugation (22000 × *g*).

Determination of ammonium, nitrite, and nitrate

Ammonium and nitrite concentrations were determined as described in Bollmann et al., 2011 (115, 118, 119). The nitrate concentration was determined using Griess reagent after the removal of nitrite with sulfamic acid (120) and the reduction of nitrate to nitrite with vanadium chloride (121) (supplemental material).

Molecular methods

DNA isolation

DNA was isolated from polycarbonate filters using the Qiagen DNeasy UltraClean Microbial Kit (Valencia, CA, USA) with the following modifications. Beads, 600 µl PowerBead solution and 100 µl Solution SL were added to the filters in a 2 ml screw cap tube. The filters were homogenized in a BioSpec Mini-Beadbeater-24 (BioSpecbead beater (Biospec Products, Inc., Bartlesville, OK) at 4800 rpm for 30 s. This procedure was repeated once. The samples were stored in-between for 10 min on ice. After bead beating the samples were treated following the manufacturer's recommendations.

High-quality genomic DNA for metagenome sequencing was isolated with Jetflex Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's recommendations.

PCR and qPCR

Standard PCR reactions were performed using GoTaq green master mix (Promega, Madison, WI) using primers presented in Table 1 and under conditions presented in Table 2. The *amoA* gene abundance in the *Nitrospira* sp. BO4 culture was determined using the Bioline SensiFAST SYBR NoROX Kit (Bioline USA, Taunton, MA, USA) in 5 µl reactions with 1 µl sample in an Illumina Eco Real-Time PCR system (Illumina, San Diego, CA, USA) with initial denaturation at 95°C for 10 min, denaturation at 95°C for 10 sec and annealing at 52°C for 30

sec for 40 cycles (Table 1, Table 2). Standard curves were constructed using a plasmid containing the *amoA* gene of *Nitrospira* sp. BO4. The efficiency of the reactions ranged from 93.5% to 99.5% and R^2 in all experiments was > 0.99.

16S rRNA gene amplicon sequencing

DNA from the enrichment culture Cmx-BO4 was amplified with barcoded primers targeting the V4 region of the 16SrRNA gene (515F-806R) (122, 123). The PCR product was sequenced on an Illumina MiSeq system at the Center for Bioinformatics and Functional Genomics (CBFG) at Miami University. The software package QIIME2 was used to analyze the 16S rRNA gene amplicon sequencing data (124). The sequences were imported into QIIME2, forward and reverse sequences were joined, and the sequences were quality controlled eliminating all sequences with QC scores below 30. The sequences were denoised with deblur and taxonomy was assigned using the Greengenes 13_8 (97%) OUT database as reference.

Sanger sequencing

PCR products were cleaned up with the Spin Column PCR Purification Kit (Amherst, NY) and sequenced with a BigDye Terminator cycle sequencing kit on an Applied Biosystems 3730xl DNA analyzer (Life Technology Corporation, Carlsbad, CA).

Table 1. Primers used in this study to amplify *amoA* and *nxrB* sequences. Data about PCR conditions and qPCR validation can be found in Table 2.

Target sequence	**Primer pairs**
Comammox *amoA* (97)	comaA-244F: 5'- TAY AAY TGG GTS AAY TA -3' comaA-659R:5'- ARA TCA TSG TGC TRT TG -3'
AOA *amoA* (44)	Arch amoA F: 5'-STA ATG GTC TGG CTT AGA CG-3' Arch amoA R: 5'-GCG GCC ATC CAT CTG TAT GT-3'
AOB *amoA* (96)	amoA-1F: 5'-GGG GTT TCT ACT GGT GGT-3' amoA-2R KS: 5'-CCC CTC KGS AAA GCC TTC TTC-3'
nxrB (125)	nxrB-19F: 5'- TGG CAA CTG GGA CGG AAG ATG -3' nxrB-1237R: 5'- GTA GAT CGG CTC TTC GAC CTG -3'
M13-cloning	M13-F: 5'-GTA AAA CGA CGG CCA G-3' M13-R: 5'-CAG GAA ACA GCT ATG AC-3'
qPCR *amoA* Cmx-BO4 (This study)	BO4-F1 5'- CTG GTG CTG GAC ATC GTC TT -3' BO4-R12 5'- GTC CGA ACA TAC GCA AAG CC -3'

Table 2. PCR conditions (temperature [°C]/time [s]) for all PCR's and validation of Cmx-BO4 *amoA* qPCR of DNA samples.

	Coma *amoA*	**AOA *amoA***	**AOB *amoA***	***nxrB***	**M13**	**Cmx-BO4 *amoA***
Denaturation (initial)	95/120	95/300	95/180	95/180	95/120	95/600
Denaturation	95/30	95/45	95/30	95/30	95/60	95/10
Annealing	55/30	53/60	54/60	56/45	50/60	52/30
Extension	72/30	72/60	72/60	72/60	72/60	
Extension (final)	72/300	72/900	72/600	72/300	72/600	
Cycles	35	35	35	35	25	40
Melting curve						55°C -95°C
Efficiency [%]						93.5-99.5
R^2						> 0.99
Concentration for calibration curve [copies per µl]						170 – $1.7x10^{-8}$

Sequence analysis

The sequences were edited with 4Peaks (https://nucleobytes.com/4peaks/index.html) and blasted against NCBI nucleotide database (nr/nt).

Library preparation for metagenome sequencing

The isolated DNA was quantified with the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA). Five (5) µg DNA was mechanically sheared using the M220 Focused-ultrasonicator Instrument (Covaris, Inc., Woburn, MA) to get 400 bp DNA fragments. The sheared DNA fragments were size selected for 400 bp using the Blue Pippin DNA size selection system (Sage Science, Beverly, CA). The selected fragments were cleaned of impurities with ProNex® Size-Selective Purification System (Promega Corp., Madison, WI). The size distribution of the DNA fragments was analyzed with Agilent High Sensitivity DNA kit in Agilent 2100 Bioanalyzer (Agilent Scientific Instruments, Santa Clara, CA). The Illumina MiSeq library was prepared using the NEXTflex™ PCR-Free DNA Sequencing Kit (PerkinElmer, Inc, Waltham, MA).

Sequencing of the metagenome

The DNA of the *Nitrospira* sp. BO4 enrichment culture was sequenced on the Illumina MiSeq system (Illumina Inc., San Diego, CA) using MiSeq reagent kit v3 (150-cycle) at Center for Bioinformatics and Functional Genomics at Miami University (CBFG).

Metagenome sequence analysis

Reads were demultiplexed in Illumina Basespace to obtain 22.7 million paired-end FASTQC reads of 2*75 bp reads. Metagenome sequences were quality controlled, assembled, and binned into metagenome-assembled genomes (MAG) on KBase (The Department of Energy Systems Biology Knowledge Base) (126) (Workflow: https://narrative.kbase.us/narrative/96801). Reads were quality controlled using Trimmomatic (v0.36). Sequence length ranged from 35-75 bp and no sequences were flagged as low-quality reads. The taxonomy of the quality-controlled reads was determined with Kaiju-v1.7.3 against NCBI BLAST nr database. The reads were assembled using metaSPAdes (v3.13.0), MEGAHIT (v1.2.9) and IDBA-UD (v1.1.3) and the quality of the assemblies was assessed by QUAST (v4.4). The metaSPAdes assembly had the highest quality and was binned with CONCOCT (v1.1), MaxBin2 (v2.2.4) and MetaBAT2 (v1.7). The bins were optimized with the DAS tool (v1.2) and the genome quality was assessed with CheckM (v1.0.18). Two high quality

(completeness > 95%; contamination < 5%) metagenome assembled genomes (MAG) were obtained. The assembled metagenome of the enrichment culture Cmx-BO4 and the MAG of *Nitrospira* sp. BO4 were uploaded to the Integrated Microbial Genomes and Microbiomes system of the Department of Energy – Joint Genome Institute (DOE-JGI-IMG). The metagenome and the MAG were annotated through the IMG annotation pipeline and further analyzed in IMG (127).

Phylogenomic tree

A phylogenomic tree including comammox and canonical nitrite oxidizing *Nitrospira* was constructed using the software package UBCG (128) (38). Whole genome sequences were obtained from NCBI genome database (Table 3). The OrthoANI similarities between selected genomes and *Nitrospira* sp. BO4 were calculated using the ANI calculator on the webpage ezbiocloud.net (129).

Nucleotide sequence accession numbers

All sequence data were deposited at Genbank. The *amoA* and *nxrB* sequences were deposited under the accession numbers ON637246 and ON637247. The next generation sequencing data were deposited under the bioproject PRJNA838966. The assembly and binning are publicly available in KBase (https://narrative.kbase.us/narrative/96801) and the metagenome of the enrichment culture Cmx-BO4 and annotated MAG in the DOE-JGI-IMG database under the genome ID's: 3300039027 (metagenome) and 2868173055 (MAG).

RESULTS AND DISCUSSION

Enrichment of *Nitrospira* sp. BO4

Here we present a study investigating the growth and genomic potential of *Nitrospira* sp. BO4 enriched from the sediment of Lake Burr Oak in Ohio. The enrichment was started by inoculating 1g sediment into 50 ml mineral salts medium (MS medium) with 0.25 mM ammonium. The culture was originally filtered through 0.45 μm filters when ammonium was

Table 3. Isolate identification and Genbank assembly accession numbers of the genomes used for the phylogenomic tree presented in Figure 2.

Strain	**Genbank assembly accession number**
Nitrospira defluvii	GCA_000196815.1
Nitrospira lenta BS10	GCA_900403705.1
Nitrospira moscoviensis NSP M-1	GCA_001273775.1
Nitrospira sp. RCB	GCA_005239475.1
Nitrospira sp. RSF3	GCA_005116835.1
Nitrospira sp. CG24E	GCA_002869895.2
Nitrospira inopinata ENR4	GCA_001458695.1
Candidatus Nitrospira nitrosa COMA1	GCA_001458735.1
Candidatus Nitrospira kreftii comreactor 17	GCA_014058405.1
Candidatus Nitrospira nitrificans COMA2	GCA_001458775.1
Nitrospira sp. SG-bin1	GCA_002083365.1
Nitrospira sp. RSF12	GCA_005116955.1

consumed to remove AOB because the culture was initially started as an enrichment culture for AOA (45, 115). The filtrate was transferred to fresh medium (5 ml filtrate into 50 ml medium). This process was repeated six times. Afterwards the culture was transferred to medium with 0.5 mM ammonium and kept as part of a larger culture collection of enrichment cultures. The enrichment cultures were transferred every 4-6 weeks to fresh medium by transferring 5 ml culture to 50 ml fresh medium. Between 2009 and 2011 DNA from the culture was isolated and amplified with AOA and AOB specific *amoA* primers (44, 96) to identify the dominant ammonia oxidizer without any results. After the publication of the papers by Daims et al., 2015 (9) and Van Kessel et al., 2015 (10), the culture was tested for the presence of comammox ammonia oxidizers. The identity of the comammox was determined by sequencing of the *amoA* gene. Blasting the DNA sequence of the *amoA* gene showed that *Nitrospira* sp. BO4 belonged to the comammox *Nitrospira* and was closest related to *Candidati* Nitrospira nitrosa (81% identity) and Nitrospira nitrificans (85% identity). *Candidatus* Nitrospira kreftii did not show up in the blast results and no match was detected when the *amoA* sequence was blasted against the genome of *Candidatus* Nitrospira kreftii in the NCBI database. The DNA of the ammonia oxidizing enrichment culture Cmx-BO4 was also amplified with AOA and AOB *amoA* primers (44, 96) but no PCR products were detected.

Genome analysis

The metagenome of the enrichment culture Cmx-BO4 was sequenced, assembled, and binned with KBase. The MAG of *Nitrospira* sp. BO4 was analyzed in IMG. The community composition of the enrichment culture Cmx-BO4 was assessed using 16S rRNA gene amplicon sequencing data and analysis of the metagenomic reads in KBase using Kaiju. *Nitrospira*-related reads represented more than 56-57% of the reads when analyzed with both methods (Table 4). Based on the 16S rRNA gene amplicon sequencing data other strains in the community included different *Alpha*- and *Betaproteobacteria* and *Bacteroidetes* (Table 4). No sequences related to AOA or AOB were detected in the 16S rRNA gene amplicon sequencing data.

Table 4. Community composition of the enrichment culture Cmx-BO4 based on 16S rRNA gene amplicon sequencing and classification of the metagenomic reads in KBase using Kaiju.

Closest relative by BLAST search	**Phylum**	**Amplicon [%]***	**Kaiju [%]**
Nitrospira sp.	*Nitrospirae*	56	57.4
Cupriavidus sp.	*Proteobacteria - Betaproteobacteriales*	10	2.5
Bradyrhizobium sp.	*Proteobacteria – Alphaproteobacteria*	11	4.4
Panacibacter sp.	*Bacteroidetes*	3	n.d.
Hyphomicrobium sp.	*Proteobacteria – Alphaproteobacteria*	10	7.3
Methylocystaceae bacterium	*Proteobacteria – Alphaproteobacteria*	3	n.d.
Flavitalea sp.	*Bacteroidetes*	1	n.d.
Unclassified			30.5

* Abundances above 1% of the overall community presented. n.d.: not detected.

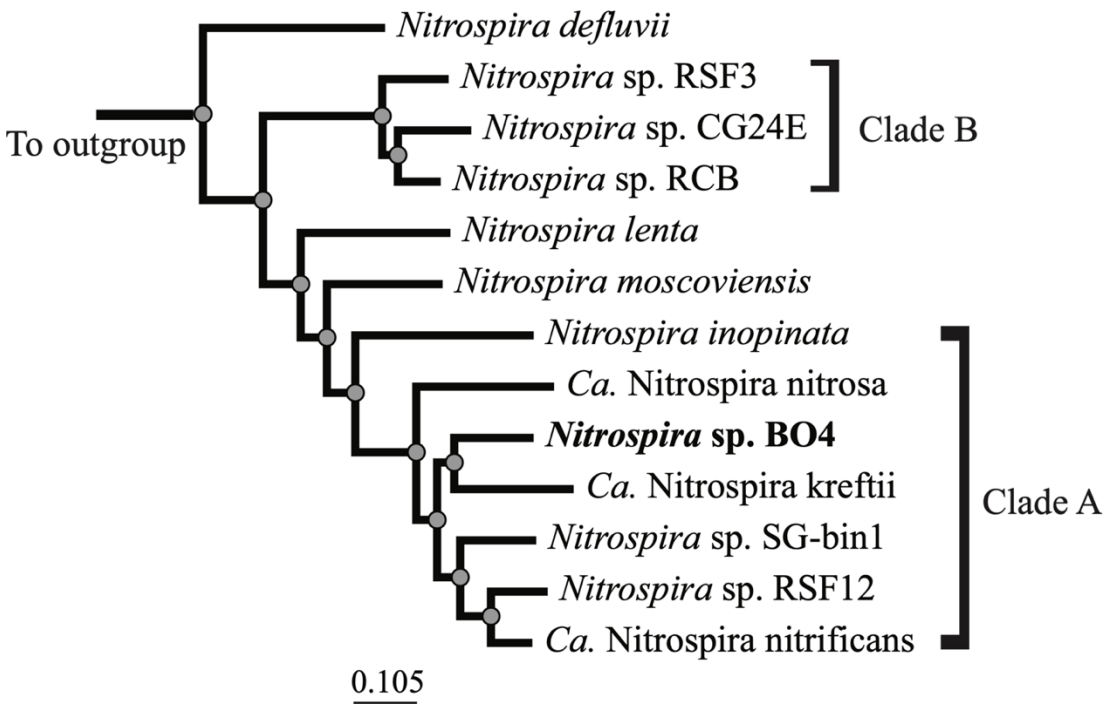

Figure 2. Phylogenomic tree of the MAG of the enriched *Nitrospira* sp. BO4 with representative comammox and canonical Nitrospira strains. The scale bar represents 10.5% sequence divergence. The Genbank assembly accession numbers can be found in Table 3.

The taxonomic affiliation of nitrogen cycle related genes in the enrichment culture Cmx-BO4 was analyzed in KBase (Table 5). More than 90% of the genes involved in ammonia (*amoCAB, haoAB*) and nitrite oxidation (*nxrAB*) were classified into the *Nitrospirae*. Less than 1% of the ammonia oxidation genes and around 5% of the nitrite oxidation genes were classified into other groups while the rest of the genes were unclassified (Table 5).

The *Nitrospira* sp. BO4 MAG was assembled with SPAdes and binned with MetaBat and submitted to JGI-IMG for further analysis. The MAG was 95.85% complete and had 3.69% contaminations (Table 6). The genome size was 4,711,400 bp and 85.8% of the DNA coded for genes (Table 7). Overall, 4889 protein-coding genes were detected of which 3031 (61%) have a predicted function (Table 7, Table 8). A phylogenomic tree was constructed using the software package UBCG based on 92 bacterial core genes (128) (Figure *2*) *Nitrospira* sp. BO4 belonged to comammox clade A. The genome of *Nitrospira* sp. BO4 showed the highest similarity to the genomes of *Ca.* Nitrospira nitrificans (ANI: 78.2%) and *Ca.* Nitrospira kreftii (ANI: 76.8%) (Table 9). Of the comammox strains that are characterized in pure and enrichment cultures, the only isolated strain *Nitrospira inopinata* was the least related to *Nitrospira* sp. BO4 (ANI: 72.1%).

Table 5. Taxonomic affiliation [%] of selected nitrogen cycle genes detected in the metagenome of the enrichment culture Cmx-BO4 analyzed in KBase.

Gene	***Nitrospirae***	**Other phyla**	**Unclassified**
Ammonia monooxygenase subunit A (*amoA*)	89.7	0.4	9.9
Ammonia monooxygenase subunit B (*amoB*)	97.1	0.2	2.7
Ammonia monooxygenase subunit C (*amoC*)	93.4	0	6.6
Hydroxylamine dehydrogenase (*hao*)	90.3	0.2	9.4
Nitrate reductase / nitrite oxidoreductase subunit A (*narG, nxrA*)	94.1	5.5	0.5
Nitrate reductase / nitrite oxidoreductase subunit B (*narH, nxrB*)	92.1	5.0	2.9
Urease subunit gamma (*ureA*)	55.7	8.4	36.0
Urease subunit beta (*ureB*)	21.7	12.8	65.6
Urease subunit alpha (*ureC*)	15.9	30.0	54.3

Table 6. Overview over *Nitrospira* sp. BO4 MAG assembled in K-Base.

***Nitrospira* sp. BO4 Assembly method**	SPAdes	MegaHit	IDBA
Binning method	MaxBin, CONCUCT, MetaBat optimized by DASTool	MaxBin, CONCUCT, MetaBat optimized by DASTool	MaxBin, CONCUCT, MetaBat optimized by DASTool
Completeness [%]	95.85	95.85	95.85
Contamination [%]	3.69	3.69	3.69
GC content [%]	55.49	55.50	55.52
Genome size [bp]	4708985	4677486	4635384
# of contigs	30	57	104

Table 7. Genome properties of the *Nitrospira* sp. BO4 MAG. Values in parentheses represent the percent (%) of total genes.

Attribute	***Nitrospira* sp. BO4 MAG**
Genome size (bp)	4,711,400
DNA coding region (bp)	4,041,582
GC-content (%)	55.5
DNA scaffolds	31
Total genes	4961
RNA genes	62 (1.3%)
rRNA operons	1
tRNA genes	57 (1.2%)
Protein coding genes	4889 (98.6%)
Genes with function prediction	3031 (61.1%)
Genes assigned to COG's	2998 (60.4%)
Genes assigned Pfam domains	3143 (63.4%)
Genes with signal peptides	386 (7.8%)
Genes with transmembrane helices	1099 (22.2%)
CRISPR repeats	1

Table 8. Number of genes associated with the general COG functional categories in the MAG of *Nitrospira* sp. BO4.

Code	**value**	**%**	**Description**
J	220	6.25	Translation, ribosomal structure, and biogenesis
A			RNA processing and modification
K	223	6.34	Transcription
L	154	4.38	Replication, recombination, and repair
B	1	0.03	Chromatin structure and dynamics
D	47	1.34	Cell cycle control, cell division, chromosome partitioning
Y			Nuclear structure
V	86	2.44	Defense mechanisms
T	285	8.1	Signal transduction mechanisms
M	284	8.07	Cell wall/membrane/envelope biogenesis
N	107	3.04	Cell motility
Z	5	0.14	Cytoskeleton
W	48	1.36	Extracellular structures
U	75	2.13	Intracellular trafficking, secretion, and vesicular transport
O	196	5.57	Posttranslational modification, protein turnover, chaperones
C	244	6.94	Energy production and conversion
G	130	3.7	Carbohydrate transport and metabolism
E	191	5.43	Amino acid transport and metabolism
F	67	1.9	Nucleotide transport and metabolism
H	170	4.83	Coenzyme transport and metabolism
I	88	2.5	Lipid transport and metabolism
P	188	5.34	Inorganic ion transport and metabolism
Q	72	2.05	Secondary metabolites biosynthesis, transport, and catabolism
R	366	10.4	General function prediction only
S	214	6.08	Function unknown
-	1963	39.57	Not in COG's

Table 9. Pairwise ANI comparisons [% similarity] of the MAG *Nitrospira* sp. BO4 and selected comammox and canonical nitrite-oxidizing *Nitrospira* genomes. The OrthoANI were calculated by ANI calculator on the webpage ezbiocloud.net (3). The red frame indicates the ANI values related to *Nitrospira* sp. BO4 and the green frame all in pure and enrichment culture cultivated comammox strains.

	***Nitrospira* sp. BO4**	***Nitrospira inopinata***	***Ca.* Nitrospira nitrificans**	***Ca.* Nitrospira kreftii**	***Ca.* Nitrospira nitrosa**	***Nitrospira lenta***	***Nitrospira moscoviensis***	***Nitrospira defluvii***
***Nitrospira* sp. BO4**	100							
Nitrospira inopinata	72.1	100						
***Ca.* Nitrospira nitrificans**	78.2	72.4	100					
***Ca.* Nitrospira kreftii**	76.8	70.9	77.0	100				
***Ca.* Nitrospira nitrosa**	74.9	71.3	75.8	74.4	100			
Nitrospira lenta	72.0	71.5	71.6	70.7	70.5	100		
Nitrospira moscoviensis	72.6	73.1	72.8	71.6	71.6	73.3	100	
Nitrospira defluvii	69.4	70.0	70.0	69.5	69.3	70.7	71.3	100

Metabolic potential of *Nitrospira* sp. BO4

The complete set of genes for ammonia and nitrite oxidation were detected in the genome of *Nitrospira* sp. BO4 (Table 10). The *amoA* gene could not be detected in the MAG of *Nitrospira* sp. BO4 but was detected by PCR and found in the metagenome of the enrichment culture. Therefore, we assume that the *amoA* is present, but *amo* operon (*amoCAB*) could not be completely assembled. In addition, an *amoC* singleton and the genes for the copper binding and copper resistance protein (*copCD*) were detected in the MAG of *Nitrospira* sp. BO4. The genome contained one cluster of genes for hydroxylamine oxidation encoding the hydroxylamine dehydrogenase (*haoAB*), the cytochromes c554 and cm552 (*cycAB*) and the cytochrome c biogenesis proteins (*ccmABCDEFGHI*). The genome of *Nitrospira* sp BO4 encoded one cluster of the nitrite oxidoreductase subunits A and B (*nxrAB*) and four copies of the gamma subunit of the nitrite oxidoreductase (*nxrC*) (Table 10). Finally, copper containing nitrite reductase (*nirK*) was found in the genome of *Nitrospira* sp. BO4. A complete set of genes for urea metabolism (urease, urease accessory protein and ABC type urea transporter) were detected in the genome of *Nitrospira* sp. BO4 indicating that this comammox strain could use urea as source for ammonium as shown for *Ca.* Nitrospira kreftii (93).

All genes for glycolysis, gluconeogenesis, oxidative and reductive TCA and pentose phosphate pathway were present in the genome of *Nitrospira* sp. BO4. The genome encoded the genes for the electron transport chain and an F-type ATPase. In addition, cytochrome P450, bacterioferritin, cupredoxin and plastocyanin were found in the genome of *Nitrospira* sp. BO4 (Table 10). A variety of genes involved in defense against reactive oxygen species such as hydrogen peroxide and superoxides were detected in the genome of *Nitrospira* sp. BO4 (Table 10). Transporters for inorganic ions such as iron (Fe^{3+}) with and without siderophore, sulfate, phosphate and molybdate were found in the genome of *Nitrospira* sp. BO4. The genome also encoded an ammonium transporter (*amtB*). However, no transporter for nitrite and/or nitrate was detected.

The evaluation of the MAG of *Nitrospira* sp. BO4 revealed no new genome features when compared to the genomes of other characterized comammox strains and enrichments such as *Nitrospira inopinata*, *Ca.* Nitrospira nitrificans, *Nitrospira nitrosa* and *Nitrospira kreftii* (9, 10, 48, 93).

Table 10. Overview of the metabolic capacity of *Nitrospira* sp. BO4. Genes were found in the MAG of *Nitrospira* sp. BO4 or in the metagenome of the enrichment culture Cmx-BO4 in IMG or by PCR with gene specific primers**.**

Ammonia and nitrite metabolism	**Genes**
Ammonia oxidation	*amoCAB, amoC* (single copy)
Hydroxylamine oxidation	*haoAB, cycAB*
Nitrite oxidation	*nxrAB,*
Copper binding/resistance protein	*copCD*
Cyt c-type biogenesis protein and heme exporter protein	*ccmABCDEFGHI*
Nitrite reductase	*nirK*
Urea metabolism	
Urease and urease accessory protein	*ureABC, ureDFG*
Hydrogen metabolism	
Ni/Fe hydrogenase	*hyd*
Central metabolism	
Glycolysis and gluconeogenesis	Genes for All enzymes
TCA	Genes for All enzymes
Reductive TCA (CO_2 fixation)	Genes for All enzymes
Pentose Phosphate pathway	Genes for All enzymes
Electron transport chain and ATP synthesis	
NADH-quinone oxidoreductase	*nuoABCDEFGHIJKLM*
Succinate dehydrogenase	*sdhABC*
Quinol cyt c reductase	present
cyt bd ubiquinol oxidase	*cydAB*
F-type ATPase	*atpABCDEFGHIR*
ROS defense	
Catalase and peroxidases	present
Superoxide dismutase	present
Transporters	
ABC type sulfate transporter	*cysAPUW*
ABC type phosphate transporter	*pstABCS*
ABC type molybdate transporter	*modABC*
ABC type Fe^{3+} transporter	*fbpABC*
ABC type Fe^{3+} siderophore transporter	*fepABCD*
ABC type urea transporter	*urtABCDE*
Ammonium transporter Rh	*amtB*
High affinity iron transporter	*efeU*
Low affinity iron/copper permease	present
Cobalt-zinc-cadmium efflux system	*czcABC*

Growth of *Nitrospira* sp. BO4

Ammonium, nitrite and nitrate concentrations along with the abundance of the *amoA* gene were followed over time (Figure 3). The culture consumed ammonium while producing nitrate and a small amount of nitrite. The nitrite accumulation started when around half of the ammonium was consumed. When the ammonium was completely consumed, the nitrite concentration increased to 65 μM and decreased again to 40 μM. The copy number of the *amoA* gene increased while ammonium was consumed, and nitrate produced indicating that *Nitrospira* sp. BO4 was growing with ammonia as substrate.

In summary, the comammox strain *Nitrospira* sp. BO4 is the sole ammonia and nitrite oxidizer in the enrichment culture Cmx-BO4. No additional ammonia and nitrite oxidizers were detected in the 16S rRNA gene amplicon sequencing library (Table 4) or by amplification with archaeal or bacteria *amoA* primers and only a small percentage of functional genes did not cluster with comammox *amo*, *hao* and *nxr* (Table 5). Finally, residual nitrite was left in the culture (Figure 3) indicating that no canonical nitrite oxidizer that would consume nitrite is present in the culture.

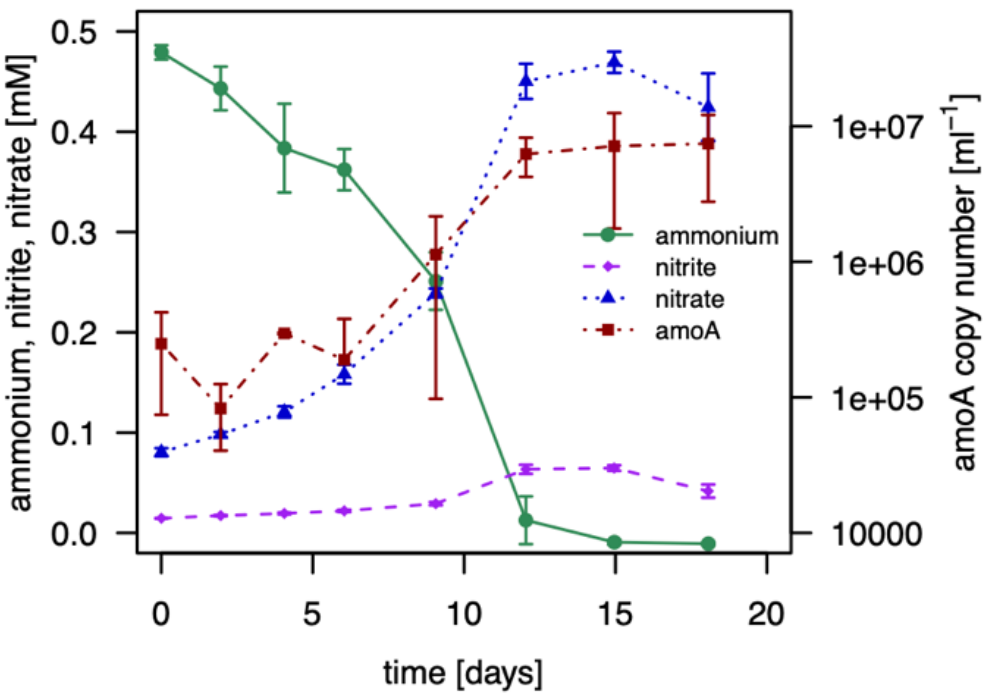

Figure 3. Conversion of ammonium to nitrate via nitrite by and *amoA* copy number of *Nitrospira* sp. BO4 in the enrichment culture cmx-BO4 (mean ± SD, n=3).

Influence of environmental factors the growth of *Nitrospira* sp. BO4

We characterized the impact of different environmental factors on the growth of *Nitrospira* sp. BO4. Ammonium concentration varies in freshwater environments depending on the trophic state of the lake. The highest growth rate of *Nitrospira* sp. BO4 was observed at the lowest ammonium concentration (0.01 mM) (Figure 4). The growth rate decreased with increasing ammonium concentrations and remained stable at ammonium concentrations above 0.05 mM. The lag phase was only one day at the lowest ammonium concentrations of 0.025 mM and increased to six days at the highest ammonium concentration of 3 mM (Figure 5) indicating that *Nitrospira* sp. BO4 needs to adapt to the higher ammonium concentrations. The closest characterized relative of *Nitrospira* sp. BO4, *Ca.* Nitrospira kreftii is inhibited by increasing ammonium concentrations (93). *Nitrospira inopinata* and *Ca.* Nitrospira kreftii have very low K_m values for ammonia indicating their advantage over other ammonia oxidizers under low ammonium conditions (48, 93). The comparison of growth rates of representative AOA, AOB and comammox strains showed that the growth rate of *Nitrospira* sp. BO4 were higher than the growth rates of AOA AC2 and AOB G5-7 at 0.01 mM and 0.025 mM ammonium (Figure 6).

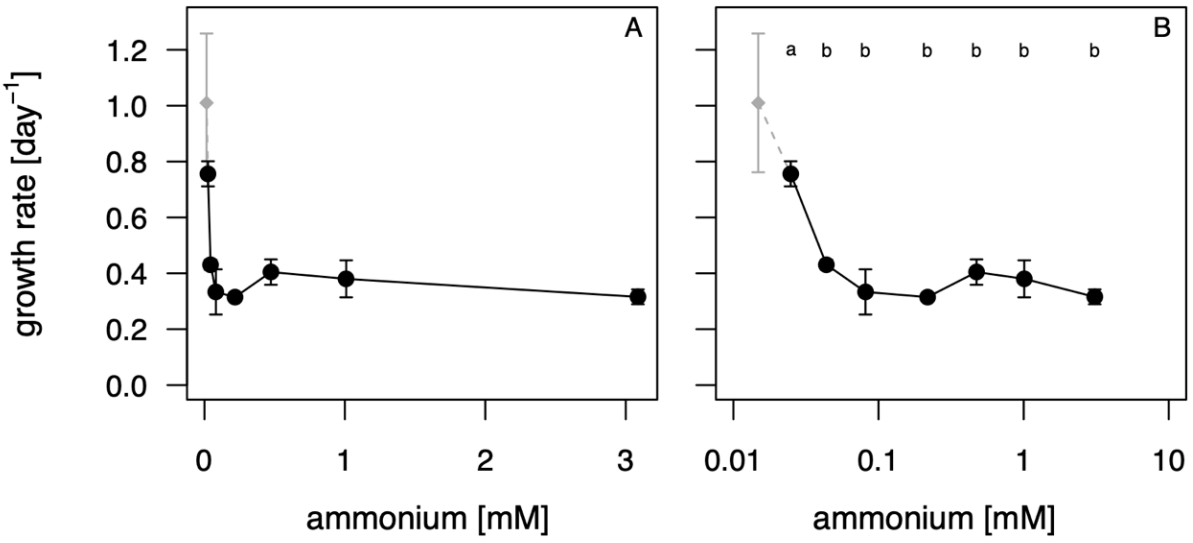

Figure 4. Influence of the ammonium concentration on the growth rate of *Nitrospira* sp. BO4 (means ± SD, n=3). Ammonium concentrations are shown on a linear scale (A) and a logarithmic scale (B). The growth rate at 0.01 mM ammonium was determined in a separate experiment, is shown in a different color than the other data and not included in the statistical analysis. Different letters above the symbols (B) indicate significant differences between values determined by one-way ANOVA followed by Tukey test ($P < 0.01$).

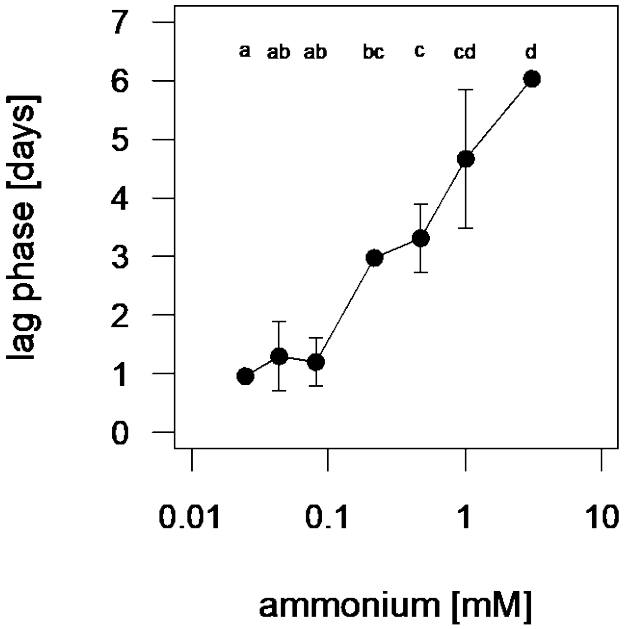

Figure 5. Influence of ammonium concentration [mM] on the lag phase [days] of the comammox strain *Nitrospira* sp. BO4 (mean ± SD, n=3-4). Different letters above the symbols indicate significant differences between values determined by one-way ANOVA followed by Tukey test ($P < 0.01$).

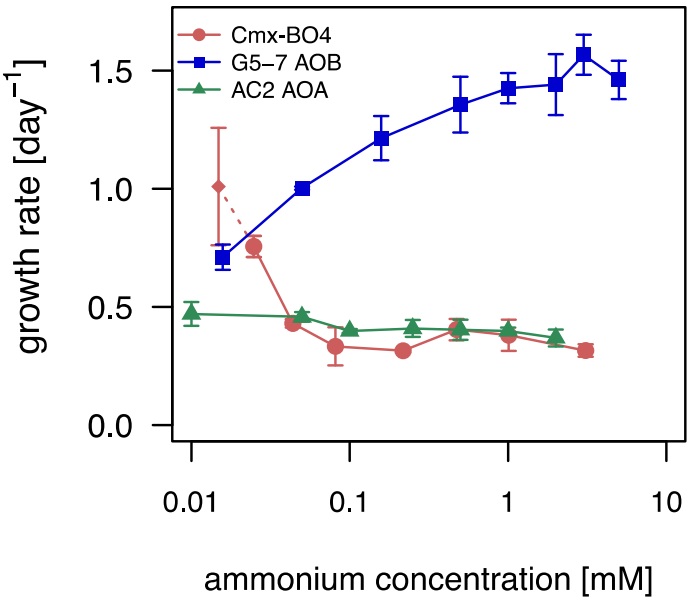

Figure 6. Influence of ammonium concentration [mM] on the growth rate [day-1] of the comammox strain *Nitrospira* sp. BO4, the AOA strain AC2 and the AOB strain G5-7 (mean ± SD, n=3-4). Ammonium concentration is shown on a logarithmic scale. Data point at 10 µM ammonium (BO4 comammox) was obtained in a separate experiment. (Data for G5-7 AOB and AC2 AOA were retrieved from French et al., 2012).

Nitrospira sp. B04 was enriched from the sediment of Lake Burr Oak. The average pH of samples taken from lake Burr Oak in the years 2007 and 2008 was 8.3 (130). Growth of *Nitrospira* sp. BO4 was investigated at different pH values with initial ammonium concentrations of 0.1 mM ammonium. The highest growth rate was measured at pH 7.2, and slightly lower growth rates were detected at pH 6.3, 7.5 and 8 (Figure 7). No growth was observed at pH 3-5 and pH 9. The growth rates of *Nitrospira* sp. BO4 were also measured in an initial ammonium concentration of 0.5 mM (Figure 8A) and compared to the growth rates of AOA AC2 and AOB G5-7 (Figure 8B). The growth rate of *Nitrospira* sp. BO4 was lower than that of the AOB G5-7, regardless of the pH (Figure 8B). However, at pH 6.5 and pH 8.5 the growth rate of *Nitrospira* sp. BO4 was higher than the growth rate of AOA AC2, while at a more neutral pH the growth rates of both cultures were almost the same. *Nitrospira* sp. BO4 is a non-acidophilic ammonia oxidizer and adapted to the ambient pH in its original environment of 8.3 (130, 131). This observation correlates with activity measurements showing that *Nitrospira inopinata* oxidizes ammonium with similar rates at pH values between 6.5 and 8.5 (54).

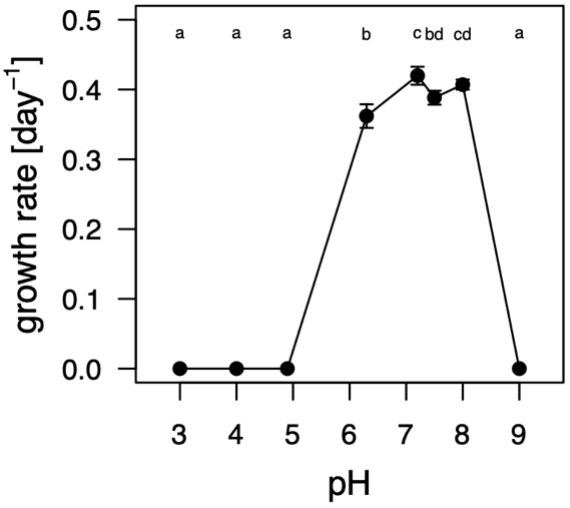

Figure 7. Influence of the initial pH on the growth rate of *Nitrospira* sp. BO4 (means ± SD, n=4). Different letters above the symbols indicate significant differences between values determined by one-way ANOVA followed by Tukey test ($P < 0.01$).

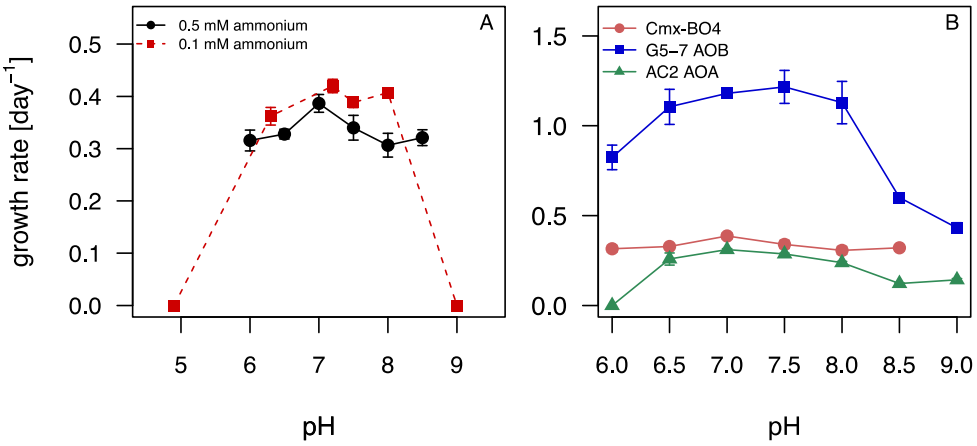

Figure 8. Influence of pH on the growth rate [day^{-1}] of ammonia oxidizers. (A) Comammox strain *Nitrospira* sp. BO4 incubated in the presence of 0.1 mM and 0.5 mM ammonium (mean ± SD, n=3-4). (B) Comammox strain *Nitrospira* sp. BO4, the AOA strain AC2 and the AOB strain G5-7 (mean ± SD, n=3-4). All cultures were incubated at 0.5 mM NH4+. (Data for G5-7 AOB = and AC2 AOA were retrieved from French et al., 2012 (45)).

Light is known to be an inhibiting factor of growth of ammonia oxidizers (85, 132). We investigated the impact of white, red, and blue light with an intensity of 30 µmol photons m^{-2} s^{-1} on the growth of *Nitrospira* sp. BO4. Blue light completely inhibited the growth of *Nitrospira* sp. BO4 (Figure 9A). In white light the growth rate of *Nitrospira* sp. BO4 was lower than the control (Figure *9*B) while no decrease of the growth rate was observed in red light (Figure 9C). This observation is similar to the results observed for the AOB-G5-7 and the AOA enrichment culture DW1 (45). The copper-containing ammonia monooxygenase of AOB is very sensitive to blue light through absorption of light by the oxygenated state of the enzyme (85, 132). It is very likely the ammonia monooxygenase of comammox *Nitrospira* is inhibited in a similar manner because the enzyme ammonia monooxygenase in AOB and comammox are very close related (9).

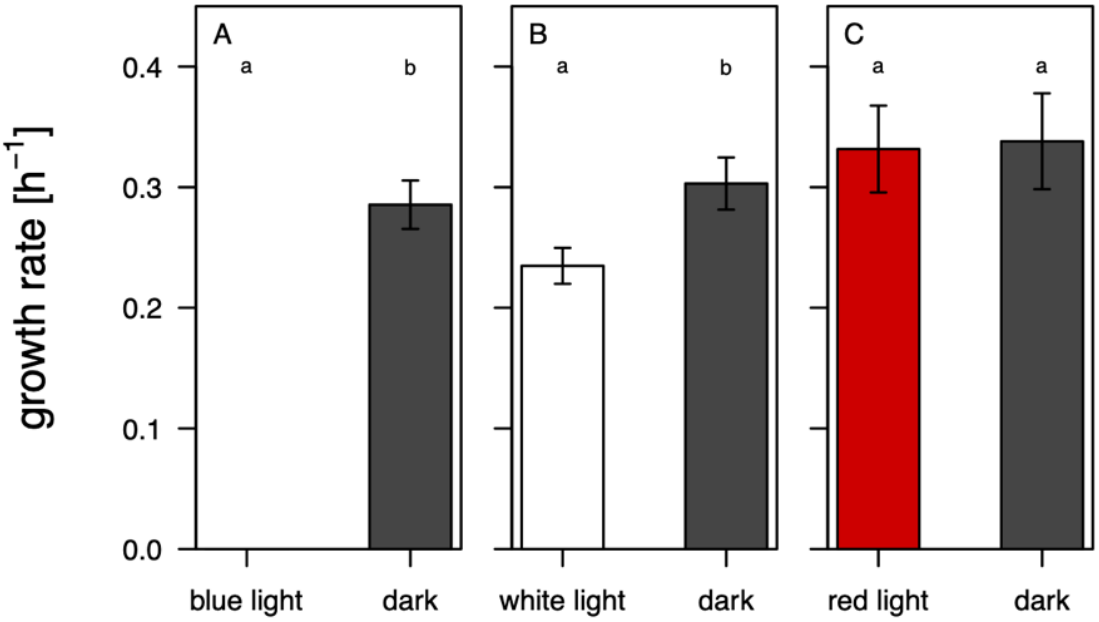

Figure 9. Influence of blue (470 ± 5nm) (A), white (5000-7000K) (B) and red (623 ± 3nm) (C) light on the growth rate of Nitrospira sp. BO4 (means ± SD, n=4). Different letters of figure above the bars indicate significant differences between values determined by one-way ANOVA followed by Tukey test ($P < 0.01$).

Complete ammonia oxidizers oxidize ammonia via nitrite to nitrate (9, 10). To test the influence of nitrite on the growth of *Nitrospira* sp. BO4, the culture was incubated in the presence of 0.5 mM ammonium alone, 0.5 mM nitrite alone and 0.5 mM of both compounds (Figure 10). The different media were inoculated with 10% (5 ml culture into 50 ml medium) of a late log phase culture. Ammonium alone was converted to nitrate with some nitrite being produced (Figure 10A). The ammonium consumption started after 5-6 days, and ammonium was completely consumed after around 3 weeks. When incubated in the presence of ammonium and nitrite, ammonium consumption started after 1-2 days of incubation and all ammonium was used up after 2 weeks (Figure 10B). Nitrite consumption started after 1 week of incubation and was slower than the ammonium consumption. Nitrite was not completely consumed after 45 days of incubation (Figure 10B). The nitrite concentration remained constant over 45 days when the cultures were incubated only with nitrite indicating that nitrite alone was not oxidized to nitrate (Figure 10C).

N. inopinata can oxidize nitrite when an actively nitrifying culture is transferred to nitrite-only medium but it is not able to sustain nitrite oxidation activity after subsequent addition of nitrite (9). The authors assume *N. inopinata* can initially oxidize the nitrite with the residual nitrite oxidoreductase enzyme activity. Eventually, however, growth and activity cease because *N. inopinata* is missing the genes for the assimilatory nitrite reductase and the nitrite transporter - two genes required for nitrite assimilation (9). *Nitrospira* sp. BO4 is also missing the assimilatory nitrite reductase and nitrite transporter for nitrite assimilation. In contrast to *N. inopinata*, *Nitrospira* sp. BO4 did not oxidize nitrite when inoculated into nitrite-only medium (Figure 10C). *Nitrospira* sp. BO4 was transferred in late-log phase and diluted 1:10 during inoculation (5 mL culture inoculated into 50 mL medium). We assume that the use of a diluted, late-log phase culture would diminish residual nitrite oxidoreductase activity, however, *Nitrospira* sp. BO4 continued to consume nitrite when incubated with ammonium and nitrite after the ammonium was completely consumed (Figure 10B). This nitrite consumption is very like due to residual activity of the nitrite oxidoreductase which is comparable to the nitrite oxidation observed by Daims et al (2015) (9) in *N. inopinata*. In summary, the experiments with *N. inopinata* and *Nitrospira* sp. BO4 were set-up differently, however, the observations for the two comammox strains were comparable. Both cultures were not able to assimilate and be metabolically active in the presence of nitrite as the only energy and nitrogen source.

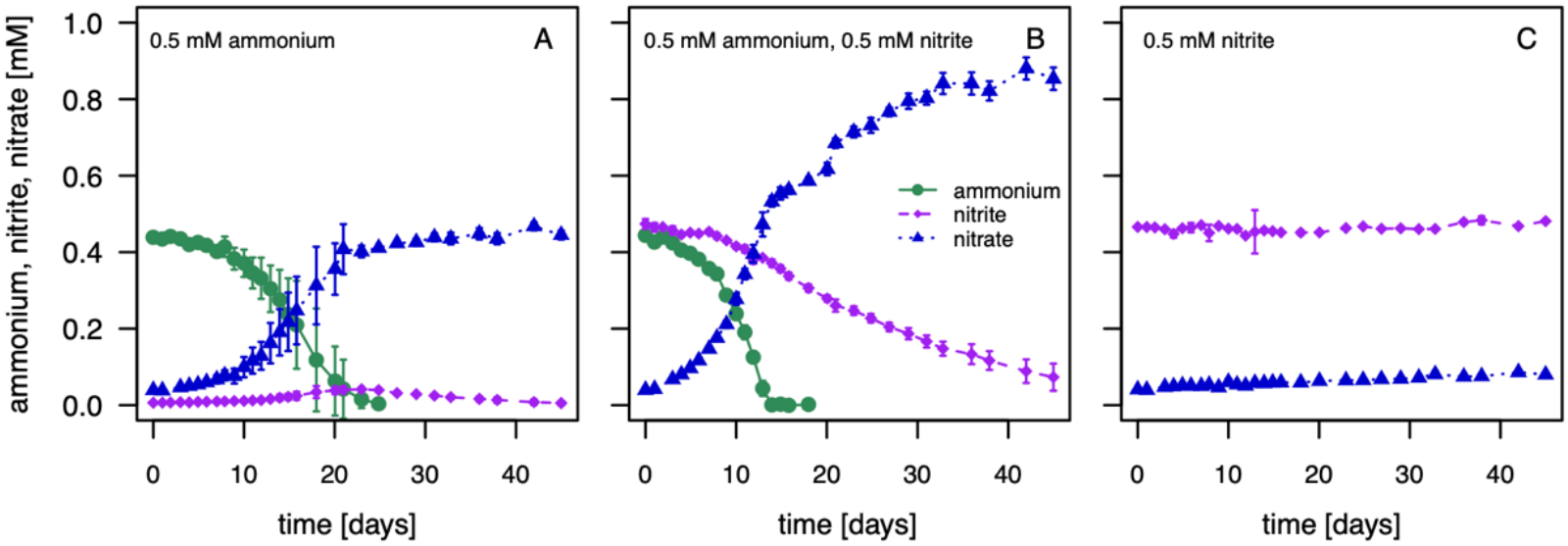

Figure 10. Conversion of ammonium and nitrite by *Nitrospira* sp. BO4 (means ± SD, n=4). Cultures were incubated in the presence of 500 µM ammonium (A), 500 µM ammonium and nitrite (B) and 500 µM nitrite (C).

In addition, we detected 20 - 30 µM of residual nitrite in our cultures. *N. inopinata* has a very low affinity to nitrite with a K_m of 372 ± 55 µM nitrite (48). If the affinity to nitrite of *Nitrospira* sp. BO4 is in the same order of magnitude, *Nitrospira* sp. BO4 might not be able to use nitrite below the measured concentrations. In contrast, *Ca.* Nitrospira kreftii, *Nitrospira nitrosa* and *Nitrospira nitrificans* show a higher affinity to nitrite (10, 93). This higher affinity is very likely due to the presence of canonical nitrite oxidizes in the cultures, while no canonical nitrite oxidizer was found in the culture *Nitrospira* sp. BO4 (Table 4).

CONCLUSIONS

To our knowledge *Nitrospira* sp. BO4 is the first described complete ammonia oxidizer from a freshwater environment. The growth rates under different environmental conditions were comparable to the growth rates of freshwater AOA and lower than the growth rates of freshwater AOB. Of the tested environmental factors, ammonium availability was the most important niche differentiating factor between the three different groups of ammonia oxidizers (Figure 4, Figure 6). High ammonium concentrations favored AOB, while lower ammonium concentrations favored AOA and even more comammox. This is in accordance with environmental surveys. Comammox have been found in a multitude of engineered systems such as drinking water treatment and distribution systems, wastewater treatment plants and aquaculture filtration systems (108, 109, 133–136). The ammonium availability in all these systems was generally low. *N. inopinata* and *Ca.* Nitrospira kreftii have a high affinity to ammonium enabling them to live under conditions with low ammonium availability (48, 93). In summary, these results indicate that complete ammonia oxidizers are well adapted to exist at low ammonium concentrations. Future research will investigate how freshwater comammox compete with AOA and AOB under nutrient limiting conditions for ammonium and which strains are most successful.

.

CHAPTER 2

Competition between ammonia-oxidizing Archaea and complete ammonia oxidizers from freshwater environments

Sabita Ghimire-Kafle, Matt E. Weaver Jr., Madisen P. Kimbrel and Annette Bollmann

We are planning to submit chapter 2 to the journal of *Applied and Environmental Microbiology*.

Author contributions:

MEW initiated experimental design with the help of AB and SG and contributed to lab work and data evaluation. MPK contributed to lab work. SG and AB contributed to lab work, data evaluation, figure representation and editing manuscript.

ABSTRACT

Aerobic ammonia oxidizers (AOs) are prokaryotic microorganisms which contribute to the global nitrogen cycle by performing the first step of nitrification. Nitrification is the oxidation of ammonium to nitrite and nitrate. While aerobic AOs are globally ubiquitous, their distribution is controlled by key environmental conditions, in particular, substrate (ammonium) availability. Ammonia-oxidizing bacteria (AOB) are generally adapted to higher ammonium concentrations while ammonia-oxidizing archaea (AOA) and complete ammonia oxidizers (comammox) are generally found in environments with lower ammonium availabilities. AOA and comammox are both found in oligotrophic environments but it is not understood how they interact within their microhabitat. We assessed competition for ammonium between an AOA and a comammox from freshwater Lake Burr Oak. The AOA enrichment culture BO1 contained *Nitrosoarchaeum* sp. as AOA (AOA-BO1) and a canonical NOB *Nitrospira* sp. (NOB-BO1). The comammox enrichment BO4 (Cmx-BO4) contained the comammox strains *Nitrospira* sp. BO4. Competition experiments were performed either in continuous cultivation with < 1 μM ammonium or in batch cultivation with 50 μM and 500 μM ammonium. Regardless of ammonium concentration, Cmx-BO4 outcompeted AOA-BO1 under all tested conditions. The dominance of Cmx-BO4 may be related the ability of comammox to yield more energy through the complete oxidation of ammonia to nitrate as well as their utilization of a more efficient carbon fixation pathway – the reductive TCA cycle. These predictions are supported by higher abundance (3.5-fold) of comammox relative to AOA in the sediments of Lake Burr Oak.

IMPORTANCE

Nitrification is a key process in the global nitrogen cycle. Aerobic ammonia oxidizers (AOs) play a central role in the nitrogen cycle by performing the first step of nitrification. Ammonia-oxidizing archaea (AOA) and complete ammonia oxidizers (comammox) are the dominant nitrifiers in environments with low ammonium availability. There is a lack of understanding on whether AOA and commamox truly coexist in low ammonium sediments which is necessary to understand the niches of AOA and comammox. Here, we present the first study characterizing a competition between freshwater AOA and comammox under varying substrate concentrations. Our results contribute to delineating the microniches of the key nitrifiers in oligotrophic freshwater sediments.

INTRODUCTION

Nitrification is a critical step of the nitrogen cycle linking the most reduced form of inorganic nitrogen – ammonia – with the most oxidized form – nitrate (6). Nitrifiers are prokaryotic chemolithoautotrophic microorganisms that derive their energy through oxidation of either ammonia to nitrite or nitrite to nitrate. In aerobic environments, nitrification is carried out by ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and complete ammonia oxidizers (comammox). AOA and AOB oxidize ammonia to nitrite only and NOB oxidize nitrite to nitrate only; however, comammox can catalyze both steps in one cell (7–10, 33, 34, 137). NOB and comammox oxidize nitrite to nitrate with the enzyme nitrite oxidoreductase (NXR) (9–11). Each group of the ammonia oxidizers has a phylogenetically distinct ammonia monooxygenase (AMO) that can be used as phylogenetic marker for ammonia oxidizers (9, 10, 44, 96). Unfortunately, the nitrite oxidoreductases of comammox and NOB cannot be phylogenetically distinguished and therefore not suitable to use as a phylogenetic marker to distinguish comammox and canonical nitrite oxidizers (9, 10, 135). Each group of ammonia oxidizers has a unique pathway to fix inorganic CO_2 for their carbon requirement. AOA, AOB and comammox fix CO_2 by 3-Hydroxypropionate pathway, Calvin-Benson-Bassham (CBB) cycle, and reductive Tricarboxylic cycle (rTCA), respectively (10, 11, 37, 39, 138).

Ammonia oxidizers are distributed ubiquitously in both terrestrial, aquatic and engineered ecosystems. The ecological niches of the different ammonia oxidizers are a function of a variety environmental factors such as ammonium concentration, pH, salinity, levels of organic matter and metals, humidity, nitrification inhibitors, light and temperature (45, 49, 139–152). Ammonium is the energy substrate of all three groups of ammonia oxidizers and each group exhibits different substrate affinity, which is a major driving factor for distribution of ammonia oxidizers. While most AOB have a rather low affinity to ammonium, AOA and comammox have a higher affinity to ammonium (9, 48, 93). AOB dominate high ammonium environments such as municipal and industrial wastewater treatment plants, eutrophic coastal environments, as well as freshwater and nutrient-rich soils in coherence with their low affinity AMO enzymes (146, 153, 154). Before the discovery of comammox, the dominant ammonia oxidizers in oligotrophic environments like the open ocean, oligotrophic freshwater environments and unfertilized natural soils were AOA (146, 155, 156). After the discovery of

comammox in 2015, comammox were reported to be the dominant ammonia oxidizers in some oligotrophic environments such as drinking water treatment plants (157), full scale waste water treatment plants (158), tertiary wastewater treatment plants (52, 159), forest soils (160) and agricultural soils (102). AOA and comammox have been detected together in several oligotrophic environments, including agricultural soils (102, 160), freshwater recirculating aquaculture systems (133) and subtropical forest soil (161).

Both AOA and comammox have a high affinity for ammonium and both groups of AO are detected in nutrient poor environments. The oligotrophic nature of AOA and comammox raises the question how AOA and comammox respond to limiting amounts of ammonium and if this response would result in competition or coexistence. Competition between different nitrifiers under limiting energy substrates have been investigated in the past. Such competition experiments have generally shown that the strains with the higher affinity for their substrate were more successful under substrate limiting conditions and the strains with the higher growth rates were more successful when the substrate was present in excess (113, 162–165). Here we investigate the competition between AOA and comammox under limiting (continuous culture) and excess ammonium (batch culture) conditions. We used two enrichment cultures from Lake Burr Oak in Ohio – Cmx-BO4 which contains the comammox strain *Nitrospira* sp. BO4 (49) and AOA-BO1 which contains the AOA strain *Nitrosoarchaeum* sp. BO1 and the canonical NOB *Nitrospira* sp. BO1 (Bollmann et al., in prep). The competition experiments between the two oligotrophic ammonia oxidizers will contribute to an understanding of the distribution of ammonia oxidizers in nutrient-limited environments.

MATERIALS AND METHODS

Medium

Mineral salts medium (MS) containing 10 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$. H_2O, 0.2 mM $MgSO_4.7H_2O$ and 1 ml per liter of trace elements solution was used for cultivation and competition experiments (115). For all experiments in batch cultures, HEPES was added as buffering agent. At ammonium concentrations of 0.1 mM and below, HEPES was added at a final concentration of 1 mM and at all other ammonium concentrations, the molar ratio of ammonium to HEPES was 1:4. Before autoclaving, pH of the MS medium was adjusted to 7.5-7.7 by adding 1M NaOH and after autoclaving, a sterile phosphate solution was added to obtain a final concentration of 0.4 mM KH_2PO_4.

Cultures

Two cultures were enriched from the sediment of mesotrophic Lake Burr Oak in Burr Oak State Park, Eastern Ohio. The comammox enrichment Cmx-BO4 contains *Nitrospira* sp. BO4, a comammox nitrospira that is closest related to *Candidatus* Nitrospira kreftii and *Nitrospira nitrificans* and was recently described (49).

The AOA enrichment AOA-BO1 contains ammonia-oxidizing archaeal strain *Nitrosoarchaeum* sp. BO1 and was enriched following the same protocol as used for the enrichment of other AOA (45, 113). AOA-BO1 is closest related to *Nitrosoarchaeum koreensis* MY1 (166). The enrichment culture AOA-BO1 also contains a canonical NOB from the genus *Nitrospira* sp (Bollmann et al., in prep). Both cultures were transferred every four to six weeks to fresh MS medium with 0.5 mM ammonium.

Sediment sample collection

Sediment samples (0.7 g) were taken from the top 1 cm of sediment cores from Lake Burr Oak (Kimbrel et al., in prep). Before sampling the cores were used for a three-day experiment to measure the denitrification in the sediments (167).

Growth experiments at different ammonium concentrations

Detecting growth of AOA and comammox at low ammonium concentrations (below 25 µM ammonium) can be difficult. To calculate growth rates at low ammonium concentrations, it is advantageous to observe 2 - 3 divisions of the microorganisms. In this experiment, we adjusted the cell density that would enable the culture to divide around three times before the substrate was fully consumed. The pre-culture for this experiment was grown in MS medium with 0.5 mM ammonium. The ammonium concentrations in the growth experiment ranged from 0.01 mM to 1 mM. Medium with 0.5 mM ammonium was inoculated with 10% of the pre-culture. The inoculum size for the other ammonium concentrations was adapted accordingly. For example, 0.05 mM ammonium was inoculated with 0.5 mL pre-culture in 50 ml of medium.

Nitrite and nitrate production by the ammonia oxidizers was monitored over time. Growth rates were calculated based on the assumption that the production of nitrite and nitrate is correlated to the growth of ammonia oxidizers (45, 49, 115, 117). The growth rates were calculated as slope of the log transformed nitrite and nitrate concentration over time. The slope was linear over several days and the R^2 was ≥ 0.98 and in most cases even ≥ 0.99.

Continuous-culture competition experiment

Continuous-culture competition experiments between AOA-BO1 and Cmx-BO4 were performed in chemostats (Eppendorf, Hamburg, Germany). Two chemostats of vessel volume 5 liters each were, filled with 3 liters of unbuffered MS medium with 0.5 mM ammonium and autoclaved. The temperature was set to 27°C, stirring to 50 rpm and the medium was bubbled with sterile air with a rate of 500 ml/min. The pH was adjusted to 7.3-7.7 using 2% (wt/vol) Na_2CO_3 solution. The chemostats were inoculated with 500 ml of late logarithmic cultures of AOA-BO1 and Cmx-BO4, respectively. Ammonium, nitrite and nitrate in the chemostats were measured daily. As the cultures started to consume ammonium, agitation was increased stepwise from 50 to 250 rpm and kept at 250 rpm throughout the experiment. When the cultures had consumed approximately 80% of the initial ammonium concentration, MS medium with 0.5 mM ammonium was added to the chemostats at a flow rate of 1.6 ml/min. The flow rate was decreased step wise to 0.5 ml/min as the starting flow rate was too high. After both of the cultures reached a steady state, 800 ml of each culture was removed from the each chemostat and inoculated into the other chemostat creating a 1:6 and 6:1 ratio of AOA-BO1 and Cmx-BO4 (vol/vol) in the two chemostats. Samples for molecular analysis were collected by filtration (0.1-μm polycarbonate filters, Sigma-Aldrich, St. Louis, MO) on a regular basis before and after mixing and stored at -80°C for further analysis. The two chemostats E1 and E2 were run 41 and 49 days after mixing, respectively. Chemostat culture competition experiment was performed to investigate competition for ammonium between AOA-BO1 and Cmx-BO4 at very low ammonium concentration (<10 μM).

Batch culture competition experiment

AOA-BO1 and Cmx-BO4 cultures were grown in MS medium with 0.5 mM ammonium until late logarithmic phase. The two cultures were mixed in a 1:1 ratio (vol/vol). Single cultures (AOA-BO1 and Cmx-BO4) and the mixed culture were inoculated into 50 mL of MS media. The inoculum size varied with initial ammonium concentration: MS medium with 0.05 mM ammonium was inoculated with 0.5 ml cultures and MS medium with 0.5 mM ammonium was inoculated with 5 ml culture. The cultures were incubated in the dark at 27°C and ammonium concentrations were monitored twice a week. After approximately 80% or more of ammonium was consumed in all cultures, 5 ml of the cultures was transferred into 50 ml fresh media with the same ammonium concentrations. The cultures were transferred three times. Samples were collected for molecular analysis after the second and fourth growth cycles by filtering the

cultures onto 0.1 µm polycarbonate membrane filters, which were stored at -80°C until molecular analysis. Batch culture competition experiment was performed to investigate the competition between AOA-BO1 and Cmx-BO4 at ammonium concentrations higher than in chemostat.

Spent media experiment

AOA-BO1 and Cmx-BO4 cultures were grown in 500 ml of MS medium with 0.5 mM ammonium at 27°C. Spent medium was produced by filtering the outgrown cultures through 0.1µm Millipore Express® PLUS Membrane Filters (Thomas Scientific, NJ, USA). The spent media from AOA-BO1 (SM_BO1) and Cmx-BO4 (SM_BO4) were supplemented with ammonium to reach a final concentration of 0.5 mM, the pH was readjusted to 7.5-7.7 and medium (45 ml) was distributed into 125-mL Erlenmeyer flasks. Mineral salts medium with 0.5 mM ammonium was used as control. Spent medium of each culture and the mineral salts medium controls were inoculated with 5 ml of late log phase culture of AOA-BO1 and Cmx-BO4, respectively. The cultures were incubated in the dark at 27°C and ammonium, nitrite and nitrate concentrations were measured regularly. Spent media experiment was performed to investigate if the AOA-BO1 and/or Cmx-BO4 secreted inhibitory or stimulatory substances in the media affect the growth of the other AO

Chemical analysis

Ammonium, nitrite and nitrate were measured using colorimetric assays (49, 115, 121).

Molecular analysis

DNA isolation. DNA was extracted from the filters and sediment using the Fast DNA spin kit for soil (MP Biomedicals, Solon, OH.

Quantitative PCR (qPCR). The *amoA* gene of Cmx-BO4 and AOA-BO1 was used to quantify the abundance of the ammonia oxidizers in the cultures and the AOA-*amoA*, AOB-*amoA* and comammox-*amoA* genes were used to quantify the ammonia oxidizers in the sediment samples (Table 11). The standards for the qPCR were prepared by cloning PCR products of the respective primer pairs.

The abundance of canonical NOB *Nitrospira* sp. in the enrichment culture AOA-BO1 was quantified using the cyanase gene (Table 11). The metagenome of AOA-BO1 showed that the genome of the canonical NOB *Nitrospira* sp. encoded the cyanase gene (Bollmann et al., in prep) while the comammox strain *Nitrospira* sp. BO4 doesn't encode a cyanase gene (49)

allowing differentiation of NOB from AOA-BO1 and Cmx-BO4 in mixed cultures. The standard for the cyanase gene was prepared by amplifying the gene with the primer cynF1 and cynR5, which resulted in a 310 bp PCR product. The PCR product was cleaned, quantified and used as standard for the qPCR. The newly designed primers cyn-F3 and cyn-R3 were used to quantify the cyanase gene. These primers amplify a162 bp PCR product from the standards. The qPCR reactions were performed using Bioline SensiFAST SYBR NoROX kit (Bioline USA, Taunton, MA, USA) in 5-µl reaction mixtures with 0.5-µl samples in an Illumina Eco real-time PCR system (Illumina, San Diego, CA, USA) with primers in the Table 11. The efficiencies of qPCR reactions ranged from 90-115% and the correlation coefficients were >0.98 in all reactions. At very low concentrations or in the complete absence of the template for each of the primer pairs, some unspecific amplification was observed. Melting curves showed different melting patterns for those PCR products compared to the standards and samples with high abundance of the targeted genes. Data from those samples with unspecific amplification were omitted.

Table 11. Primers used for quantification of ammonia and nitrite oxidizers in the DNA samples from the competition experiment.

Target gene	**Sequence**
AOA-BO1 *amoA*	BO1-F1 5'- GCT TTG CGT AGG TGC AAC AT -3' BO1-R1 5'- ACA AGT ACG CCT CCA AAC AGT -3'
Cmx-BO4 *amoA* (49)	BO4-F1 5'- CTG GTG CTG GAC ATC GTC TT -3' BO4-R12 5'- GTC CGA ACA TAC GCA AAG CC -3'
Cyanase of *Nitrospira* sp. BO1	cyn-F1 5'-AGA AGG TGA CGA TTG CCG AG -3' cyn-R5 5'-GGC TTC CGG TCA CTT TTT CC-3' cyn-F3 5'-CGA CGA AGC CAG AAA AGT CG-3' cyn-R3 5'-CTG TTC TCG CAT CGA ATC GC-3'
AOB *amoA* ((96)	amoA-1F, 5′-GGG GTT TCT ACT GGT GGT-3′ amoA-2R KS, 5′-CCC CTC KGS AAA GCC TTC TTC-3′
AOA *amoA* (44)	Arch amoA F, 5′-STA ATG GTC TGG CTT AGA CG-3′ Arch amoA R, 5′-GCG GCC ATC CAT CTG TAT GT-3′
Comammox *amoA* (168)	comamoA F (5′-AGGNGAYTGGGAYTTCTGG-3′) comamoA R (5′-CGGACAWABRTGAABCCCAT-3′)

RESULTS

Enrichment of AOA-BO1

AOA-BO1 was enriched from the sediment of Lake Burr Oak in MS medium with 0.25 mM ammonium as energy source and without the addition of carbon. The enrichment culture stoichiometrically oxidized ammonium to nitrate. Based on the sequence similarity of the *amoA* gene, the ammonia oxidizer in the AOA-BO1 enrichment culture is closest related to *Nitrosoarchaeum koreense* MY1 (98.76 % identity), an AOA isolated from garden soil (166). The culture also contains a nitrite oxidizer belonging to the genus *Nitrospira* and heterotrophic bacteria.

Influence of ammonium on the growth of AOA *Nitrosoarchaeum* sp. BO1 and Comammox *Nitrospira* sp. BO4

The growth of AOA-BO1 and Cmx-BO4 were characterized at different ammonium concentrations by cultivating both cultures in MS media with ammonium concentrations between 10 μM and 1 mM. This growth experiment has been conducted differently than previous growth experiments (45, 49, 113, 115) (material and methods section because the large inoculum size of the old experimental design made it often difficult to determine the growth rates at very low ammonium concentrations. The growth rates of AOA-BO1 were higher than those of Cmx-BO4 through 25- to 500 μM but were only significantly different at some of the ammonium concentrations (Figure 11). At 10 μM and 1000 μM ammonium AOA-BO1 and Cmx-BO4 grew at similar rates (Figure 11).

Growth experiments were performed to analyze if either of Cmx-BO4 or AOA-BO1 secreted stimulatory or inhibitory substances to the media that would influence the growth of the other ammonia oxidizer. AOA-BO1 and cmx-BO4 were grown in regular MS media (control) and spent medium of AOA-BO1 and Cmx-BO4, respectively. The ammonium consumption by AOA-BO1 and Cmx-BO4 in spent media was similar to the ammonium consumption in regular MS media (Figure 12, Figure 13) indicating that both cultures are not excreting substances that have an inhibitory or stimulatory effect on the other ammonia oxidizers.

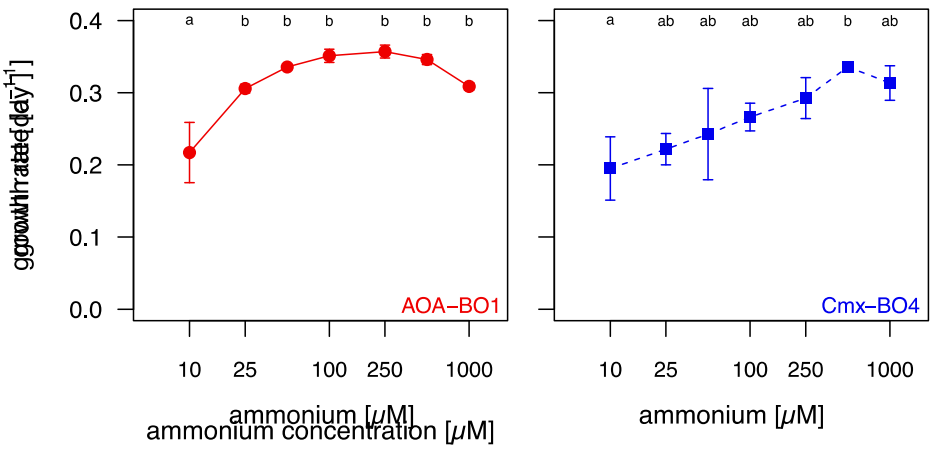

Figure 11. Growth rate of AOA-BO1 and Cmx-BO4 at different ammonium concentrations (average ± SD, n=3). Different letters above the symbols indicate significant differences between values determined by one-way ANOVA followed by the Tukey test (P <0.01).

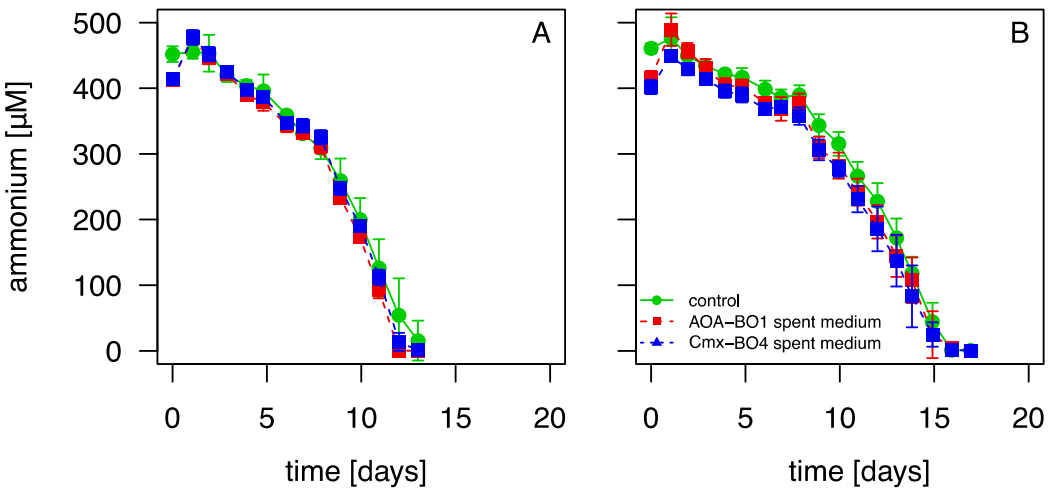

Figure 12. Ammonium consumption over time by AOA-BO1 (A) and Cmx-BO4 (B) cultures. The cultures were incubated in mineral salts medium (control) and in spent medium of AOA-BO1 and Cmx-BO4 (average ± SD; n=3).

Competition at intermediate and high ammonium concentrations

AOA-BO1 and Cmx-BO4 were cultivated as mixed batch cultures in MS medium with 50 and 500 μM ammonium to investigate their competition at intermediate and high ammonium concentrations. When the ammonium in the media was consumed, each of the cultures was transferred to fresh medium with the same ammonium concentration. Transfers were repeated three times. After the second and fourth growth cycles, the relative abundance of AOA-*amoA* and Cmx-*amoA* were quantified (Figure 13). Cmx-BO4 was dominant in all mixed cultures after 2 and 4 growth cycles in the presence of either 50 or 500 μM ammonium (Figure 13). AOA-*amoA* was only detected in 500 μM ammonium after 2 growth cycles, but its abundance was lower than that of Cmx-*amoA* by 5 orders of magnitude but does not cause competitive exclusion of AOA (Figure 16). Canonical NOB *Nitrospira* sp. (NOB-BO1) *cynase* was detected in the mixed cultures in low abundances around 1000 – 10000 copies/ml (Figure 13).

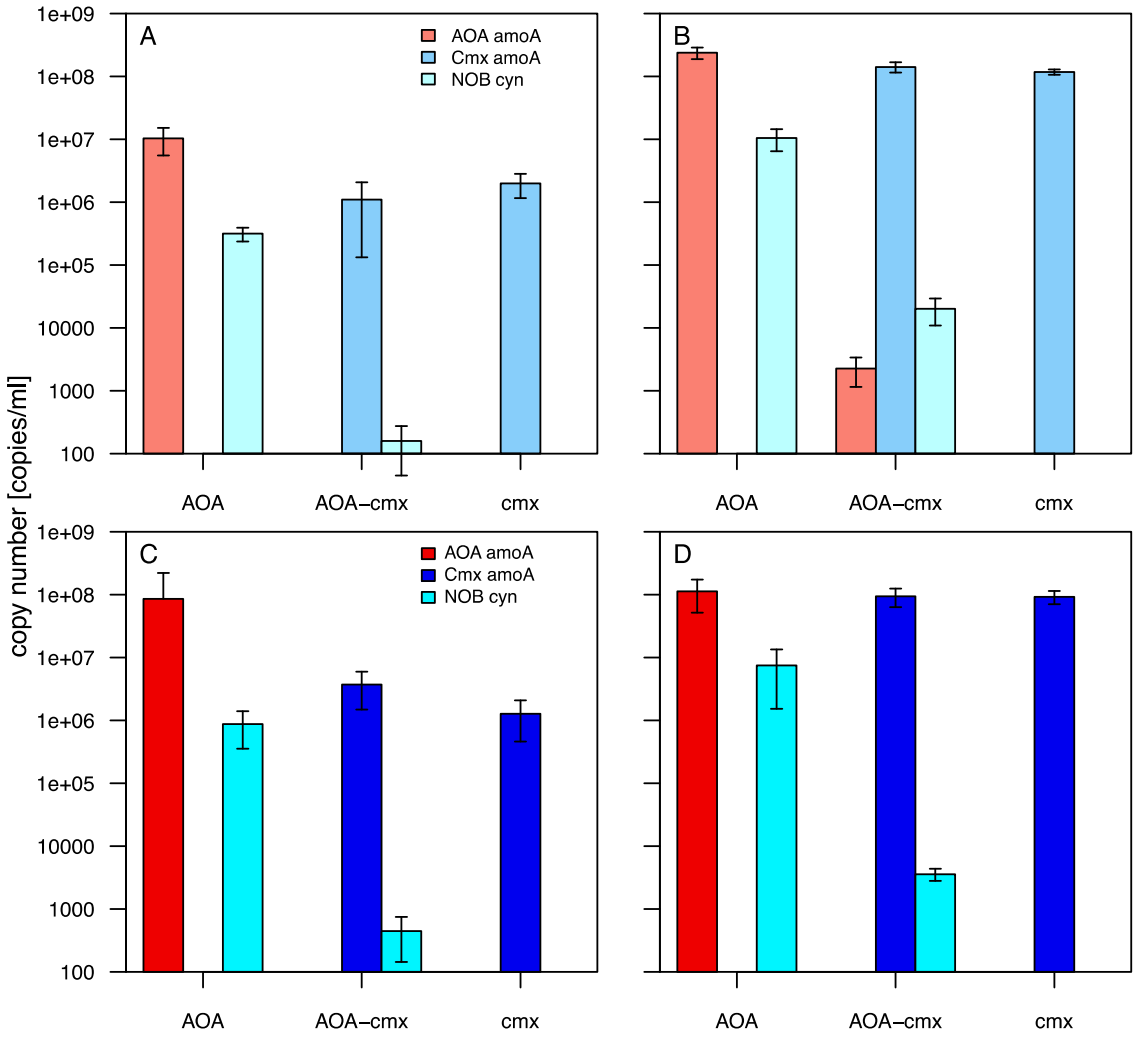

Figure 13. amoA of AOA (AOA-BO1), Comammox (Cmx-BO4) and cyanase (NOB-BO1) gene abundance in batch culture competition experiments at initial ammonium concentrations of 50 µM (A and C) and 500 µM (B and D) after 2 (A and B) and 4 (C and D) growth cycles (average ± SD, n=5).

Competition between AOA and comammox under limiting ammonium concentrations

Continuous cultures were used to conduct competition experiments with ammonium as growth-limiting substrate. Chemostats were inoculated with AOA-BO1 (Chemostat E1) and cmx-BO4 (Chemostat E2), respectively Figure 14, Figure 15). AOA-BO1 consumed the provided ammonium within 15 days (Figure 14A). When the pumps were initially started at a rate of 1.2 ml/min, ammonium accumulated indicating that AOA-BO1 was not able to grow as fast as the dilution rate in the chemostat. The dilution rate was decreased to 0.5 ml/min and AOA-BO1 completely oxidized ammonium to nitrate with no detectable nitrite (Figure 14A, Figure 15). Under the same conditions, in chemostat E2, cmx-BO4 oxidized ammonium to nitrate with some residual nitrite (Figure 14B, Figure 15B). Cmx-BO4 needed around 20 days until most of the ammonium was consumed. When the pumps were started at a flow rate of 0.8 ml/min ammonium addition, the ammonium concentration increased indicating that the growth rate was too high for Cmx-BO4. The dilution rate was decreased to 0.5 ml/min and Cmx-BO4 converted most of the ammonium to nitrate (Figure 14B), with 30 – 50 µM of nitrite remaining.

After 8 weeks, both chemostats had reached steady state conditions and the cultures were mixed by transferring 800 ml from one chemostat to the other chemostat. Ammonium consumption in chemostat E1 which was initially inoculated with AOA-BO1 stopped after the addition of cmx-BO4 (Figure 14, Figure 15). The pumps were stopped for 15 days until the cultures had consumed the ammonium completely. On day 15 the pumps were started at 0.1 ml/min and the flowrate was increased over the next 10 days to 0.45 ml/min. Ammonium was consumed and completely converted to nitrate without any detectable nitrite. There was no interruption of the ammonium consumption in chemostat E2 after mixing the cultures. Prior to mixing, around 30 µM nitrite was detectable in chemostat E2 which was consumed within 10 days after mixing and no nitrite was no longer detectable (Figure 14, Figure 15).

Prior to mixing, the abundance of the AOA-BO1 *amoA* gene was approximately 10^9 copies/ml in chemostat E1 while the abundance of the cmx-BO4 *amoA* gene in chemostat E2 was around 10^8 copies/ml. The NOB-BO1 *cyn* gene was detected with an abundance of 10^7 copies/ml in chemostat E1 showing that AOA-BO1 was growing in a co-culture with NOB-BO1. No NOB *cyn* genes were detected in cmx-BO4 (49). Mixing AOA-BO1 and cmx-BO4 resulted in the presence of all three organisms in both chemostats: AOA-BO1, cmx-BO4 and NOB-BO1.

In chemostat E1 the AOA-BO1 *amoA* abundance was approximately 10^9 copies/ml after mixing the cultures. The AOA-BO1 *amoA* abundance decreased to 10^5 copies/ml at the end of the experiment after 49 days. The cmx-BO4 *amoA* abundance in the chemostat E1 was 10^7 copies/ml directly after mixing and decreased to 10^4 copies/ml within the first few days after mixing. At the same time the ammonium concentration increased indicating that the ammonia oxidizers were inactive. Therefore, the pumps were stopped until day 15. Over time ammonium consumption started again and the abundance of cmx-BO4 increased. When the pumps were started again the abundance of cmx-BO4 increased over time while the abundance of AOA-BO1 decreased. At the end of the experiment cmx-BO4 was more abundant than AOA-BO1. The abundance of the NOB-BO1 *cyn* gene decreased to around 10^7 to 10^5 copies/ml but increased again when ammonium consumption started again. At the end of the experiment the abundance of NOB-BO1 was around 10^4 copies/ml.

In chemostat E2 the abundance of the cmx-BO4 *amoA* gene decreased to 10^7 copies/ml and stayed stable for the rest of the experiment (Figure 15D). The abundance of the AOA-BO1 *amoA* gene decreased rapidly and at the end of the experiment, where roughly 1000 copies/ml were detectable. The abundance of the NOB-BO1 *cyn* gene decreased to around 10^5 copies and remained stable until the end of the experiment (Figure 15D). At the same time the nitrite in the culture was completely consumed (Figure 15B).

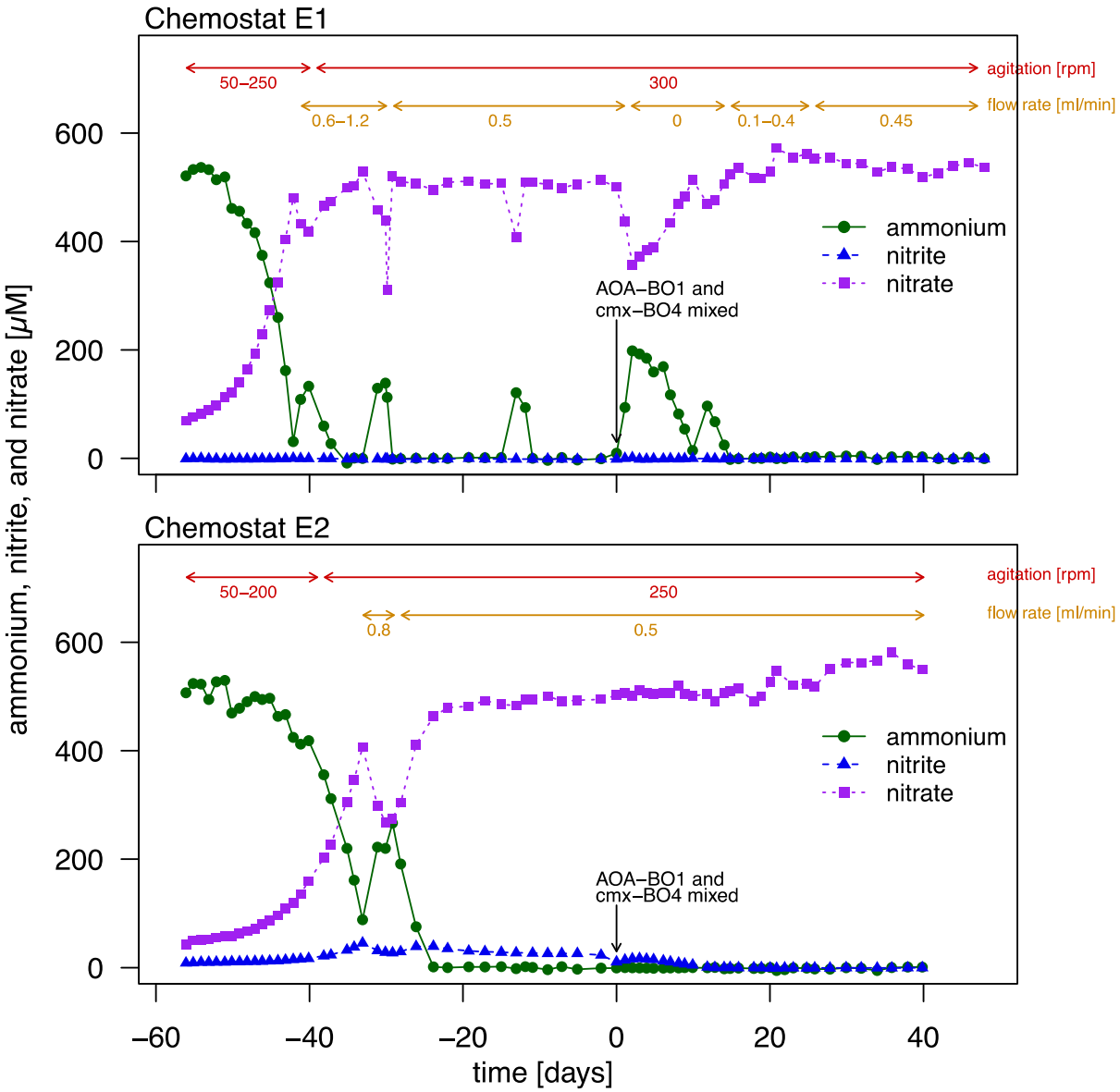

Figure 14. Ammonium, nitrite and nitrate over time in the chemostats. Chemostat E1 was inoculated with the culture AOA-BO1 and chemostat E2 with the culture cmx-BO4. The cultures were started as batch cultures and the pumps were started when the ammonium was almost completely consumed. The cultures were mixed at time point 0.

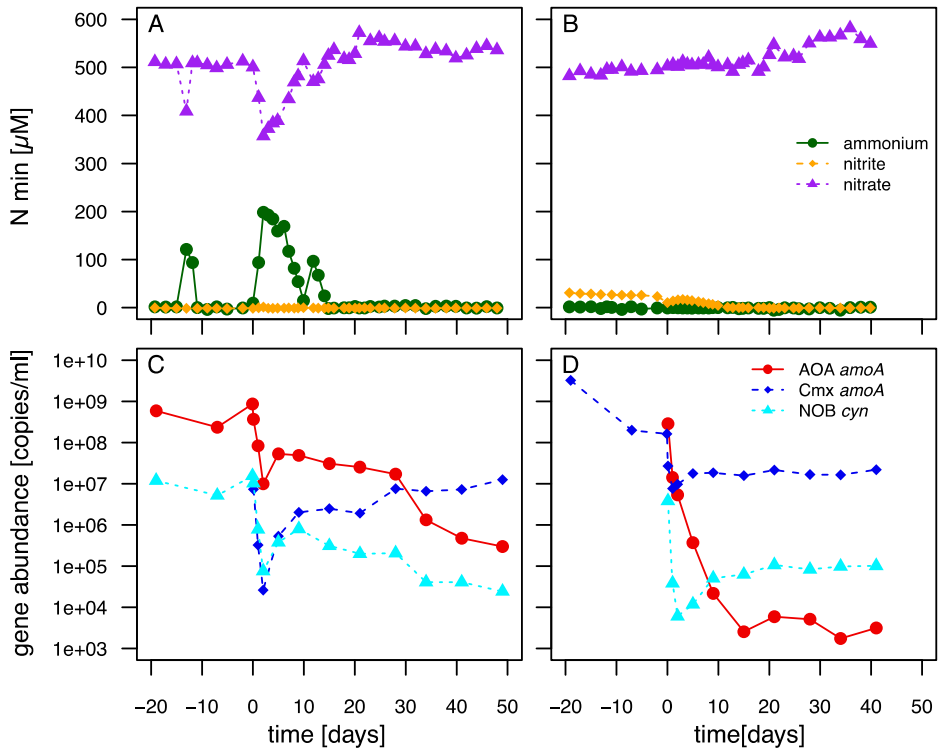

Figure 15. Ammonium, nitrite, and nitrate concentrations (A and B) and AOA-BO1 and cmx-BO4 *amoA* and NOB-BO1 *cyn* gene abundance (C and D) during competition between AOA-BO1 and cmx-BO4 for limiting ammonium. Chemostat E1 was inoculated with AOA-BO1 and chemostat E2 with cmx-BO4. The cultures were grown as single cultures and mixed on day 0.

Biofilms in chemostats

Long-term experiments with ammonia oxidizers in chemostats often result in the formation of biofilms on the glass walls of the chemostats (165). Biofilms were detected and harvested in chemostat E1 at the air-liquid interface, in the middle of the vessel and at the bottom of the vessel and at the bottom of the vessel of chemostat E2. Abundances of AOA-BO1, cmx-BO4 and NOB-BO1 in the biofilm samples were determined. However, the absolute numbers of the three nitrifiers had no real meaning because they could not be related to volume or area. Therefore, the ratio between mx-BO4 and AOA-BO1 was calculated to determine if the same ammonia oxidizer was dominant in the biofilm and in the culture liquid (Figure 16B). In chemostat E1, AOA-BO1 was dominant in the biofilm at the bottom of the chemostat, while in the middle and at the top Cmx-BO4 was dominant. In chemostat E2 cmx-BO4 are also dominant in the biofilm. The ratios between Cmx-BO4 and AOA-BO1 were always lower in the biofilm than in the culture liquid (Figure 16) indicating that the proportion of AOA-BO1 was higher in the biofilms than in the liquid culture. AOA may be able to avoid competition with comammox by forming a biofilm on the walls of chemostat.

Comammox vs canonical nitrite oxidizers

After mixing the cultures in the chemostats and in batch culture for the competition experiments, all cultures contained cmx-BO4 and NOB-BO1, two strains that can oxidize nitrite. The ratios of the cmx-*amoA* to cyanase were calculated to get an insight into the possible contribution of cmx-BO4 and NOB-BO1 to nitrite oxidation (Figure 17). The highest ratios between cmx-BO4 and NOB-BO1 were observed in the batch cultures (Figure 17C) while the lowest ratio was detected in the biofilms (Figure 17B). The chemostats had around 10-50 times lower cmx-*amoA* to cyanase ratio than the batch incubations, indicating that NOB-BO1 contributed more to the nitrite oxidation in the chemostats than in the batch cultures (Figure 17A, Figure 17C).

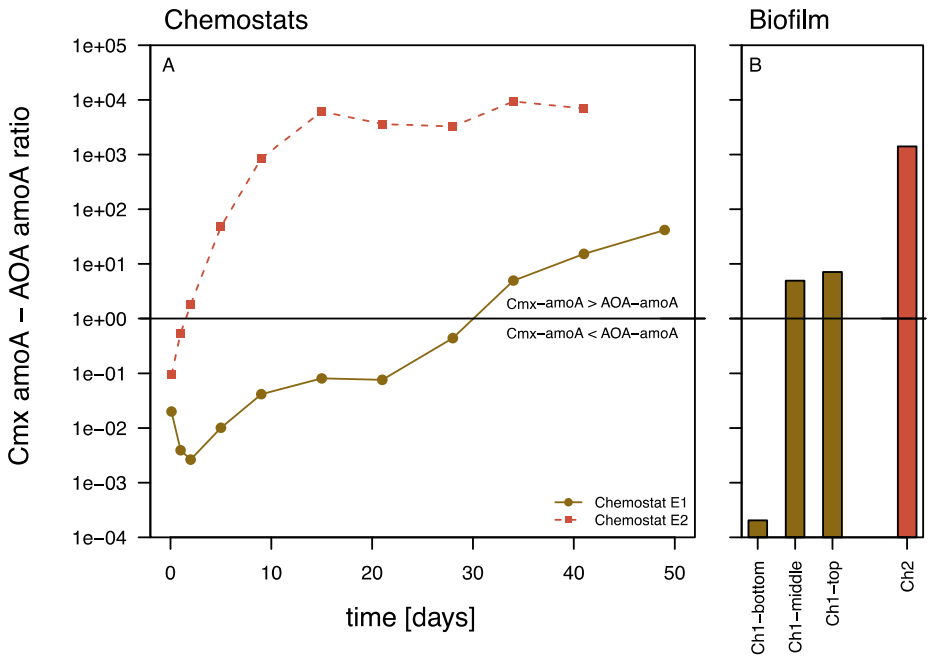

Figure 16. Cmx-BO4 *amoA* to AOA-BO1 *amoA* ratio in chemostat E1 and E2 (A) and in the biofilms in the chemostats (B).

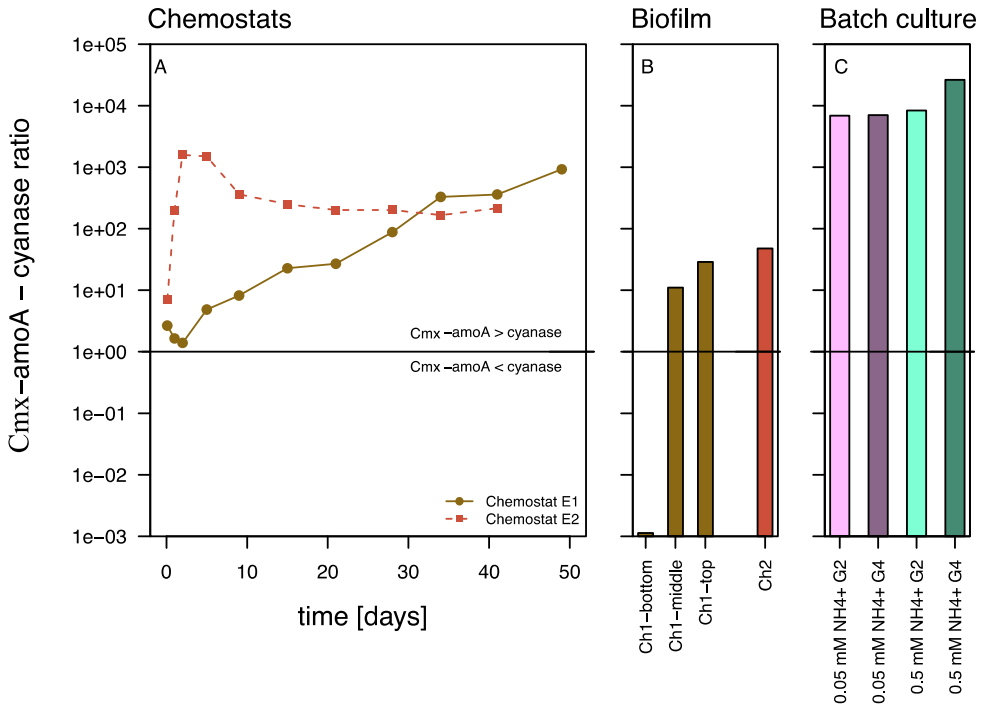

Figure 17. Cmx-BO4 *amoA* to NOB-BO1 cyanase ratio in chemostat E1 and E2 (A), in the biofilms in the chemostats (B) and in batch culture (C).

Abundance of ammonia oxidizers in the sediment of Lake Burr Oak

The abundance of AOA, AOB and comammox was determined in the sediment of Lake Burr Oak as part of a project analyzing the microbial community of two freshwater reservoirs in Ohio (Kimbrel et al, unpublished). Comammox was the most abundant, while AOA were the least abundant ammonia oxidizer in the sediment of Lake Burr Oak (Figure 18).

DISCUSSION

When the enrichment cultures AOA-BO1 and cmx-BO4 were cultivated as mixed cultures competing for ammonium in chemostats and in batch cultures, the abundance of *Nitrospira* sp. BO4 was several orders of magnitude higher than the abundance of *Nitrosoarchaeum* sp. BO1 under all tested conditions (Figure 13, Figure 15). This observation indicates that *Nitrospira* sp. BO4 can outcompete *Nitrosoarchaeum* sp. BO1 under limiting and elevated ammonium concentrations. Interestingly, *Nitrosoarchaeum* sp. BO1 grew faster than *Nitrospira* sp. BO4 in growth experiments determining the growth rate at different ammonium concentrations (Figure 11).

Competition experiments between different nitrifiers were often described by r and K selection or oligotrophic and copiotrophic lifestyles, respectively (113, 162, 163, 165). r and k selection theory states that under nutrient-limited condition, a slow growing species with higher affinity to substrate outcompetes the faster growing species with a lower affinity to the substrate (169). Factors that can determine if competing microorganisms would be classified as r- and K-strategists or oligo- and copiotrophs include but are not limited to affinity to the substrate and growth rate (169, 170). AOA and comammox generally have a higher affinity to ammonium than AOB (48, 93). *Nitrosoarchaeum* sp. BO1 is closest related to *Nitrosoarchaeum koreensis* MY1, which has a K_m for ammonia of 5 nM NH_3 (54). The K_m for ammonia of *N. inopinata* is 30-40 nM NH_3 and 40 nM NH_3 for *Candidatus* Nitrospira kreftii (48, 54, 93). Based on these literature data the affinity of the AOA used in this study was almost one order of magnitude higher than the affinity of comammox. This observation indicates an advantage for comammox in this competition experiment. The growth rates of *Nitrosoarchaeum* sp. BO1 and *Nitrospira* sp. BO4 at 50 µM ammonium (elevated ammonium concentrations) were not significantly different, but the growth rate of *Nitrosoarchaeum* sp. BO1 was higher than the growth rate of *Nitrospira* sp. BO4 (Figure 11). At 500 µM ammonium both cultures had the same growth rate (Figure 11).

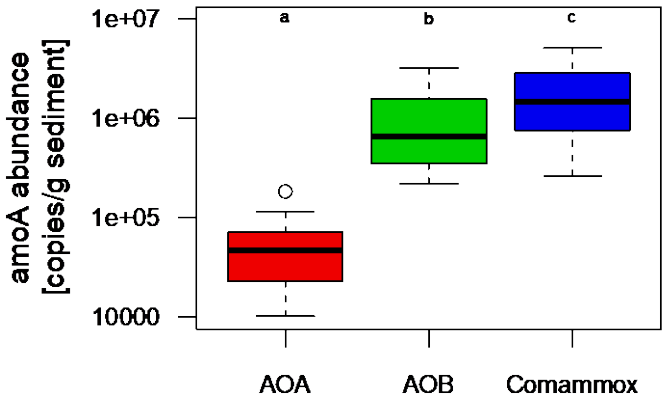

Figure 18. AOA, AOB and cmx *amoA* gene abundance (copies/g sediment) in Lake Burr Oak. Samples were taken in June, August and November 2016 at the inflow, the middle, and the outflow of the Lake (n=15-18). All data for each ammonia oxidizers were summarized to give a general insight into the abundance of the different microorganisms. Different letters indicate significant differences determined by one-way ANOVA followed by Tukey test ($P < 0.01$).

Therefore, the main characteristics that were used in the past to determine if a microorganism belonged to r- or K-strategists or the oligotrophic/copiotrophic group were contradictory to the outcome of to the competition between *Nitrosoarchaeum* sp. BO1 and *Nitrospira* sp. BO4. These observations raise the question what physiological advantages *Nitrospira* sp. BO4 has over *Nitrosoarchaeum* sp. BO1 enabling the former to outcompete the latter.

Nitrospira sp. BO4 yields more energy per molecule of ammonia than *Nitrosoarchaeum* sp. BO1 because comammox yields energy from complete oxidation of ammonium to nitrate through nitrite ($\Delta G^{0'}$ = -348.9 kJ/mol) while AOA oxidize ammonia to nitrite only ($\Delta G^{0'}$ = -274.7 kJ/mol) (8–10, 38, 92). Kinetic theory predicts that comammox would have a lower maximum growth rate than other ammonia oxidizers but a higher yield due to the additional step in the oxidation of ammonium to nitrate (92). *Nitrospira* sp. BO4 and *Nitrosoarchaeum* sp. BO1 have different carbon fixation pathways, namely the reductive citric acid cycle (reductive TCA) and 3-hydroxypropionate pathway, respectively (39, 49). The reductive TCA cycle requires 2 Ferredoxin, 3 NAD(P)H and 2 ATP while 3-hydroxypropionate cycle requires 7 NAD(P)H and 9 ATP to fix 3 CO_2/HCO_3^- (171, 172). The ability to gain more energy per molecule ammonia and the lower energy requirements for carbon fixation of comammox could be important factors to explain the dominance of *Nitrospira* sp. BO4 under ammonia limited and elevated conditions.

Nitrospira sp. BO4 was dominant in most of the biofilms, apart from the biofilm in chemostat E1 at the bottom of the vessel. While *Nitrospira* sp. BO4 took over both chemostats, *Nitrosoarchaeum* sp. BO1 decreased slower in chemostat E1 than in chemostat E2 (Figure 15C, Figure 15D). This slower decrease in chemostat E1 could be due to constant feeding of *Nitrosoarchaeum* sp. BO1 from the biofilm into the culture liquid. Future experiments will focus on the impact of surfaces for attachment on the outcome of competition experiments between different ammonia oxidizers.

The comammox have been reported to be the most abundant AO in several environments (52, 157, 158). The freshwater lake Burr Oak sediment samples also has comammox as the most abundant AO. The abundance of comammox is approximately 3.5-fold higher than that of AOA (Figure 18). This suggests that the result from this in vitro competition experiment may apply in vivo in the natural environment as well. A higher abundance of comammox have been observed in oligotrophic environments like agricultural soil and biofilter sand samples (102, 133). The

acidic subtropical forest soil, in contrast had a higher abundance of AOA than comammox, however the pH of the soil could have affected the distribution of ammonia oxidizers (161). These observations indicate that comammox are the more robust AO in oligotrophic environments in non-acidic environments.

Before mixing, the NOB *Nitrospira* sp. BO1 was only detected in the enrichment culture AOA-BO1 (Figure 13). Cmx-BO4 did not contain a canonical nitrite oxidizer (49) (Figure 13, Figure 15C). The abundance of *Nitrospira* sp. BO1 decreased after mixing of the two enrichment cultures AOA-BO1 and cmx-BO4 in the chemostats and in batch culture (Figure 13, Figure 15C, Figure 15D). In a co-culture with AOA or AOB, NOB can use all the nitrite produced by the ammonia oxidizer as energy substrate (7, 11). The comammox strain *Nitrospira* sp. BO4 only produces a very small amount of nitrite while most of the ammonia is oxidized to nitrate (49) (Figure 15B). Therefore, the abundance of *Nitrospira* sp. BO1 decreased when the comammox strain *Nitrospira* sp. BO4 outcompeted *Nitrosoarchaeum* sp. BO1 in the different competition experiments. The NOB *Nitrospira* sp. BO1 was able to use the low levels of nitrite present in the enrichment culture Cmx-BO4 because canonical nitrite oxidizers have a higher affinity to nitrite than comammox strains (48, 93).

The ratio between the copy number of the *Nitrospira* sp. BO4 *amoA* gene and the *Nitrospira* sp. BO1 cyanase gene was higher in batch cultures than in continuous cultures indicating that the relative abundance of *Nitrospira* sp. BO1 is higher in the continuous culture than in batch culture (Figure 17). In contrast, in the chemostat competition experiment, nitrite is produced constantly by *Nitrospira* sp. BO4 (Figure 15B) resulting in a constant supply of nitrite to *Nitrospira* sp. BO1 and an equilibrium between the comammox and the canonical nitrite oxidizer in the chemostats (Figure 15, Figure 17). This relationship and its impact on the nitrite oxidation by comammox and canonical nitrite oxidizers will be investigated in more detail in the future.

CONCLUSION

Comammox *Nitrospira* sp. BO4 outcompeted AOA *Nitrosoarchaeum* sp. BO1 in competition experiments at low and elevated ammonium concentrations. *Nitrospira* sp. has a higher yield from ammonia oxidation than *Nitrosoarchaeum* sp. BO1 because of the ability of the former to perform complete oxidation of ammonia to nitrate through nitrite while the latter only oxidizes ammonia to nitrite. Comammox *Nitrospira* sp. BO4 also has energetically more

efficient carbon fixation pathway, reductive TCA cycle, compared to 3-hydroxypropionate pathway in *Nitrosoarchaeum* sp. Hence, comammox may be the dominant ammonia oxidizers in Lake Burr Oak which is supported by higher abundance of commamox compared to AOA in the sediments of Lake Burr Oak. Comammox are also the dominant AO in several other oligotrophic environments. In summary, comammox may be playing the major ecological function of ammnonia-oxidation in oligotrophic environments. Future research will investigate the role of nitrite oxidizers in oligotrophic environments.

Chapter 3

Underlying mechanism of high ammonium-toxicity in ammonia-oxidizing bacteria.

Sabita Ghimire and Annette Bollmann

ABSTRACT

Ammonium in elevated concentrations is toxic to all domains of life. High ammonium toxicity is also observed in AO as indicated by a decrease in the growth rate of AO with increasing ammonium concentration. However, the mechanism of ammonium toxicity in AO is not yet understood. In mammalian cells, there is a correlation between high ammonium and an increase in oxidative and nitrosative stress. We investigated the underlying mechanism of high ammonium toxicity in ammonia-oxidizing bacteria (AOB) *Nitrosomonas* sp., high-ammonium toxicity of which is environmentally relevant in eutrophic environments. *Nitrosomonas* spp., within cluster 6a (eg: *Nitrosomonas* sp. Is79) are less tolerant to ammonium than *Nitrosomonas* cluster 7 (eg: *N. eutropha)*. Tolerance of *Nitrosomonas* sp. Is79 to ammonium concentration can be enhanced by co-cultivation with the heterotroph *Pseudomonas* sp. H19 in a coculture. Here, we investigate i) the effect of high ammonium on *Nitrosomonas* sp. Is79 and *N. eutropha* and ii) the role of heterotroph *Pseudomonas* sp. H19 on relieving ammonium toxicity on *Nitrosomonas* sp. Is79. Through growth experiments, we determined ammonium was toxic to *N. eutropha* and *Nitrosomonas* sp. Is79 at 400 mM and 10 mM respectively. To study the response of *Nitrosomonas* sp. to high ammonium, cluster 6a *Nitrosomonas* sp. Is79 (pure and a coculture) and *N. eutropha* were cultivated at low, intermediate and high ammonium concentrations and their gene expression will be assessed through RNA-seq.

SIGNIFICANCE

Ammonia oxidizers (AO) are chemolithoautotrophs that generate energy through oxidation of ammonia to nitrite and/or nitrate. Of the aerobic ammonia oxidizers, ammonia-oxidizing bacteria (AOB) is adapted to grow at high ammonium e.g., in eutrophic environments while ammonia-oxidizing archaea (AOA) and complete ammonia oxidizers (comammox) are adapted to grow at low ammonium e.g., in oligotrophic environments. Understanding high ammonium toxicity in AO helps predict the distribution of AO in an environment with known ammonium concentrations.

INTRODUCTION

All forms of life require nitrogen. Autotrophs like plants, algae and bacteria fulfil their nitrogen requirement by assimilating inorganic forms of nitrogen like ammonium or nitrate. When both ammonium and nitrate are present, plants prefer ammonium over nitrate because uptake and assimilation of ammonium requires less energy than the usage of nitrate as nitrogen source (173). Most heterotrophs acquire nitrogen in organic forms such as amino acids and nucleotides (18–20).

Above certain concentrations, ammonium has been recognized to be toxic to all domains of life including plants and algae (174–177). High ammonium causes stunting and chlorosis in plants (57). An increase of the ammonium chloride (NH_4Cl) concentration in media from 50 to 100 μM decreased the growth rates of oeanic phytoplankton (178). High ammonium in human serum causes irreversible brain damage in early childhood leading to cognitive impairment, seizures and cerebral palsy (58). Ammonium toxicity was first linked to an uncoupling effect in phosphorylation eliminating ATP synthesis by abolishing proton gradient across chloroplast and mitochondria (179), however, this theory was later disproved (180). Recent studies on the toxicity of ammonium in plants and mammalian cells have shown a correlation of ammonium toxicity with increased oxidative and nitrosative stress (59–61). Oxidative stress is a condition when a biological system loses a balance between production of reactive oxygen species (ROS) and the ability of the system to detoxify the ROS (181). ROS – superoxide, hydrogen peroxide and hydroxyl radicals – are highly reactive molecules which cause harm to cells and tissues by oxidizing biomolecules like amino acids and lipids and causing cell damage or death (182–184). Superoxide (O_2^-) molecules combine with nitric oxide (NO) to form Reactive Nitrogen Species (RNS) peroxynitrite anion ($ONOO^-$). Peroxynitrite anion are potent RNS causing nitration of several biomolecules such as proteins, lipids, and DNA, resulting in lipid peroxidation, DNA strand breaks, cell membrane damage, enzyme inactivation and activation of signal cascades of cell death (185).

Ammonia-oxidizing bacteria (AOB) are chemo-lithotrophic microorganisms that utilize ammonium as an electron donor to generate energy as well as assimilate ammonium to biomass. High ammonium concentrations are toxic to AOB despite their ability to oxidize ammonium as supported by a decrease in growth rate of AOB at high ammonium concentrations (62). All aerobic AO: ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA) and

complete ammonia oxidizer (comammox) show a similar phenomenon - they can only oxidize ammonium within a certain concentration range. Within the AOB, the genus *Nitrosomonas* is divided into different clusters (94). Members of cluster *Nitrosomonas* cluster 7 (relatives of *Nitrosomonas eutropha*) are more tolerant to increasing ammonium concentrations, than members of Nitrosomonas cluster 6a (relatives of *Nitrosomonas oligotropha*) (47, 62). *N. eutropha* has the highest growth rates at ammonium concentrations ≥ 5 mM while *Nitrosomonas* sp. Is79 has highest growth rates at ≤ 1 mM ammonium (62). It is not understood what mechanism causes high ammonium toxicity in AOB and why some strains of *Nitrosomonas* sp. are more tolerant to ammonium than the others.

Presence of specific heterotrophic bacteria improve the growth rate of AOB and decrease the half-saturation constant of growth (K_s) of the AOB (186). Specifically, *Pseudomonas* sp. H19 increased the growth rates of the AOB *Nitrosomonas* sp. Is79 by 60%. Proteins involved in oxidative stress response were also reduced in *Nitrosomonas* sp. Is79 by the presence of the heterotrophic bacteria indicating a role of the heterotroph in overcoming oxidative stress (186). Recent publications indicated a correlation between oxidative stress and ammonium toxicity (59–61). There is a knowledge gap if the heterotroph *Pseudomonas* sp. H19 helps reduce oxidative and nitrosative stress caused by high ammonium in *Nitrosomonas* sp. Is79.

Here, we assessed if high ammonium concentration toxicity in AOB is correlated to an increase in oxidative and nitrosative stress, as seen in other life systems (59–61). We also investigated why some strains of *Nitrosomonas* sp. are more tolerant of ammonium than others and if heterotrophic bacteria alleviate oxidative and nitrosative stress associated with high ammonium in AOB. To answer these research questions, we grew the AOB *N. eutropha*, *Nitrosomonas* sp. Is79 in pure culture and N*itrosomonas* sp. Is79 in a co-culture with *Pseudomonas* sp. Is19 at different ammonium concentrations and will sequence the transcriptome. We will compare the differential expression of all genes, with a focus on genes related to oxidative and nitrosative stress to investigate the effect of high ammonium on the gene expression of AOB. We will also analyze the effect of the heterotrophic bacterium in the co-culture to determine if heterotrophic bacteria change the effect of high ammonium concentrations on the physiology of *Nitrosomonas* sp. Is79.

MATERIALS AND METHODS

Medium

Mineral salts medium (MS) containing 10 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$. H_2O, 0.2 mM $MgSO_4.7H_2O$ and 1 ml per liter of trace elements solution was used for cultivation and competition experiments (115). For growth experiments, pH of media was buffered by adding 10 mM and 20 mM HEPES buffer for 2.5- and 10 mM ammonia oxidation by *Nitrosomonas* sp. Is79 (pure and coculture) and *N. eutropha* respectively (115). Before autoclaving, the pH of the MS medium was adjusted to 7.5-7.7 with 1M NaOH and after autoclaving, separately autoclaved phosphate solution was added to obtain a final concentration of 0.4 mM KH_2PO_4.

Cultures

Nitrosomonas eutropha C91, originally isolated from sewage disposal plant in Chicago, IL, USA (Koops et al., 1991), is a eutrophic AOB. The genomic features of *N. eutropha* have been characterized by Stein et al., (2007). A pure culture of *Nitrosomonas* sp. Is79 and coculture of *Nitrosomonas* sp. Is79 + *Pseudomonas* sp. Is19 were obtained from an ammonia-oxidizing enrichment from the sediment of Lake Drontermeer (The Netherlands) (187, 188). All three cultures were transferred every 3-4 weeks to fresh MS media with 5 mM ammonium.

Growth experiments

The growth of *N. eutropha*, *Nitrosomonas* sp. Is79 (pure) and *Nitrosomonas* sp. Is79 + *Pseudomonas* sp. Is19 (coculture) at different ammonium concentrations was screened to determine the threshold of toxic ammonium concentration for the respective culture. The growth experiments were performed in MS media with different ammonium concentrations buffered with HEPES at pH 7.5-7.7 in a ratio of 1:4 for below 10 mM ammonium and 40 mM HEPES for all higher ammonium concentrations.

Nitrite production by the ammonia oxidizers was measured throughout the growth cycle and growth rates were calculated based on the assumption that the production of nitrite and nitrate is correlated to the growth of ammonia oxidizers (45, 49, 115, 117). Nitrite and nitrate concentrations were ln transformed (natural log) and plotted against time. The growth rates were calculated as the slope of the log transformed data over time. The slope was linear over several days and the R^2 was ≥ 0.98 and in most cases ≥ 0.99.

The ammonium concentration at which the growth rate of an AO culture decreased compared to the tested lower ammonium concentration was defined as the threshold for toxicity to the respective AO culture. Based on the determined toxic ammonium concentration, low, intermediate and high ammonium concentrations were selected to study the effect of ammonium

concentrations on gene regulation. Cultures were also cultivated with nitrite and methyl viologen as controls for nitrosative and oxidative stress respectively to evaluate if there is a correlation between high ammonium toxicity and increase in oxidative and nitrosative stress. The conditions of cultivation are comprehensively listed in Table 12. Ammonium and nitrite concentrations and pH of media were monitored regularly. The ammonium concentrations were maintained stable by replenishing it after 15-25% of the ammonium was depleted and the pH of media was maintained at 7.5 ± 0.2 by adding NaOH. Ammonia (NH_3) and ammonium (NH_4^+) exist in a pH dependent equilibrium with a pKs value of 9.25 and maintaining pH is important to keep ammonia and ammonium concentrations reproducible (63).

Chemical analysis

Ammonium and nitrite were measured using colorimetric assays (115).

RNA isolation

After the *N. eutropha* and *Nitrosomonas* sp. Is79 (pure and coculture) cultures had consumed ammonium to produce 5- and 2.5 mM nitrite, respectively, cell biomass was collected through 0.22-µm membrane filter. The filter was suspended in RLT buffer (Qiagen, Valencia, CA, USA) + DTT, homogenized and RNA extracted immediately. If RNA was not extracted immediately, the cell homogenate in RLT buffer + DTT was stored at -80°C.

RNA was isolated from biomass of cultures incubated at all conditions in Table 12 using Qiagen RNeasy Mini Kit (Valencia, CA, USA) with following modifications. The membrane filters with the biomass were suspended in 700 µL of RLT buffer with (40 µL of 1M DTT in 1 mL RLT buffer) and homogenized in a BioSpec Mini-Beadbeater-24 (BioSpec Products, Inc., Bartlesville, OK, USA) at 4,800 rpm for 30 s. After bead beating, the samples were treated following manufacturer's recommendation. The samples were kept on ice in between treatments and RNA was eluted in 40 µL RNase-free water.

Table 12. Conditions of cultivation of *N. eutropha*, *Nitrosomonas* sp. Is79 (pure), and *Nitrosomonas* sp. Is79 + *Pseudomonas* sp. Is19 (coculture).

Culture	**Conditions**	**NH_4^+ [mM]**	**NO_2^- [mM]**	**Additional supplements**
N. eutropha	Low ammonium	5		
	Intermediate ammonium	100		
	High ammonium	400		
	Control for nitrosative stress	5	5	Sodium nitrite as nitrite source
	Control for oxidative stress	5		Methyl viologen as ROS source
***Nitrosomonas* sp. Is79**	Low ammonium	1		
	Intermediate ammonium	5		
	High ammonium	10		
	High ammonium	20		
	Control for nitrosative stress	1	2.5	Sodium nitrite as nitrite source
	Control for oxidative stress	1		Methyl viologen as ROS producer
***Nitrosomonas* sp. Is79 + *Pseudomonas* sp. H19**	Low ammonium	1		
	Intermediate ammonium High ammonium	5 10		

The isolated RNA samples were DNase-treated, cleaned, and concentrated using a ZYMO RESEARCH RNA Clean & ConcentratorTM-5 kit following the manufacturer's manual selecting for total RNA (Zymo Research, Irvine, USA). Highly concentrated DNase-free RNA samples were eluted 35 µL of RNase-free water. The quality of RNA samples was monitored by performing agarose gel electrophoresis in 1% non-denaturing agarose gel in 1x TAE. RNA samples were quantified using Qubit™ RNA HS assay (Thermo Fisher Scientific, Waltham, MA).

RESULTS

Effect of ammonium on the growth and activity of *Nitrosomonas sp.* Is79 and *N. eutropha*

To investigate the effect of different ammonium concentrations on growth and activity of AOB *Nitrosomonas* sp., growth experiments were performed with *N. eutropha* and *Nitrosomonas* sp. Is79 at different ammonium concentrations. The growth and ammonia oxidation activity of the cultures at different ammonium were investigated by calculating growth rates and lag phase of growth. Of the tested ammonium concentration range of 1 mM to 70 mM ammonium, *Nitrosomonas* sp. Is79 oxidized ammonium to nitrite at up to 50 mM ammonium (Figure 19). At 1- and 5 mM ammonium, all of the ammonium was oxidized to nitrite. At 20 mM ammonium and above, the nitrite production was inhibited after some nitrite was produced (Figure 19). For example, at 30 mM ammonium, nitrite production was 0.5-fold lower than that in 5 mM ammonium medium. Nitrite production further decreased at higher ammonium concentrations while there was no nitrite produced at 70 mM ammonium (Figure 19). Effect of ammonium on *N. eutropha* were tested at 1- to 500 mM ammonium concentrations. *N. eutropha* oxidized ammonium, producing nitrite, in all the tested ammonium concentrations (Figure 20). Of the tested ammonium concentrations, nitrite production by *N. eutropha* plateaued at 100 mM around 6 mM and the nitrite production decreased with increasing ammonium concentrations (Figure 20). At 400 mM ammonium, *N. eutropha* produced approximately 0.5-fold lower nitrite than at 50 mM ammonium (Figure 20).

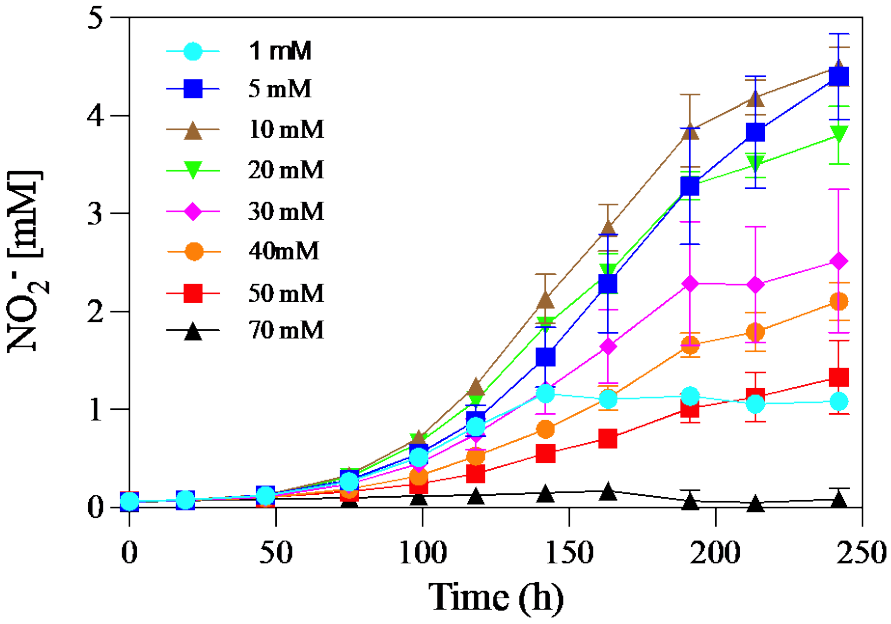

Figure 19. Nitrite production by *Nitrosomonas* sp. Is79 at different ammonium concentrations (mean ± SD, n=3).

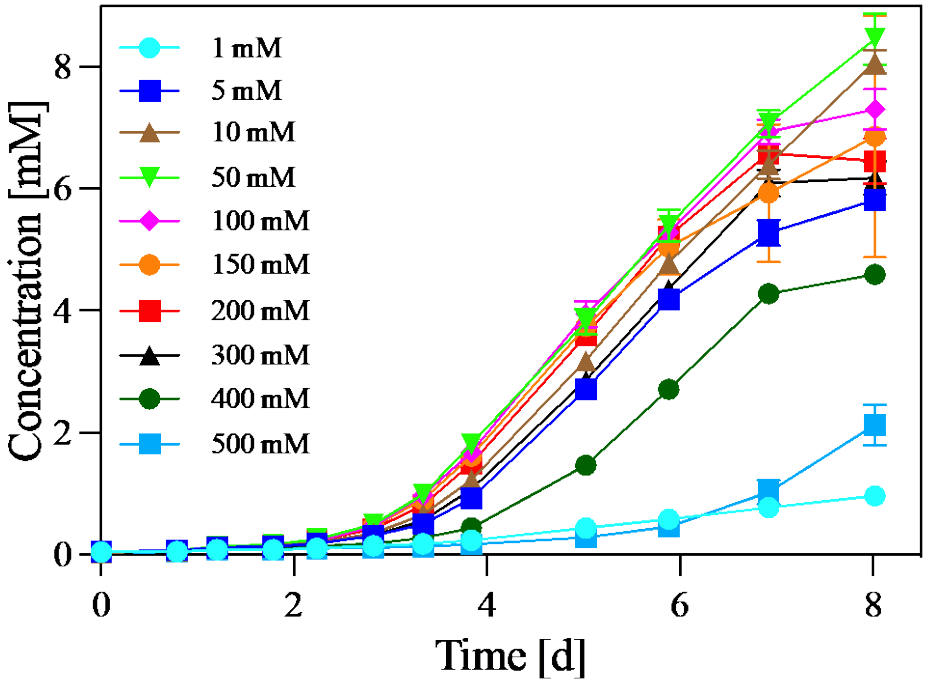

Figure 20. Nitrite production by *Nitrosomonas eutropha* at different ammonium concentrations (mean ± SD, n=3).

Both AOB strains followed Monod kinetics i.e., the growth rates increased with increasing in ammonium concentration until a high ammonium threshold was reached, above which ammonium was inhibitory to their growth (Figure 21). The maximum growth rate of *Nitrosomonas* sp. Is79 occurred at 10 mM ammonium and declined from 20- to 40 mM ammonium. There was some nitrite production activity at 50 mM ammonium, but the absence of logarithmic nitrite production indicated no growth. Both growth and activity of *Nitrosomonas* sp. Is79 culture were completely inhibited at 70 mM ammonium (Figure 21). *N. eutropha* had the highest growth rate at 100 mM ammonium and the growth rate decreased by 0.67-fold at 400 mM ammonium. *N. eutropha* had some nitrite production but no growth at 500 mM ammonium.

Cultivation of *N. eutropha* at low, intermediate and high ammonium to study gene expression

Low, intermediate and high ammonium concentrations to study gene regulation in *N. eutropha* were selected based on the growth experiment testing the effects of different ammonium concentrations on growth (Figure 20). *N. eutropha* was cultivated at 5-, 100- and 400 mM ammonium to study gene expression at low, intermediate and high ammonium concentrations respectively. *N. eutropha* was also cultivated with 100 mM in the presence of 2 μM methyl viologen or 5 mM nitrite as controls for oxidative and nitrosative stress, respectively (Table 12). Methyl viologen produces reactive oxygen species (ROS) causing oxidative stress to cells (189). Nitrite was *N. eutropha* oxidized ammonium at all tested conditions (Figure 22). Of the low, intermediate, and high ammonium conditions, the growth rate of *N. eutropha* was the lowest at 400 mM ammonium (Figure 22B). The nitrite production at 400 mM was delayed by 2 days compared to 5- and 100 mM ammonium reflecting a longer lag phase at 400 mM ammonium compared to other tested ammonium concentrations (Figure 22A).

The growth rate of *N. eutropha* was approximately 0.36-fold lower at 100 mM ammonium compared with 100 mM ammonium in presence of 5 mM nitrite added. *N. eutropha* grew at a similar rate at 100 mM ammonium with or without a 2 μM methyl viologen background (Figure 22).

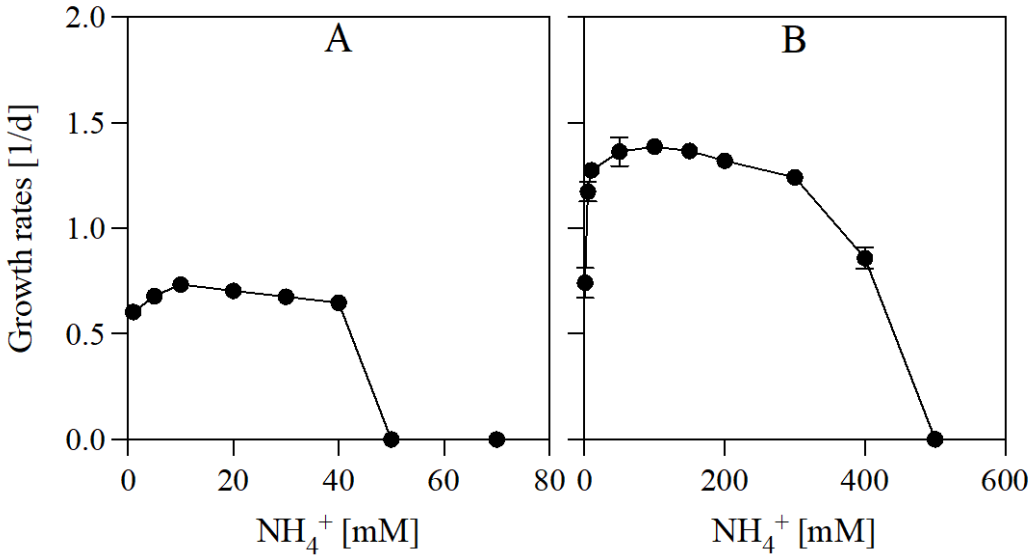

Figure 21. Nitrite production rates of (A) *Nitrosomonas* sp. Is79 and (B) *N. eutropha* at different ammonium concentrations (mean ± SD, n=3).

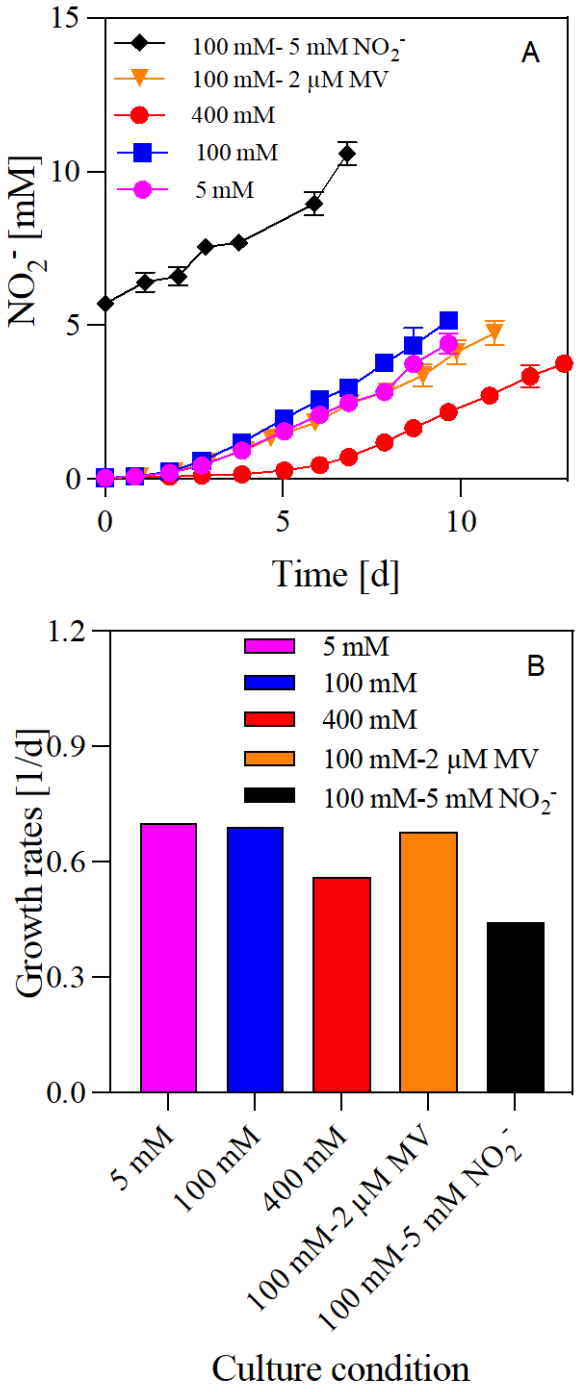

Figure 22. (A) Nitrite production by *N. eutropha* at 5-, 100-, 400 mM ammonium only, 100 mM ammonium + 0.2 µM MV and 100 mM ammonium + 5 mM nitrite (mean ± SD, n=4) (B) Ammonia-oxidation rates of *N. eutropha* at 5-, 100-, 400 mM ammonium only, 100 mM ammonium + 0.2 µM MV and 100 mM ammonium + 5 mM nitrite (mean ± SD, n=4)

Cultivation of *Nitrosomonas* sp. Is79 at low, intermediate and high ammonium to study gene expression

Low, intermediate and high ammonium concentration to study gene regulation in *Nitrosomonas* sp. Is79 were selected based on the growth experiment (Figure 19). 10 mM was selected as the high ammonium because at and above 20 mM ammonium, the culture did not produce 5 mM nitrite in a logarithmic fashion (Figure 19). Hence, *Nitrosomonas* sp. Is79 was cultivated at 1-, 5- and 10 mM ammonium to study gene expression at low, intermediate and high ammonium concentrations respectively. *Nitrosomonas* sp. Is79 was also cultivated at 5 mM ammonium in the presence of 0.2 μM methyl viologen and 2.5 mM nitrite as controls for oxidative and nitrosative stress, respectively (Table 12). *Nitrosomonas* sp. Is79 oxidized ammonium at all tested conditions (Figure 23A). The growth rate of *Nitrosomonas* sp. Is79 was 0.75-fold at 1 mM ammonium compared with the growth rates at 5- and 10 mM ammonium (Figure 23).

The presence of 2.5 mM nitrite in 5 mM ammonium resulted in a 0.25-fold reduction in the growth rate of *Nitrosomonas* sp. Is79 compared to cultures grown in 5 mM ammonium without the 2.5 mM nitrite supplement (Figure 23B). The growth rate of *Nitrosomonas* sp. Is79 at 5 mM ammonium only was similar to the growth rate of cultures grown in the presence of 5 mM ammonium and 0.2 μM methyl viologen (Figure 23B).

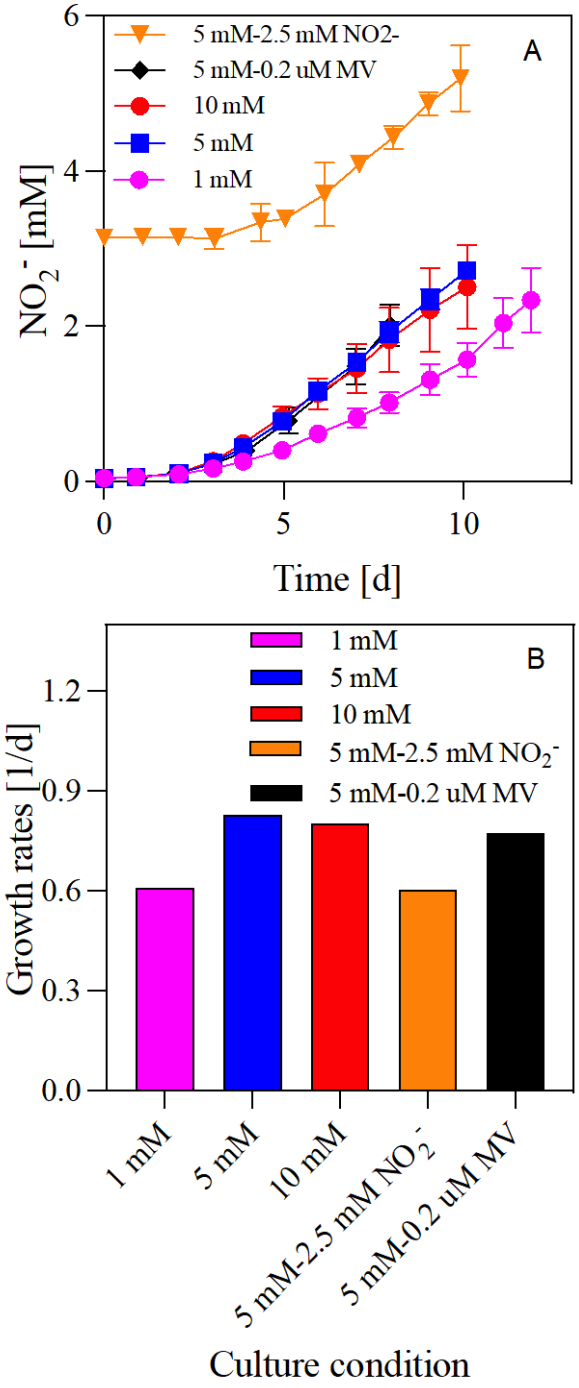

Figure 23. Ammonia-oxidation activity of *Nitrosomonas* sp. Is79 at low, intermediate and high ammonium: (A) Nitrite production by *Nitrosomonas* sp. Is79 at 1-, 5-, 10 mM ammonium only, 5 mM ammonium + 0.2 µM MV (mean ± SD, n=4), (B) Ammonia-oxidation rates of *Nitrosomonas* sp. Is79 at 1-, 5-, 10 mM ammonium only, 5 mM ammonium + 0.2 µM MV (mean ± SD, n=4).

Cultivation of *Nitrosomonas* sp. Is79 + *Pseudomonas* sp. H19 at low, intermediate and high ammonium to study gene expression

Nitrosomonas sp. Is79 + *Pseudomonas* sp. coculture was cultivated at 1-, 5- and 10 mM ammonium to study gene regulation at low, intermediate and high ammonium concentrations and compare them to the pure culture of *Nitrosomonas* sp. Is79. The coculture oxidized ammonium at all three ammonium conditions (Figure 24). The growth rate of *Nitrosomonas* sp. Is79 + *Pseudomonas* sp. coculture was similar at low, intermediate and high ammonium concentration (Figure 24B). Surprisingly, the growth rate of the coculture was higher at 1 mM ammonium and similar at 5- and 10 mM ammonium compared to the pure culture of *Nitrosomonas* sp. Is79 (Figure 24).

DISCUSSION

Ammonium impacts the growth of *Nitrosomonas* sp. negatively at high concentrations even though it is essential as an energy substrate for these chemolithotrophic bacterium. Ammonium is one of the factors affecting the distribution of AO in the environment. Understanding the ammonium toxicity in an AO can be beneficial in predicting the distribution and abundance of the specific AO in a known environment. Wastewater treatment plants receive large amounts of human-produced urea, which can lead to high levels of ammonium. Understanding ammonium toxicity in AO is also beneficial in selecting AO in wastewater treatment plants.

The sensitivity of *Nitrosomonas* sp. towards ammonium concentration depends on the strain of *Nitrosomonas* sp. as observed with the high sensitivity of *Nitrosomonas* sp. Is79 compared to *N. eutropha* (Figure 19, Figure 20, Figure 21). High ammonium affects growth rate as well as the lag phase of growth (Figure 22, Figure 23). *N. eutropha* is tolerant to higher concentration of methyl viologen and nitrite (Figure 22, Figure 23) indicating a correlation between high ammonium and oxidative and nitrosative stress. This observation also reflects the ability of *N. eutropha* to adapt to high oxidative and nitrosative stress.

Unexpectedly, the growth rate of *Nitrosomonas* sp. Is79 was not increased by the presence of heterotroph *Pseudomonas* sp. H19 at the tested high ammonium concentration of 10 mM. However, the growth rate of the strain was higher at 1 mM ammonium in coculture than in pure culture of *Nitrosomonas* sp. H19. The presence of the heterotroph likely increases the K_s of *Nitrosomonas* sp. Is79 to ammonia, resulting in a faster growth rate at low ammonium concentration. We may further be able to make speculations about the effect of heterotroph on the influence of ammonium on *Nitrosomonas* sp. Is79.

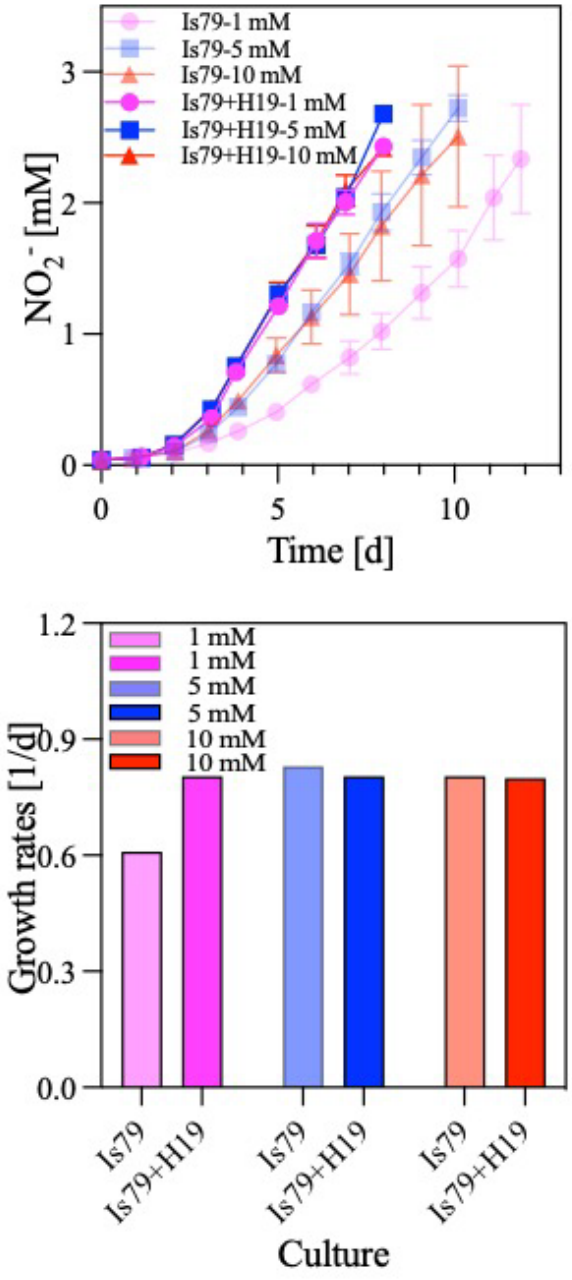

Figure 24. Ammonia oxidation activity of *Nitrosomonas* sp. Is79 pure and coculture compared: (A) Nitrite production by *Nitrosomonas* sp. Is79 pure and *Nitrosomonas* sp. Is79 + H19 coculture at 1-, 5- and 10 mM ammonium (mean ± SD, n=4), (B) Ammonia oxidation rates of *Nitrosomonas* sp. Is79 pure and *Nitrosomonas* sp. Is79 + H19 coculture at 1-, 5- and 10 mM ammonium (mean ± SD, n=4).

CONCLUSION

In this chapter, I studied the impact of high ammonium concentration on the growth and physiology of AOB *Nitrosomonas* sp. and the impact of heterotroph on alleviating the high ammonium toxicity in the AOB. We have investigated the concentrations of ammonium that are toxic to *Nitrosomonas* sp. Is79 and *N. eutropha.* We have determined *N. eutropha* to be more tolerant to ammonium than *Nitrosomonas* sp. Is79. *Pseudomonas* sp. H19 increased the growth rate of *Nitrosomonas* sp. Is79 at low (1 mM) ammonium concentrations. RNA-seq data will be help us further understand how the physiological states of the *Nitrosomonas* sp. might be different at different ammonium concentrations and in the presence of heterotroph.

SUMMARY

With the introduction of significant amount of human-produced nitrogen into the environment in the form of synthetic fertilizers (approximately 150 Tg annually), it is increasingly crucial to predict, model, and regulate nitrification processes in both natural and engineered settings (190). The excessive application of fertilizers has led to the eutrophication of terrestrial, inland, and coastal aquatic environments (191), resulting in substantial shifts in microbial communities (192, 193). Along with the shifts in the environment, it is necessary to understand the impact of eutrophication on ammonia oxidizers.

The process of nitrification involves the conversion of ammonium to nitrate, which occurs through a two-step process facilitated by microorganisms. Previously, nitrification was believed to be carried out by two groups of microorganisms. Firstly, ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) catalyze the oxidation of ammonia (NH_3) to nitrite (NO_2^-) under aerobic conditions. Secondly, nitrite-oxidizing bacteria (NOB) carry out the oxidation of nitrite to nitrate (NO_3^-), thus completing the nitrification process. However, this belief was rebuked in 2015 after the discovery of a third group of microorganisms performing complete ammonia oxidation commonly called comammox (9, 10).

The discovery of comammox added a new dynamic to the field of nitrification. Some important ecological questions were raised following the discovery of comammox including where are comammox found, which nitrifier is numerically and functionally dominant at different environments, what are the effects of abiotic factors on distribution of AO and how the nitrifying microbes interact in nature.

The goal of this dissertation was to characterize the effect of biotic and abiotic environmental factors on AOs. A comammox enrichment BO4 from mesotrophic freshwater Lake Burr Oak was genomically and physiologically characterized (49). Physiological activity of the enrichment was characterized through growth at different environmental conditions and the genomic characterization was utilized to predict the metabolic potential of the comammox strain. Competition experiments were utilized to investigate the competitive interaction between the comammox enrichment and an AOA enrichment from the same lake. In addition, the toxic effect of high ammonium concentrations on pure cultures of *Nitrosomonas eutropha* and *Nitrosomonas* sp. Is79 and coculture of *Nitrosomonas* sp. Is79 with *Pseudomonas* sp. was characterized by cultivating them at different ammonium concentrations. The summary of findings of this

dissertation has been presented in Figure 25. The gene expression of the cultures at high ammonium will be evaluated by analyzing the differential gene expression when RNA-seq data is available.

Genomic and ecophysiological characterization of comammox

Ecophysiological characterization of comammox enrichment BO4 was performed through a series of growth experiments at different environmental conditions like ammonium concentration, pH, light and nitrite concentration. Comammox enrichment BO4 grew the best at the lowest tested ammonium concentration (25 μM) and grew at up to 3 mM ammonium, the highest tested ammonium concentration. The enrichment grew at pH 6 to 8, highlighting its neutrophilic nature. The BO4 enrichment was completely and partially inhibited by blue and white light respectively indicating the light sensitivity of the AMO enzyme, as observed in AOA and AOB (85, 132). The enrichment was able to oxidize provided ammonium to nitrate with a small leftover of nitrite. However, the culture was not able to oxidize nitrite only, in the lack of the ability to assimilate nitrite as speculated by Daims et al. (2015) (9). In addition, the growth data were used to compare the ecophysiological response of the comammox enrichment with AOB G5-7 and AOA-AC2 enrichments. Overall, AOB G5-7 were observed to grow at higher ammonium concentrations with higher growth rates than comammox BO4 and AOA-AC2. AOA-AC2 grew through pH 6.5-9 and AOB G5-7 grew through pH 6-9. All three tested ammonia oxidizers are non-acidophilic. Photoinhibition was observed in all the three ammonia oxidizers tested.

This dissertation produced the first metagenome assembled genome (MAG) of a comammox *Nitrospira* sp. BO4 from a freshwater environment (49). The MAG has gene clusters for ammonia and nitrite oxidation, in addition to the genes of the reductive TCA cycle for carbon fixation. The MAG also indicates its potential to oxidize urea which could not be tested in the lack of pure culture. The MAG of *Nitrospira* sp. BO4 also lacks assimilatory nitrite reductase and nitrite transporter in correlation with its inability to grow in nitrite only. Overall, this dissertation asserts that growth experiments together with metagenomic study is a powerful tool in characterizing enrichment cultures of comammox.

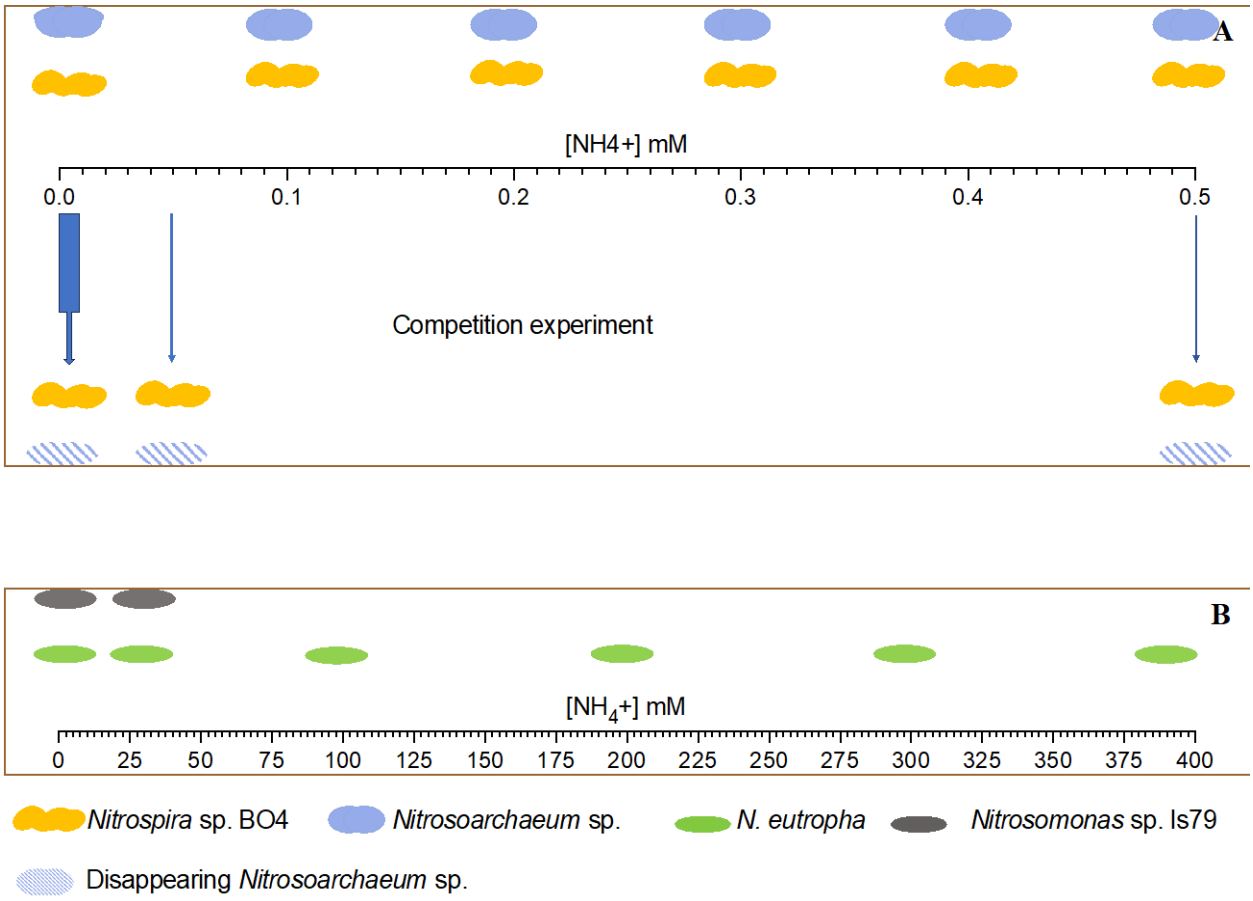

Figure 25. Ammonium concentraton influences the ammonia-oxidation activity of ammonia-oxidizers (A) ammonia oxidation activity and competition for ammonium between comammox *Nitrospira* sp. BO4 and AOA *Nitrosoarchaeum* sp. (B) ammonium oxidation activity of AOB *N. eutropha* and *Nitrosomonas* sp. Is79.

Outlook and future directions

The discovery of comammox refuted the long-held belief that nitrification is a two-step process carried out by two groups of microorganisms, AOA/AOB and NOB. Following the discovery of comammox, it is critical to understand the ecological function and niches occupied by the comammox along with other nitrifiers. To investigate the roles of nitrifiers in pristine and disturbed natural environments and engineered ecosystems, researchers often employ culture-independent techniques such as metaproteomics (194), metatranscriptomics (195) and metagenomics (196, 197). While these -omics approaches can identify microbial communities, they cannot account for the critical information like microbial function, their microniches and microbial interactions**.** By exploring and correlating physiological adaptations within specific groups of ammonia oxidizers with genomic information, we can gain insights into how environmental disturbances influence microbial community succession and overall ecosystem function. However, only 4 comammox cultures originating from engineered ecosystems have been characterized physiologically and genomically (9, 10, 38, 93). This dissertation presents ecophysiological and genomic characterization of a comammox enrichment from freshwater environment. Enriching and isolating more comammox from diverse environments in correlation with their genomic and physiological study could be utilized to understand the physiological and niche diversity of comammox in nature.

The challenges of cultivating ammonia oxidizers are well understood. However, with continuous and ingenuous efforts, it is not impossible to obtain enrichment and pure cultures of comammox. Comammox are known for their oligotrophic nature. It may be helpful to attempt to culture comammox in chemostats at low ammonium concentrations excluding other eutrophic ammonia oxidizers. Microbes live in close interactions and may not be able to be separated from their codependent partners. In such case, it might be logical to cultivate the comammox as enrichments or cocultures. Genomic and physiological study of monoclonal culture of comammox living in association with heterotrophs could describe a lot about the physiology and ecological function of the comammox compared to the -omics based studies of environmental samples inundated with a wide variety of ammonia oxidizers.

Competition between ammonia-oxidizing Archaea and complete ammonia oxidizers from freshwater environments

AOA and comammox generally are oligotrophic in nature and they coexist in several oligotrophic environments such as drinking water treatment plants (157), wastewater treatment plant (158, 159) and agricultural soils (102). It is interesting to understand the interaction between AOA and comammox at low ammonium environments to interpret if AOA and comammox live together in close association or only one occupies at a microniche. This dissertation investigated the competitive interaction between comammox enrichment BO4 and AOA enrichment BO1 at low (≤1 μM), intermediate (50 μM) and high (500 μM) ammonium concentrations.

Comammox BO4 outcompeted AOA at all tested ammonium concentrations indicating that comammox BO4 has a competitive advantage over AOA BO1. Firstly, comammox have a higher energy yield per molecule of ammonia because of their ability to perform two-step oxidation of ammonia to nitrate through nitrite. Secondly, comammox BO1 has the most energy efficient carbon fixation pathway, reductive TCA compared to less efficient 3-hydroxypropionate carbon fixation pathway in AOA BO1 (9, 10, 39). This dissertation highlights that when two oligotrophic ammonia oxidizers are competing for limiting ammonium concentrations, the one with better metabolic advantages outcompetes the other. Competition experiments are a great tool in characterizing the interactions between competing microbes which can facilitate the understanding the niche occupied by the microbes.

Outlook and future directions

AOA, AOB and comammox have phylogenetically distinct AMO enzymes. The ammonia oxidizers can be differentiated based on the gene sequence of *amoA* gene subunit, ammonia oxidizers. The AMO enzymes of AOB generally have low affinities to ammonia while comammox and AOA have higher affinities to ammonia (48). In accordance with the affinities of AMO enzymes, AOB are generally eutrophic in nature while AOA and comammox are oligotrophic in nature. The different microniches cannot be differentiated by standard sampling method that can include multiple microniches in a small area. Competition experiments are a very useful tool to simulate interaction between microbes in microniches. Previous competition experiments for ammonium between AOB-heterotroph and AOA-AOB show that AOB outcompete heterotrophs when organic carbon is limited (< critical C/N ratio) (116, 198) and AOA outcompete AOB at low ammonium concentrations (113).

This dissertation investigated the competitive interaction between oligotrophic AOA-BO1 and comammox-BO4 from freshwater Lake Burr Oak. Comammox-BO4 had a competitive advantage over AOA-BO1 indicating that comammox and AOA maybe be separated in microniches. Similar competition experiments between other strains of comammox and AOA will help investigate if all comammox have a competitive advantage over AOA. Nitrite-oxidizers are also found in association with the oligotrophic AOA and comammox. It will be interesting to observe if nitrite oxidizers play a role in the comammox outcompeting AOA in oligotrophic environment by removing nitrite.

High ammonium-toxicity in ammonia-oxidizing bacteria *Nitrosomonas* sp.

Ammonium is toxic to AOB at high concentrations. *Nitrosomonas* cluster 7 is more tolerant to ammonium than *Nitrosomonas* cluster 6a. *Nitrosomonas* sp. Is79 (cluster 6a) and. *N. eutropha* (cluster 7) were cultivated at different ammonium concentrations to observe if they were differently affected by ammonium. Ammonium concentrations above 10 mM were toxic to *Nitrosomonas* sp. Is79 decreasing its growth rate and increasing lag phase (Figure 23). Ammonium concentrations above 400 mM were toxic to *N. eutropha* (Figure 22). *Nitrosomonas* sp. Is79 + *Pseudomonas* sp. H19 grows faster than the pure culture of *Nitrosomonas* sp. Is79 (186) (Figure 25). We have collected RNA samples of pure cultures of *N. eutropha*, *Nitrosomonas* sp. Is79 and coculture of *Nitrosomonas* sp. Is79 + *Pseudomonas* sp. H19 to investigate effect of ammonium on gene expression of the cultures.

Outlook and future directions

High ammonium is toxic to AOB *Nitrosomonas* sp. The mechanism by which high ammonium causes toxicity is yet to be understood. The tolerance of *Nitrosomonas* sp. towards ammonium varies depending on the bacterial strain. *N. eutropha* is more tolerant to ammonium than *Nitrosomonas* sp. Is79. We are yet to understand the differential ammonium tolerance. Also, some heterotrophs may increase ammonium tolerance in AOB. This dissertation is attempting to address the ammonium toxicity phenomenon in AOB. We do not have the data from RNA-seq yet and we look forward to dissecting the phenomenon.

Overall summary

This dissertation presents ecophysiological and genomic characterization of a comammox from a freshwater mesotrophic Lake Burr Oak. In 2023, eight years past the discovery of comammox, only 5 comammox have been characterized and all except *Nitrospira* sp. BO4 of the

characterized comammox originated from bioengineered systems like hot water pipes and recirculating aquaculture system (9, 10, 49, 93). We enriched a comammox from the sediment of mesotrophic Lake Burr Oak and investigated its effect of environmental conditions such as ammonium concentration, pH, light and nitrite (49). We sequenced the metagenome of comammox enrichment and obtained metagenome assembled genome (MAG) of *Nitrospira* sp. BO4 to analyze the metabolic potential of the comammox strain. The second focus of this dissertation was to investigate competition for ammonium between the comammox and an AOA originating from the same freshwater Lake Burr Oak.

Additionally, this dissertation investigated the effect of high ammonium on AOB *Nitrosomonas* sp.

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