Ecology of Ammonia-oxidizing Archaea and Bacteria in Freshwater Biofilters

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

Aquarium biofilters are designed to promote the growth and activity of nitrifying microorganisms, which are primarily responsible for the removal of toxic nitrogen-cycle intermediates. Ammonia is a natural waste product excreted by fish that is lethal to aquatic life at relatively low concentrations. Ammonia-oxidizing archaea (AOA) outnumber ammonia-oxidizing bacteria (AOB) in biofilters of mature freshwater aquaria with low-ammonia conditions. However, no study has investigated the early establishment of AOA and AOB within biofilter communities, especially when aquarium ammonia concentrations are elevated. My thesis research investigated the relative abundance of AOA and AOB in freshwater aquarium biofilters through early aquarium establishment. AOA and AOB genes were detected in DNA extracts from the biofilters of 14 start-up freshwater aquaria with increasing fish biomass loads (Experiment 1), as well as from 12 biofilters of start-up aquaria treated with AOA and AOB supplements (Experiment 2). In start-up aquaria, early ammonia concentrations increased with fish biomass, and AOB *amoA* genes were strongly detected over AOA marker genes in all filters without initial AOA inoculation. Inoculation of AOA-dominated supplements into newly established biofilters improved early ammonia oxidation rates in comparison to filters supplemented with AOB or those lacking supplements. Inoculated AOA thrived in filter biofilm during and beyond stabilization of low-ammonia conditions in aquaria. Microbial activity experiments demonstrated that AOA were present and active in the biofilters eight months after inoculation, when aquaria were fully established.

In addition, AOB and AOA populations were monitored in new aquaria in three unregulated home environments. Thaumarchaeal 16S rRNA genes were detected in all

aquarium filters within one month of aquarium development. In one filter, AOA were the only ammonia-oxidizers detected in the biofilm during aquarium development, suggesting that AOA were the sole contributors to nitrification in this aquarium. The results from these experiments suggest that AOA may be key players in early aquarium nitrification once introduced into the aquarium environment. Further, this research provides insight into the ecology of AOB and AOA in engineered freshwater environments.

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1 INTRODUCTION

1.1 BACKGROUND

1.1.1 AQUARIUM NITROGEN CYCLING AND BIOFILTRATION

The health of aquatic species is governed by the water chemistry of their individual habitats, analogous to how air quality affects the health of humans and other terrestrial organisms. Water properties such as temperature, pH, dissolved gases, and nitrogen compounds (ammonia, nitrite, nitrate), all directly impact the physiology of aquatic organisms. Stabilization of these properties in the environment is imperative for establishing a balanced aquatic ecosystem. In closed aquatic environments, such as home aquaria and re-circulating aquaculture systems, efficient nitrogen cycling is vital for aquatic health; certain nitrogenous compounds, such as ammonia and nitrite, are toxic to aquatic species. Ammonia is a metabolic waste product excreted by fish that can be lethal to aquatic life at concentrations exceeding only 0.02 mg/L (Lawrence 2007). In solution, equilibrium is established between unionized ammonia (NH_3) and ionized ammonium (NH_4^+) compounds (Guerdat *et al.* 2010). Temperature and pH affect the equilibrium, with a higher pH associated with a higher percentage of the molecule in the unionized form. Because unionized ammonia is approximately 300-400 times more toxic than ionized ammonium (Thurston and Russo 1981), the toxicity of total ammonia (NH_3 and NH_4^+) increases with higher pH levels. Other factors, such as low dissolved oxygen (O_2) levels and high dissolved carbon dioxide (CO_2) levels, can also increase the toxicity of ammonia in the water (Lloyd 1961). In low oxygen levels, there is a decrease in fish respiration at

the gills, resulting in less expulsion of carbon dioxide and an increase in pH of the surrounding water, ultimately driving a rise in toxic, unionized ammonia (Lloyd 1961).

Nitrifying microorganisms within aquatic ecosystems reduce total ammonia concentrations through nitrification, a multi-step oxidative process in which microorganisms gain energy by oxidizing ammonia to nitrate via hydroxylamine and nitrite (Figure 1; Whalen and Sampedro 2010).

Ammonia monooxygenase   *Hydroxylamine oxidoreductase*   *Nitrite oxidoreductase*

$NH_3/NH_4^+ + O_2 + 2H^+ + 2e^-$ → $NH_2OH + H_2O + E$ → $NO_2^- + 5H^+ + 4e^-$ → $NO_3^- + 2H^+ + 2e^- + E$

1. Ammonia Oxidation   **2. Hydroxylamine Oxidation**   **3. Nitrite Oxidation**
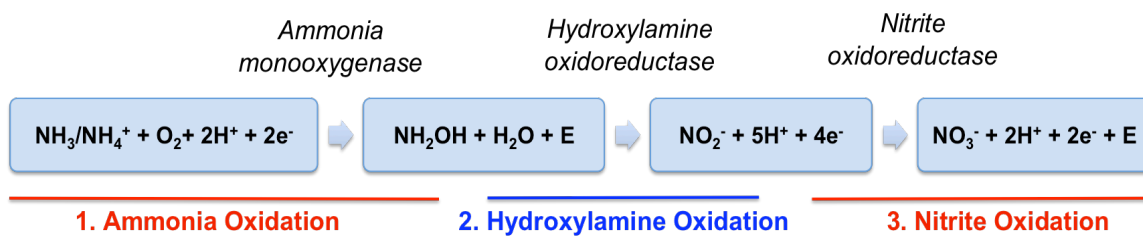
Figure 1. Schematic diagram of the nitrification process. Ammonia monooxygenase, hydroxylamine oxidoreductase and nitrite oxidoreductase are the enzymes in nitrifying microorganisms that catalyze these reactions. E represents energy released from this process (Whalen and Sampedro 2010).

The oxidized intermediates and products of nitrification, including hydroxylamine, nitrite and nitrate, are less toxic than ammonia to aquatic organisms, but high accumulations of these compounds can still pose risks to ecosystem health (Camargo *et al.* 2005; Meade 1985). For some aquatic species, nitrite concentrations exceeding 1 mg/L can impact organism health (Lawrence 2007). Nitrate is considered non-toxic, yet health risks can occur with chronic exposure to high concentrations (i.e., > 200 mg/L; Camargo *et al.* 2005).

Biofiltration is an engineered process that uses microorganisms to break down waste compounds and pollutants in the environment. In a biofilter, microorganisms grow on a fixed substrate medium, forming a biofilm community. Biofiltration is commonly used in closed aquatic systems to regulate aspects of water chemistry, including

concentrations of toxic nitrogen species. Engineered aquarium biofilters are particularly

designed to promote the growth and activity of nitrifying microorganisms, to help establish

low ammonia and nitrite levels and contribute to a balanced aquatic environment (Guerdat

et al. 2010). Most mechanical aquarium filters house filtration media within a casing that

allows aquarium water to flow through the system. A variety of natural and synthetic

biofiltration media are available for aquarium filters; the general purpose of these media is

to provide a substrate with high surface area for growth of nitrifying organisms. In a newly

established aquarium, such microbial populations in the biofilter develop in response to the

availability of nitrogenous compounds. Phases of accumulated ammonia and nitrite are

seen in aquarium water during the early establishment of nitrifying microorganisms in the

filters (Figure 2).
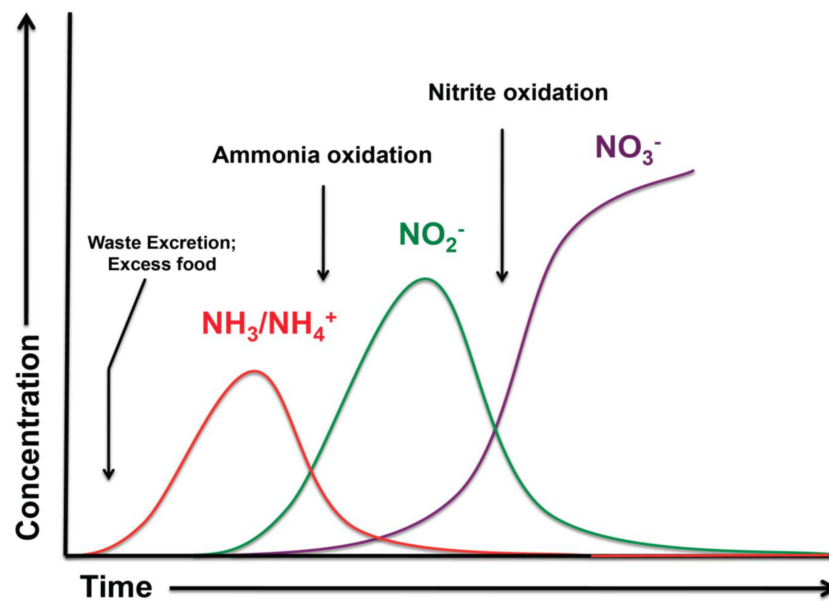
Figure 2. Nitrogen cycling in a new aquarium. Stages of accumulated ammonia, nitrite and nitrate during the establishment of nitrifying microorganisms in an aquarium biofilter are shown in this idealized plot.

Ammonia and nitrite concentrations are eventually reduced and stabilized within aquaria due to the combined activities of chemolithoautotrophic microorganisms growing in the filters. Once a microbial consortium is established in a filter biofilm, efficient nitrification prevents a build-up of ammonia and nitrite in the water. Nitrate accumulates within a closed aquatic system in the absence of applied mechanisms involved with its depletion, such as plant/algal growth, water replacement, or denitrification.

1.1.2 AMMONIA-OXIDIZING ARCHAEA

In the past, bacteria were thought to be solely responsible for chemolithoautotrophic nitrification. More specifically, members of the *Beta-* and *Gammaproteobacteria* (e.g., *Nitrosomonas* within the *Betaproteobacteria*), termed ammonia-oxidizing bacteria (AOB), were considered solely responsible for mediating aerobic oxidation of ammonia to nitrite, whereas nitrite-oxidizing bacteria (e.g., *Nitrobacter*) exclusively mediated the oxidation of nitrite to nitrate. Ammonia monooxygenase catalyzes the oxidation of ammonia to hydroxylamine in the first step of nitrification (Bock and Wagner 2006), and genes encoding this enzyme (*amo* genes) are commonly used as biomarkers for the detection of AOB. Research within the past decade has revolutionized this understanding by revealing evidence for the existence and activity of ammonia-oxidizing archaea (AOA) in a range of habitats worldwide. Initial evidence for potential ammonia-oxidizing capabilities within the archaeal domain was a homolog of the bacterial ammonia monooxygenase gene found on an archaeal scaffold in sequenced metagenomic DNA extracted from the Sargasso Sea (Venter *et al.* 2004). A subsequent study revealed two *amo* genes located contiguously upstream from archaeal 16S and 23S ribosomal RNA genes in a fosmid clone library carrying DNA extracted from a grassland

soil sample (Treusch *et al.* 2005). The existence of AOA was confirmed when an

ammonia-oxidizing marine archaeon, *Nitrosopumilus maritimus* strain SCM1, was isolated

in pure culture from gravel in a marine fish tank in Seattle, Washington (Könneke *et al.*

2005). Since these initial discoveries, AOA have been found in many habitats, some of

which include soils (Leininger *et al.* 2006), natural and engineered aquatic environments

(Wuchter *et al.* 2006; Beman *et al.* 2008; Francis *et al.* 2005), estuarine sediments (Beman

and Francis 2006), and geothermal habitats (de la Torre *et al.* 2008). In addition to *N.*

maritimus, another pure culture of an ammonia-oxidizing archaeon, *Nitrososphaera*

viennensis, was isolated from a garden soil in Vienna, Austria (Tourna *et al.* 2011).

Although only two AOA organisms have been isolated in pure culture to date, this falls far

short of the archaeal *amo* gene diversity detected worldwide. Multiple enrichment cultures,

closed genome assemblies, and draft genomes of AOA from a range of environmental

samples have also been reported (Table 1). Successful AOA enrichments indicate that

further AOA organisms may be cultivable and amenable to genome sequencing provided

the correct conditions are identified for their growth (Table 1).

Table 1. AOA cultivations and genome assemblies found worldwide.

Name	Culture	Environment	Location
Nitrosopumilus maritimus strain SCM1 (Könneke *et al.* 2005; Walker *et al.* 2010)	Pure culture/ complete genome	Marine tropical aquarium	Seattle, USA
Nitrososphaera viennensis strain EN76 (Tourna *et al.* 2011)	Pure culture/ draft genome	Garden soil	Vienna, Austria
Candidatus Nitrososphaera gargensis (Hatzenpichler *et al.* 2008; Spang *et al.* 2012)	Enrichment/ complete genome	Garga hot spring sediment	Buyrat Republic, Russia
Candidatus Nitrosocaldus yellowstonii (de la Torre *et al.* 2008)	Enrichment	Terrestrial hot spring sediment	Yellowstone National Park, USA
Candidatus Nitrosoarchaeum koreensis MY1 (Jung *et al.* 2011; Kim *et al.* 2011)	Enrichment/ draft genome	Soil from rhizosphere of *Cargana sinica*	Chungbuk National University, South Korea
Candidatus Nitrosotalea devanaterra (Lehtovirta-Morley *et al.* 2011)	Enrichment	Agricultural soil	Craibstone, Scotland
CN25, CN75, CN150 (Santoro and Casciotti 2011)	Enrichment	Water column	North Eastern Pacific Ocean
Candidatus Nitrosoarchaeum limnia strain SFB1 (Blainey *et al.* 2011)	Enrichment/ draft genome	Estuarine sediment	San Francisco Bay, CA, USA
Candidatus Nitrosoarchaeum limnia strain BG20 (Mosier *et al.* 2012a)	Enrichment/ draft genome	Estuarine sediment	San Francisco Bay, CA, USA
Candidatus Nitrosopumilus salaria BG20 (Mosier *et al.* 2012b)	Enrichment/ draft genome	Estuarine sediment	San Francisco Bay, CA, USA

Candidatus Cenarchaeum symbiosum (Hallam *et al.* 2006a; Hallam *et al.* 2006b)	Full genome	*Axinella Mexicana* (marine sponge) symbiont	Unstated
Nitrosopumilus maritimus strain NM25 (Matsutani *et al.* 2011)	Enrichment	Estuarine sediment	Tanoura Bay of Shinoda, Shizouka, Japan
strain JG1 (Kim *et al.* 2012)	Enrichment	Soil from *Capsicum annum* cultivation field	Chungbuk National University, South Korea
Candidatus Nitrosopumilus sediminis strain AR2 (Park *et al.* 2010; Park *et al.* 2012a)	Enrichment/ draft genome	Marine sediment	Svalbard, Arctic Circle
Candidatus Nitrosopumilus koreensis AR1 (Park *et al.* 2012b)	Enrichment/ draft genome	Marine sediment	Svalbard, Arctic Circle
Candidatus Nitrosofontus exaquare (Laura Sauder, unpublished)	Enrichment	Wastewater treatment plant	Guelph, ON Canada
Candidatus Nitrosopurus aquariensis (Laura Sauder, unpublished)	Enrichment	Freshwater aquarium biofilter	University of Waterloo Waterloo, ON Canada

All known AOA are affiliated with the newly proposed phylum Thaumarchaeota

(Brochier-Armanet *et al.* 2008), and are distinguishable phylogenetically by their distinct

16S rRNA and *amo* gene sequences (Stahl and de la Torre 2012). Early discoveries of

AOA were previously grouped within the phylum Crenarchaeota; however, analyses of

recently available genomes showed phylogenetic differences between AOA and other

crenarchaeal organisms, suggesting the group Thaumarchaeota (Brochier-Armanet *et al.*

2008; Spang *et al.* 2010**).** All current laboratory AOA cultures carry *amo* genes, and AOA

have shown a general ability to gain energy from ammonia and fix inorganic carbon (Stahl

and de la Torre 2012). However, diversity amongst discovered AOA extends beyond these generalizations, especially in terms of metabolism and optimal growth conditions. For example, certain AOA grow optimally in environments ranging from acidic to slightly alkaline conditions; *Ca.* Nitrosotalea devanaterra grows at pH 4 (Lehtovirta-Morley *et al.* 2011), *N. maritimus* and *N. viennensis* were cultured in neutral pH of 7.0-7.5 (Könneke *et al.* 2005; Tourna *et al.* 2011), and some soil AOA grow well at pH 8 (Bengtson *et al.* 2012). Furthermore, recent findings suggest that some AOA may be capable of mixotrophic or heterotrophic growth. Although organic compounds inhibit ammonia oxidation in *N. maritimus* (Tourna *et al.* 2011) and *Ca.* Nitrosocaldus yellowstonii (de la Torre *et al.* 2008), the growth rate of *N. viennensis* increases with added pyruvate (Tourna *et al.* 2011). In a study analyzing AOA abundance in peat soil, AOA abundance was higher with added organic carbon (Wessén *et al.* 2010), further suggesting that these organisms could be using organic carbon for growth. In addition, genes for urea transport systems and urease enzymes are encoded by AOA genomes, such as that of *Ca.* Cenarchaeum symbiosum (Hallam *et al.* 2006a). Activation of archaeal nitrification by urea has also been demonstrated in some soil environments, measured by an increase in abundance of archaeal *amoA* genes and uptake of radiolabelled $^{13}CO_2$ by archaea in urea-amended soils (Lu and Jia 2013). Finally, although it is unclear whether these organisms are able to oxidize ammonia, two large microorganisms (i.e., *Candidatus* Giganthauma karukerense and *Candidatus* Giganthauma insulaporcus), showing cell diameters of 10 and 13 µm, respectively, were recently added to the phylum Thaumarchaeota (Muller *et al.* 2010). The 16S rRNA genes of these archaeal organisms share 98.4% sequence homology with one another, and 97.7% sequence homology with *N. maritimus* (Muller *et al.* 2010), which

ranges in cell size from 0.17-0.22 µm (Könneke *et al.* 2005). This extraordinary finding, in addition to a plethora of new information on AOA since their initial discovery, demonstrates that there is much more to learn about these organisms and the Thaumarchaeota phylum as a whole.

1.1.3 AOA/AOB ABUNDANCE AND NICHE SEPARATION

In addition to the aforementioned discoveries that AOA inhabit a wide range of terrestrial and aquatic environments, several studies have quantified the relative abundances of AOA and AOB in these environments. Numerical dominance of archaeal *amoA* genes over bacterial *amoA* genes is typical of DNA extracted from several habitats, further challenging previous understandings of ammonia oxidation. Some environments where AOA have been found as the dominant ammonia-oxidizers include soils (Leininger *et al.* 2006), estuarine sediments (Herrmann *et al.* 2008), and marine habitats (Church *et al.* 2010). However, AOA are not always the dominant ammonia-oxidizers, and their specific contributions to ammonia oxidation within each environment have not yet been defined. For instance, AOB are numerically dominant over AOA in some engineered environments, such as certain industrial (Limpiyakorn *et al.* 2011) and municipal (Ye and Zhang 2011; Zhang *et al.* 2011) wastewater treatment plants (WWTPs).

A recent study analyzing AOA abundance in rotating biological contactors (RBCs) of WWTPs (Sauder *et al.* 2012) demonstrated niche separation between AOA and AOB, with AOA dominance corresponding with low-ammonia conditions and AOB dominance with high-ammonia conditions. In this study, AOA were reported dominant over AOB in RBCs, but only at low ammonia concentrations (Sauder *et al.* 2012). This suggestion of niche separation has been previously reported in other published studies (Erguder *et al.*

2009; Schleper 2010), such as those analyzing ammonia oxidation in ammonium-amended soils (Di *et al.* 2010; Verhamme *et al.* 2011).

1.1.4   AOA IN ENGINEERED AQUARIUM ENVIRONMENTS

AOA have been detected in a number of engineered environments, such as WWTPs (Sauder *et al.* 2012) and drinking water treatment plants (van der Wielen *et al.* 2009), suggesting that AOA play a dominant role in nitrification in large-scale biofiltration systems. However, few studies have analyzed their contributions to nitrification in small-scaled, engineered systems, such as aquarium biofilters. It is well known that AOA exist in aquarium environments, as *N. maritimus* was cultured from marine aquarium sediment (Könneke *et al.* 2005), and AOA have been detected in marine aquarium biofilter systems (Urakawa *et al.* 2008). Sauder *et al.* (2011) first looked at AOA in biofilters of freshwater aquaria. In that study, DNA was extracted from sponge in biofilters of established freshwater and saltwater aquaria, and bacterial and archaeal *amoA* and 16S rRNA genes were enumerated using quantitative real-time PCR (qPCR). AOA were numerically dominant over AOB in most of the freshwater and saltwater aquarium biofilters tested, particularly those in aquaria with low ammonia concentrations (Sauder *et al.* 2011). Meanwhile, AOB dominated in aquaria with high ammonia concentrations (Sauder *et al.* 2011). This study reinforced the existing concept of differing niches for AOA and AOB based on ammonia concentration (section 1.1.3), extending it to ammonia-oxidizers in freshwater environments.

Within the aquarium industry, AOB are currently considered the primary ammonia-oxidizers. In fact, start-up aquaria are often sold with bacterial supplements of AOB to help expedite nitrification in new aquaria. However, the recent studies of aquarium and

RBC biofilter environments challenge the concept that AOB are key to the depletion of toxic ammonia from freshwater aquarium environments. The prevalence of AOA observed in established aquaria gives rise to important questions. Firstly, when do AOA establish in aquarium biofilters? How are AOA introduced into the aquarium environment? Do AOA contribute to early nitrification in newly established aquaria? Answering these questions within controlled aquatic environments will shed light on the role of AOA in freshwater nitrogen cycling and provide new insight into the ecology of nitrifying biofilm development in aquaria. Furthermore, it could potentially shift focus of the aquarium industry from solely AOB-based ammonia oxidation to the development of both AOB and AOA in biofilter communities for an overall improvement in aquatic health.

1.2   RESEARCH OBJECTIVES AND HYPOTHESES

In closed aquarium systems where ammonia stabilization is vital for ecosystem health, findings of AOA dominance have major implications for nitrogen cycling in these environments. Studies discussed in the preceding sections provide valuable insight into the ecology of AOA and AOB in freshwater aquarium environments. However, little is known about the roles of AOA and AOB in early aquarium nitrification, specifically regarding depletion of the high ammonia concentrations common to start-up aquaria. My thesis research examined the relative contributions of AOB and AOA towards nitrification in newly established freshwater aquaria. Three experiments were designed to test my hypotheses.

1.2.1 VARIABLE FISH BIOMASS EXPERIMENT: AOB/AOA IN NEW AQUARIA

A long-term experiment was performed to analyze the presence of AOB and AOA in freshwater aquarium biofilters over an early aquarium establishment period, and how abundances of these organisms change in response to ammonia fluxes that take place during new aquarium development. This study tested the hypothesis that AOB dominate freshwater aquarium filters when there is a high level of accumulated ammonia in the water, specifically during the initial aquarium development period. I also predicted that AOA would dominate filters when ammonia levels were subsequently depleted from early peaks, helping to maintain low ammonia concentrations in established aquaria. In addition, I hypothesized that higher ammonia concentrations during the early phases of high-biomass aquarium development would be associated with longer durations of AOB dominance within biofilters. To test these hypotheses, the presence of AOA and AOB were monitored in biofilters of newly set up freshwater aquaria, over the time of initial aquarium set-up until the aquarium became fully "cycled". A range of early peak ammonia concentrations was obtained using differing fish loads in the aquaria.

1.2.2 SUPPLEMENT EXPERIMENT: AOA/AOB INOCULATION

In follow-up, a second experiment was conducted to analyze the abundance of AOB and AOA in start-up aquarium biofilters that received initial supplements of ammonia-oxidizers. The objective of this study was to observe and compare enhancements in ammonia oxidation during early aquarium development, as well as differences in microbial populations in biofilm communities, between aquaria treated with separate AOA and AOB supplements. I hypothesized that aquaria inoculated with AOA supplements

would show an increase in ammonia oxidation efficiency in comparison to those treated with AOB, predicting that AOA would prevent high ammonia accumulations commonly seen during early development periods. To test this hypothesis, newly set up aquaria were treated with commercial and laboratory supplements of AOB, AOA, or a combination of AOB and AOA. Differences in early water chemistry and biofilm microbial communities were compared between treatment groups.

1.2.3   HOME AQUARIUM EXPERIMENT: AOB/AOA IN HOME AQUARIA

A third experiment tested early establishment of AOB and AOA populations within biofilter communities of three independent start-up aquaria. The objective of this study was to observe whether AOA, AOB, or both, dominate aquarium biofilters during early start-up phases in unregulated home environments. I hypothesized that AOA would dominate freshwater aquarium biofilters in home aquaria during or shortly following early phases of high ammonia. To test this hypothesis, independent freshwater aquaria were established in the homes of three individual participants. The presence of AOB and AOA in biofilters over early aquarium development was analyzed and compared between households.

2 EXPERIMENTAL DESIGN AND METHODS

2.1 AQUARIUM SET-UPS AND MAINTENANCE

2.1.1 VARIABLE FISH BIOMASS EXPERIMENT

The variable fish biomass experiment included 12 aquaria with differing densities of *Danio rerio* (zebrafish), plus an additional 2 aquaria lacking fish to serve as negative controls. Three treatment groups were classified as "low", "medium", and "high" ammonia concentrations, which corresponded to variable fish stocking densities. Aquarium tanks housing 1 and 3 fish were considered as a low ammonia group, 6 and 9 fish were considered as a medium ammonia group, and 12 and 15 fish were considered as a high ammonia group. Duplicate aquaria were set up with each fish biomass load, for a total of four tanks per low, medium, and high ammonia treatment groups. All aquaria were set up in the Aquatic Facility ("wet lab") at the University of Waterloo, and were maintained for a 10-month duration. Two aquaria containing 15 fish as part of the high ammonia treatment group were maintained for an additional 6 months, alongside the supplement study. It is important to note that one aquarium then housed all 30 fish for an additional 4 months, after the other tank began leaking and was taken down. Samples collected at the end of the variable fish biomass experiment (16 and 20-month time points; discussed in section 2.2.1) were collected from this aquarium, and thus, were associated with changes to the experimental set-up.

All aquarium equipment and fish used for the variable fish biomass experiment were purchased from Big Al's Aquarium Services in Kitchener, Ontario. The aquarium tanks were 10 gallons in volume, and were each set up with one AquaClear 30 power filter,

glass lid, and an artificial *Cacomba* plant. All tanks were oxygenated with an airline and air stone. Aquarium filters were filled with biofilter materials purchased as part of a complete filter kit, and included sponge (bottom), carbon (middle), and ceramic biofilter beads (top). A digital thermometer was placed in one of the tanks. All aquaria and associated equipment were placed on two heavy-duty plastic racks, containing eight shelves per rack. Replicate tanks were placed side-by-side on aquarium shelves. All aquarium equipment and materials were purchased new and all pieces were thoroughly rinsed with tap water prior to set-up. All tanks were additionally rinsed with 10% hydrochloric acid prior to a tap water rinse. Municipal water from Waterloo, Ontario was used to fill each tank. Big Al's Multi-Purpose Water Conditioner (10 mL) was added to 37.5 L of tap water, as directed by the manufacturer, to neutralize chloramines and promote fish health. Although aquarium water was not changed throughout the duration of the experiment, pre-treated tap water was regularly added to maintain a high water level in each of the tanks. Aquarium water was maintained at a temperature between 20-22°C and a pH range from 8-9. Fish were fed daily with New Life SPECTRUM Community Fish Formula, which was pre-measured to two pellets per fish (approx. 5 mg). After 10 months, the fish in the tanks that remained alongside the supplement experiment began receiving three pellets each to accommodate fish growth. Light cycles varied throughout the study because of other research projects occurring in the same room; however, lights were on for a minimum of 12 hours per day. Aquarium maintenance, feeding and fish health observations were recorded daily, in addition to any changes in the facility or aquarium set-up.

Prior to commencing this experiment, an Animal Utilization Project Proposal (AUPP) was submitted and approved by the Animal Care Committee (ACC) at the University of Waterloo, requesting the use of 95 *Danio rerio* (zebrafish) specimens for this research (AUPP #11-23).

2.1.2   SUPPLEMENT EXPERIMENT

The supplement experiment included 10 aquaria subject to 5 microbial supplement groups. Each newly established aquarium housed six zebrafish that were previously used in the variable fish biomass experiment (section 2.1.1). Supplement treatments included sources of AOB and AOA, alone or in combination, and supplements were inoculated into filters of duplicate aquaria during initial set-up. The five treatments were as follows: AOB from a commercially available supplement, Nutrafin Cycle Biological Aquarium Supplement (Hagen Industries; "Cycle"), AOA from an enrichment culture maintained in the Neufeld Lab by Laura Sauder (Table 1; "*Ca.* Nitrosopurus aquariensis; unpublished), a combination of Cycle and AOA (enrichment culture), or AOA from an established aquarium biofilter sponge from an aquarium belonging to Josh Neufeld (designated aquarium FW27 in Sauder *et al.* 2011). Two tanks were also set up as control groups lacking supplements. The inoculation schedule for AOB (Cycle) and AOA (enrichment culture) followed the manufacturer's directions for the Cycle supplement. After initial set-up and introduction of fish into the tanks (Day 1), 25 ml of Cycle and AOA culture were inoculated into the filters for these treatments. On the subsequent two days (Days 2-3), 10 mL of each supplement were inoculated. Cycle was mixed by inversion prior to inoculation. AOA from an established sponge was inoculated into tanks on Day 1 by adding a piece of this sponge underneath the new sponge in the filter casings.

All aquarium equipment used in this experiment was recycled from the variable

fish biomass experiment. Prior to set-up, all tanks, glass lids, and filters were rinsed with

10% hydrochloric acid, then 10% bleach, and then thoroughly rinsed with tap water in

between and after the two chemical rinses. Plastic tubing for airlines was wiped with 100%

ethanol and new air stones were attached to the ends. Aquaria were placed on the plastic

shelving racks, but the duplicate tanks were dispersed on different shelves, as opposed to

side-by-side as in the variable fish biomass experiment. Aquarium water was treated as

previously discussed, and water temperature, water pH, and lighting were maintained as

for the variable fish biomass experiment (section 2.1.1). Fish were fed daily with Tetramin

Tropical Flakes, pre-weighed to a mass of 45 mg per tank, which was an approximate

equivalent to the weight of 18 pellets from the previous food source (3 pellets per fish).

Alongside the supplement experiment, two additional tanks were set-up lacking

supplements and were fed with the pellet food used in the variable fish biomass

experiment, as opposed to the tropical flakes. These tanks were set up to demonstrate the

impact, if any, that food type has on AOB and AOA establishment in aquarium filters.

2.1.3   HOME AQUARIUM EXPERIMENT

The home aquarium experiment studied microorganisms established in biofilters

from new aquaria in the homes of three participants. For this citizen science experiment,

participants were provided with a research package that included set-up guidelines,

sampling instructions, documentation sheets and sampling materials. All participants

received a 20-gallon aquarium, LED-light lid, AquaClear 150 aquarium filter, and a plastic

thermometer. Participants had the freedom to further build their aquarium to their liking

(e.g., gravel, plants, fish, water treatment). They received a $50 gift certificate to

Aquariums By Design, located in Waterloo, Ontario, to contribute to their choice of fish,

fish food, aquarium treatment products, and accessories (Table 2). Home Aquarium 1 and

2 did not use bacterial supplements, while Cycle was added to Home Aquarium 3.

Table 2. Contents of participating aquaria in home aquarium experiment.

Home Aquarium #	Fish Added	Food Type	Water Source/Temp	Water Conditioner	Accessories
Aquarium 1	3 Burmese spotted danios; 4 rainbow fish	Tetramin tropical fish food flakes	Municipal water/27 °C	Tetra Aquasafe water conditioner	Live plants: Java fern, *Anubias*; natural gravel
Aquarium 2	1 *Betta splendens*; 3 rasboras (week 2); 6 *Corydoras* (week 3); 6 fish (other)	Cobalt Aquatics color flakes	Municipal water/23°C	Marineland aquarium conditioner	Gravel; driftwood; rock; live plants
Aquarium 3*	3 rummy-nose tetra; 3 red-eyed tetra; 1 chocolate oranda goldfish	TetraColor tropical crisps; OmegaOne freeze dried tubifex worms	Municipal water/24°C	Nutrafin Aquaplus tap water conditioner	Gravel; toy plane; fake pottery; artificial plants

* Cycle was added during start-up as per manufacturer's instructions.

2.2   SAMPLING TIMELINE AND PROCEDURE

2.2.1   VARIABLE FISH BIOMASS AND SUPPLEMENT EXPERIMENTS

Sampling schedules and procedures were very similar for the first two experiments.

For the variable fish biomass experiment, ceramic biofilter beads were first sampled prior

to the introduction of fish into the tanks. Following the initial time point, bead samples

were collected weekly from aquarium filters for three months, then sampled monthly for

the remaining seven months. Water was sampled from aquaria every three days for three months, then sampled monthly for seven months.

 For the supplement experiment, aquarium biofilter beads were sampled prior to the first inoculation of supplements, one day following the final supplement inoculation, and then weekly for two months. Samples were collected monthly after the two-month time point for the remaining six months of the study. Water samples were collected twice weekly for the first two months of the study, then sampled monthly for the last six months of the study.

Tweezers were wiped with 100% ethanol and used to collect 2-3 biofilter beads from the tops of the filters. The beads were stored in 5-ml tubes. Water was collected from aquaria in 50-ml conical tubes. Both aquarium beads and water were stored at -20°C until analysis. Water temperature was recorded daily and the pH of all tanks was checked regularly, usually in association with the collection of each water sample.

Towards the end of the variable fish biomass experiment, the available beads in some of the filters were depleted. Beads were no longer available in the filter of one aquarium in the "medium" treatment group (9 fish), as well as filters of two aquaria in the "high" treatment group (12 and 15 fish) by the 8-month time point. Beads were also depleted in one aquarium that remained alongside the supplement experiment by the 11-month time point. Sponge was collected from these filters in a similar fashion using ethanol-cleansed tweezers and new razor blades. Sponge samples were stored at -20°C.

2.2.2 HOME AQUARIUM EXPERIMENT

Participants in the home aquarium experiment were instructed to sample ceramic biofilter beads, biofilter sponge, and water on a weekly basis. The first set of samples was collected prior to the addition of fish, the second set 48 hours after introducing fish into the environment, and then weekly thereafter. Three biofilter beads were collected in 5-ml tubes using flame-sterilized tweezers. Sponge samples roughly of size 2.5 x 1 x 1 cm were collected with flame-sterilized scissors and placed in 5-ml tubes. Water samples were collected in 50-ml tubes. All samples were stored at -20°C until analysis. Aquarium water temperature, observable fish health, and any changes made to the aquarium environment were documented with each sample collection, in addition to providing a photo of the aquarium.

2.3 WATER CHEMISTRY MEASUREMENTS

For the variable fish biomass and supplement experiments, total ammonia ($NH_4^+ + NH_3$) and nitrite (NO_2^-) concentrations were measured in the collected water samples.

2.3.1 AMMONIA MEASUREMENTS

Total ammonia concentrations were directly measured with a microplate-based, fluorometric assay adopted from Holmes *et al.* (1999) and Poulin and Pelletier (2007). This assay measures a fluorescent blue signal that is formed when O-phalaldehyde (OPA) combines with ammonia in the water samples. A working reagent consisting of OPA, sodium sulfite, and borate buffer solutions was prepared as previously described (Poulin and Pelletier 2007). Sodium sulfite is added to decrease the sensitivity of OPA to amino acids, thus enabling a higher sensitivity to ammonia (Holmes *et al.* 1999). Borate buffer

was necessary in the published study to initially evaluate background fluorescence of the working reagent (Holmes *et al.* 1999). Published protocols were altered to measure samples in a 96-well plate with smaller sample volumes, as opposed to tubes or a 48-well plate. Standards (100 μL) and water samples (100 μL) were added in triplicate to black plastic 96-well microtiter plates, and combined with 200 μL of working reagent. MilliQ water was used as a blank reagent (negative control). Prepared microplates were incubated at room temperature for approximately 3-5 hours (Holmes *et al.* 1999). Fluorescent signal was measured at wavelengths λ_{ex} = 360 nm and λ_{em}= 465 nm using the FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices, LLC) and SoftMax Pro 6.1 software (Molecular Devices, LLC). Using this software, background fluorescence from the blank reagents was averaged and subtracted from each absorbance measurement. Triplicate readings were averaged and compared with standard measurements to determine the resulting ammonia concentrations.

Ammonium chloride (NH_4Cl) standard stock solutions were prepared at 1,000 μg/L of nitrogen, aliquoted, and stored at -20°C. These concentrations used for standard preparation were calculated for only nitrogen mass in the ammonium chloride compound ($N-NH_4Cl$), as opposed to the mass of the entire NH_4Cl compound. Stock solutions were thawed and diluted with MilliQ water to appropriate concentrations. Standard curve ranges varied depending on prospective analyte concentrations in the samples. As required, samples with high concentrations were diluted in MilliQ water to fit within standards that covered the range of the majority of samples measured simultaneously.

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2.3.2 NITRITE MEASUREMENTS

Nitrite concentrations were measured using a micro-plate based, colorimetric detection assay outlined in Miranda *et al.* (2001). This assay uses a Greiss reagent system for nitrite detection. The Greiss reagent system incorporates a 1:1 ratio of sulfanilamide (SULF) and N-(1-Napthyl)-ethylenediamine (NEDD) to react with nitrite to form a detectable chromophore. Sulfanilic acid (acidic SULF) reacts with nitrite to produce a diazonium salt, which combines with NEDD to produce a bright pink azo dye (Greiss 1879). Absorption of the azo dye is measured spectrophotometrically.

The experimental protocol for this assay and preparation of solutions followed the procedures in Miranda *et al.* (2001), with few deviations from the published method. This assay was performed in a clear, 96-well microtiter plate. Sample (100 µL) was added to plate wells in triplicate, followed by the addition of 100 uL of Greiss reagent (50 µL of SULF and NEDD each). SULF and NEDD solutions were prepared as previously described (Miranda *et al.* 2001). Sodium nitrite ($NaNO_2$) standard stock solutions were prepared at 1,000 and 10,000 µg/L of nitrogen, and aliquots were stored at -20°C. These concentrations used for standard preparation were calculated from the mass of only nitrogen in the sodium nitrite compound (N-$NaNO_2$), as opposed to the mass of the entire $NaNO_2$ compound. Stock standard solutions were thawed and diluted with MilliQ water. MilliQ water was used as a blank, or negative control, to adjust for background absorbance. Standard curve ranges varied depending on prospective analyte concentrations in the samples. As required, samples with high nitrite concentrations were diluted in MilliQ water to fit within standards that covered the range of the majority of samples measured simultaneously. Prepared plates, including all samples, standards, blank

solutions, and reagents, were incubated at 37°C for 30-45 minutes, and absorbance of the produced dye was measured at $\lambda=550$ nm. Readings were taken with the FilterMax F5 plate reader, as discussed in section 2.3.1. Blank readings were subtracted from all sample and standard readings to eliminate background absorbance.

2.3.3. STATISTICAL ANALYSIS OF WATER CHEMISTRY MEASUREMENTS

Variance in ammonia concentration between treatment groups in the supplement experiment was statistically analyzed for water samples collected on Day 13 of the experiment. Variance was analyzed using a One-Way Analysis of Variance (ANOVA) test, with a confidence interval set to 95%. The test was conducted using Microsoft Excel (Microsoft), in combination with Statplus statistical software (AnalystSoft Inc.).

2.4   GENOMIC DNA EXTRACTION

In all three experiments, genomic DNA was extracted from ceramic biofilter beads sampled from the aquarium filters. The extraction method was modified from the procedures discussed in Somerville *et al.* (1989) and Murray *et al.* (1998). SET buffer (extraction buffer) was prepared by mixing 40 mM EDTA (pH 8), 50 mM Tris-HCl (pH 9), and 0.75 M sucrose, followed by autoclaving and storage of the mixture at room temperature. The SET buffer (800 µL) and 90 µL of freshly prepared lysozyme (1 mg/ml in 1 M Tris-HCl, pH 8) were added to 5-mL polypropylene tubes, each containing one ceramic bead. The tubes were incubated at 37°C for 30 minutes by rotating on a carousel platform in Shake n' Stack hybridization ovens (Thermo Fischer Scientific Inc.). To ensure that the beads were well submerged in buffer and had room to rotate, the tubes were

inverted and propped with paper towels in the oven bottles on an upward angle such that the beads rotated near the tube caps. This helped to avoid the beads wedging into the conical bottoms of the tubes. Following incubation, 100 μL of 10% SDS and 27.5 μL of fresh proteinase K (20 mg/ml in 1 M Tris-HCl, pH 8) were added to the tubes, and the units were incubated inverted at 55°C for 2 hours in the rotating hybridization ovens. Crude lysates (500 μL) were added to 2.0 mL MaXtract High Density tubes (Qiagen), and extracted once each with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8) and chloroform:isoamyl alcohol (24:1). In both steps, the solvent mixes were added directly to the MaXtract tubes, each followed by centrifugation at 15,000 rpm for 5 minutes. The aqueous phase was transferred to 2.0 mL microfuge tubes, mixed with 4 μL of linear polyacrylamide (5 μg/μL) and 2 volumes of polyethylene glycol solution (30% PEG 6000, 1.6 M NaCl), and incubated at room temperature overnight. Following incubation, mixtures were centrifuged at 15,000 rpm for 30 minutes at room temperature, decanted and rinsed with 500 μL of 80% ice-cold ethanol, and centrifuged for an additional 20 minutes at 15,000 rpm. The ethanol was aspirated from the tubes, and the DNA pellets were suspended in 50 μL of elution buffer (10 mM Tris-Cl, pH 8.5) or UV-treated water (variable fish biomass experiment only). DNA suspensions were precipitated on ice for approximately one hour and were frequently mixed. Genomic DNA samples (2 μL) were subjected to electrophoresis on a 1% agarose gel run at 80-100V for 20-30 minutes. Double-stranded DNA concentrations were also measured using the Qubit 2.0 Fluorometer (Life Technologies). All genomic DNA samples were stored at -20°C, with a working aliquot stored at 4°C during analysis.

DNA extractions were performed on sponge samples from several points in the variable fish biomass, supplement, and home aquarium experiments, using an identical protocol. However, instead of one ceramic bead in the tube, sponge was cut into tiny pieces and added to the tubes. All other steps were performed as described above.

2.5 PCR AMPLIFICATION

Amplification of AOB *amoA* genes used primers AmoA-1F and AmoA-2R (Rotthauwe *et al.* 1997). Amplification of AOA *amoA* genes used primers Arch-amoAF and Arch-amoAR (Francis *et al.* 2005), as well as CrenamoA23F and CrenamoA616R (Tourna *et al.* 2008). Thaumarchaeal and bacterial 16S rRNA genes were detected with primers 771F and 957R (Ochsenreiter *et al.* 2003), and primers 341F and 518R (Muyzer *et al.* 1993), respectively. Bacterial 16S rRNA genes were not amplified in all sample sets; these amplifications were only performed as needed for troubleshooting. Each 25-μL reaction mixture contained 2.5 μL of 10X *Taq* DNA polymerase buffer, 0.5 μg of bovine serum albumin, 0.05 pmol of deoxynucleotides, 0.05 pmol of forward and reverse primers, 1.25 U of *Taq* DNA polymerase, 1-10 ng of template genomic DNA, and a remaining volume of UV-treated water. For AOA *amoA* gene amplification, the PCR conditions were 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minute, with a final elongation period at 72°C for 10 minutes. Samples were held stably at 12°C until stored at 4°C temporarily, followed by long-term storage at -20°C. Thaumarchaeal 16S rRNA and AOB *amoA* genes were amplified with the cycling conditions described above, except that the annealing temperature was raised to 55°C. General bacterial 16S rRNA gene amplification had an annealing temperature of 55°C and

ran for only 30 cycles in total. PCR products (5 μL) were run on a 1% agarose electrophoresis gel for 20-30 min at 80-100V to check for amplification in relation to controls. Positive controls for AOB *amoA* gene detection included genomic DNA extracted from an established aquarium filter sponge (source of sponge inoculum in the supplement experiment), a plasmid vector with a bacterial *amoA* gene insert (prepared by Laura Sauder), or an experimental sample known to have strong AOB *amoA* gene amplification. Positive controls for thaumarchaeal 16S rRNA and AOA *amoA* gene amplifications included DNA extracted from an established aquarium filter sponge (source of sponge inoculum in the supplement experiment) or a plasmid vector with an AOA *amoA* gene insert (prepared by Laura Sauder). The positive control used for bacterial 16S rRNA gene amplification was genomic DNA extracted from a pure culture of *Escherichia coli*.

2.6   DENATURING GRADIENT GEL ELECTROPHORESIS

Denaturing gradient gel electrophoresis (DGGE) analysis of AOA *amoA* genes was performed as described in Sauder *et al.* (2011). Only samples from aquaria in the supplement experiment that were inoculated with AOA-enriched sponge or the AOA laboratory culture alone were analyzed by DGGE, because they were the only samples that yielded consistent AOA *amoA* gene detection by PCR. The *amoA* genes were PCR-amplified using CrenamoA23F and Crenamo616R, as described in section 2.5. Samples were loaded into 6% polyacrylamide gels prepared with a denaturing gradient from 15-55%. Volumes of loaded PCR product ranged from 3-15 μL, based on visual inspection of relative PCR amplification. Gels were run at 60°C and 85V for 15 hours in the DGGEK-2401 (CBS Scientific Company, Inc.), following technical protocols previously outlined in

Green *et al.* (2010). Gels were post-stained with SYBR Green (Invitrogen) for 1 hour, and then scanned on the Typhoon 9400 Variable Mode Imager (GE Healthcare Life Sciences). Fingerprints were compared to a standard sample using GelCompar II (Applied Maths), and patterns were analyzed between samples. A PCR-amplified AOA *amoA* gene product from genomic DNA extracted from an AOA-enriched sponge was included as a standard. The AOA-enriched sponge that yielded this standard was the source of sponge inoculum used in the supplement experiment. Genomic DNA was originally extracted from this sponge sample in September 2011 using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.)

2.7   MICROBIAL ACTIVITY EXPERIMENTATION

An experiment was conducted to analyze the activity of AOB and AOA on the biofilter beads in filters from the supplement experiment. This experiment involved incubating biofilter beads in medium containing ammonia both with and without inhibitors, and measuring the change in ammonia concentration in the medium over a 5-day period. Fresh water medium (FWM) for microbial growth and activity was prepared as previously described for AOA cultivation in Tourna *et al.* (2011). Ammonium chloride was added to the FWM at a concentration of 1000 μg/L, and bromothymol blue was used as a pH indicator. Instead of autoclaving the base medium as previously discussed (Tourna *et al.* 2011), the final medium, including all components, was filter-sterilized using a 0.2-μm filter. Glass serum flasks were pre-washed with 10% HCl solution, rinsed thoroughly, and autoclaved. FWM (25 mL) was added to the flasks. Autoclaved aluminum foil was used to cover all flasks, because previous troubleshooting experiments demonstrated that

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rubber stoppers prevented microbial activity. Microbial inhibitors were added directly to flasks containing FWM. AOB ammonia-oxidizing activity was inhibited with 10 μM of N-allylthiourea (ATU), and AOA ammonia-oxidizing activity was inhibited with 100 μM of 2-Phenyl-4,4,5,5- tetramethylimidazoline-1-oxyl 3-oxide (PTIO). ATU chelates the copper of the ammonium monooxygenase active site, preventing the production of hydroxylamine (Bédard and Knowles 1989). PTIO is an NO scavenger that inhibits a previously suggested metabolic pathway of AOA, where AOA convert ammonia to nitrite via nitroxyl, as opposed to hydroxylamine (Ellis *et al.* 2001; Shen *et al.* 2013; Walker *et al.* 2010). However, *N. maritimus* has recently demonstrated the ability to convert ammonia to hydroxylamine (Vajrala *et al*. 2013), opposing this suggested pathway. Thus, the mode of inhibition of PTIO on archaeal ammonia oxidation is unknown. Inhibitor concentrations were selected based on previous studies demonstrating their effects on AOB and AOA at different concentrations (Lehtovirta-Morley *et al.* 2013; Shen *et al.* 2013). Ceramic biofilter beads were collected from duplicate tanks treated with pre-enriched aquarium sponge (AOA source, tanks A6 and A10) and Cycle supplement (AOB source, tanks A1 and A8). Two beads from each tank were added to flasks in duplicate for each separate inhibition treatment, as well as duplicate flasks lacking inhibitors (total of twelve beads from each tank). Duplicate control flasks were set up lacking biofilter beads for each treatment to ensure stability of FWM components. Flasks were incubated at 21.5°C for 5 days, rotating at 75 rpm. The incubation temperature was chosen to reflect general aquarium temperatures from the supplement experiment. The FWM (1.2 mL) was sampled every 12 hours and stored at -20°C until further use. Ammonia concentrations were measured in the samples collected using the methods described in section 2.3.1.

2.8 QUANTITATIVE REAL-TIME PCR

Quantitative real-time PCR (qPCR) was performed by Laura Sauder to quantify thaumarchaeal 16S rRNA genes in DNA extracted from two beads sampled at 8 months from aquaria enriched with AOA-populated sponge (A6 and A10) in the supplement experiment. Primers 771F/957R were used for quantification of thaumarchaeal 16S rRNA genes (Ochsenreiter *et al.* 2003). The qPCR was performed using the CFX96 thermocycler system (Bio-Rad). Real-time PCR amplification for both samples was performed in duplicate. Reaction mixtures (10 µL) consisted of 5 µL of 2X SYBR Green IQ mix (Bio-Rad), 0.5 µg of bovine serum albumin, 2 pmol of each primer, 3.5 ng (A6) and 2.7 ng (A10) of genomic DNA, and a remaining volume of UV-treated water. The PCR conditions were 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute. Fluorescence values were recorded after each cycle. Melting curves from 65°C to 95°C were performed at the end of the PCR run, with increments of 0.5°C for 2 seconds. DNA copy numbers were reported per ng of genomic DNA.

Standards were prepared from thaumarchaeal 16S rRNA gene amplicons produced using primers Arch21F (DeLong 1992) and a modified version of 1492R (Lane 1991). Genomic DNA from an enrichment culture of *Ca.* Nitrosopurus aquariensis (Sauder, unpublished) was used as template DNA to prepare standards. Standard curves were prepared from serial dilutions of template DNA, and were quantified using the Qubit fluorometic assay. Amplification efficiency of standards was 74%, with a coefficient of determination value (R^2) of 0.994. Melting curves for the 16S rRNA gene sequence

showed single peaks. Reported copy numbers were calculated using a linear regression

equation of the standard curve.

3 RESULTS

3.1 VARIABLE FISH BIOMASS EXPERIMENT

3.1.1 WATER CHEMISTRY MEASUREMENTS AND ANALYSIS

Ammonia and nitrite concentrations were measured in aquarium water for all tanks over a 3-month aquarium development period (Figure 3).
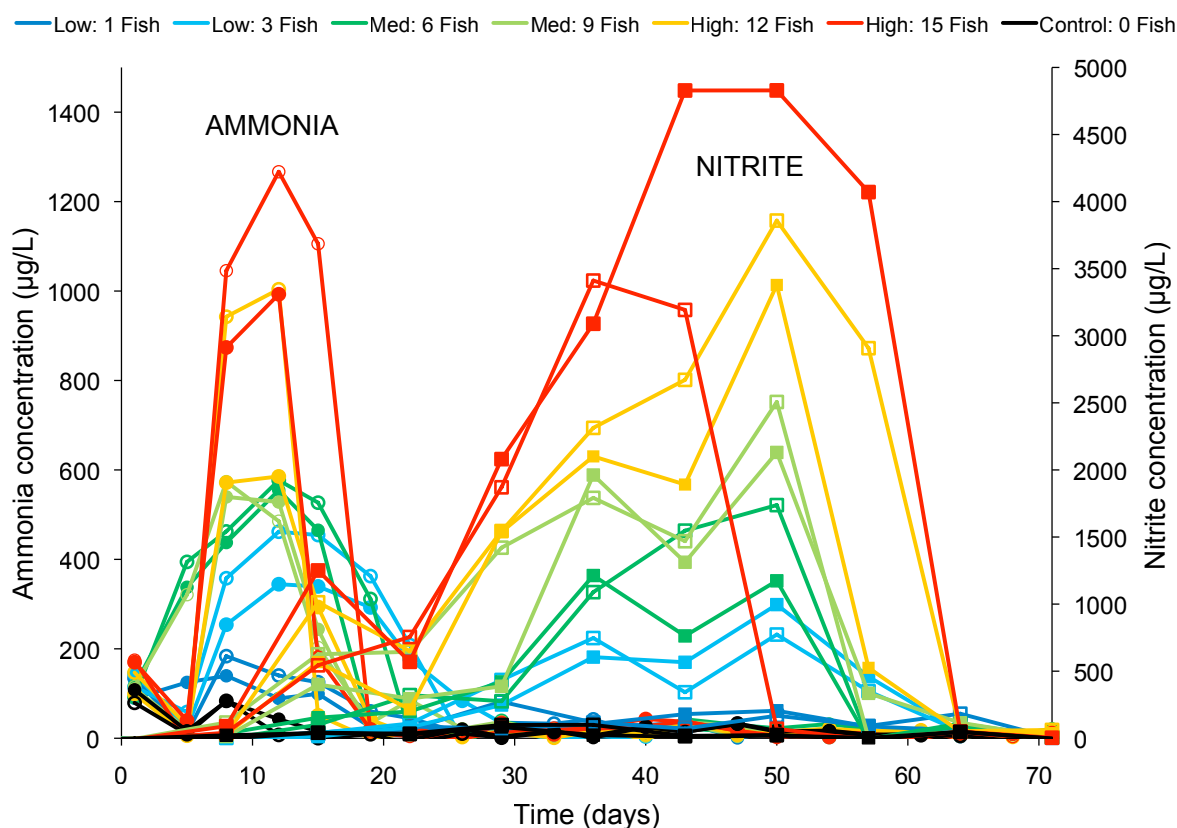
Figure 3. Ammonia and nitrite concentrations in water samples from newly set-up aquaria in the variable fish biomass experiment. Triplicate technical measurements were averaged for each time point. Biological replicates are distinguished by filled and hollow markers. Circle and square markers represent ammonia and nitrite concentrations, respectively.

There was a strong link between fish biomass and ammonia/nitrite concentrations during early aquarium development (Figure 3). Aquaria that housed only 1 fish (low) had peak ammonia concentrations around 100 µg/L, while aquaria that contained 15 fish (high) had peak concentrations at and above 1000 µg/L; ammonia concentrations in the high

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treatment groups were approximately 10X that of the low treatment groups. All other fish loads were associated with peaks between 300 and 600 μg/L, with the exception of one replicate tank housing 12 fish that reached a peak of 1000 μg/L, approximately 400 μg/L above the similarly treated tank. Most aquaria showed highest ammonia concentrations by the second week of the experiment, and all ammonia was depleted to a concentration below 100 μg/L by one month.

Ammonia concentrations in water sampled prior to adding fish (time point zero) ranged from 0-600 μg/L (data not shown). No observable trends were seen between initial ammonia concentration and aquarium number. Municipal water used to fill the tanks was at an initial pH of 7.6, and increased to pH 8.7-9.0 after the addition of water conditioner and fish. An additional experiment demonstrated that the addition of water conditioner to municipal water caused a very small immediate increase in water pH, but that unregulated municipal water stabilizes at a higher pH with or without water treatment. The municipal water likely contained high chloramine (NH_2Cl or $NHCl_2$) concentrations, as chloramine is commonly used as a disinfectant to treat tap water and render it safe to drink. Chloramine is toxic to aquatic life (Kuhns 1987); thus, commercial water conditioners are commonly used to hydrolyze chloramine, releasing ammonia (Kuhns 1987). Other compounds are added to various water conditioner treatments to bind free ammonia, or ammonia is converted to nitrite by ammonia-oxiders in the biofilter. The time zero water samples were collected shortly after water conditioner was added to the aquaria, and thus it is likely that chloramines in the water had been broken down, yet the released ammonia had not been completely bound or converted to nitrite in all aquaria, resulting in variable ammonia concentrations.

Similar trends were seen for nitrite concentrations as were for ammonia (Figure 3). Aquaria with 1 fish showed peak nitrite concentrations at approximately 300 and 500 µg/L, while aquaria with 15 fish had nitrite concentrations at approximately 3400 µg/L and 4800 µg/L. As seen for ammonia, there was a 10X difference in nitrite concentration between the lowest and highest treatment groups. All other groups showed a general increase in peak nitrite concentration with an increase in fish biomass. An exception to this trend was one replicate aquarium housing 15 fish, which had a peak nitrite concentration below those seen for the aquaria housing 12 fish. Nitrite concentration in this aquarium was also at its highest one week prior to its replicate, and decreased to a negligible concentration (zero) two weeks before the other replicate. Nitrite concentrations were generally highest between 6-7 weeks, and were depleted to levels below 200 µg/L by approximately 2 months.

3.1.2 PCR ANALYSIS

PCR was performed on genomic DNA extracted from ceramic biofilter beads to yield thaumarchaeal 16S rRNA gene amplicons, as well as AOB and AOA *amoA* gene amplicons. PCR was conducted for AOB and AOA targeting marker genes using four primer sets (Table 3): amoA-1F/amoA-2R (AOB *amoA*), 771F/957R (thaumarchaeal 16S rRNA), Arch-amoAF/Arch-amoAR (AOA *amoA*), and CrenamoA23F/CrenamoA616R (AOA *amoA*). Two primer sets were used to amplify AOA *amoA* genes to improve success of *amoA* gene detection, because information about the specific types of AOA found in these particular environmental samples is limited. All results are based on visual inspection of PCR products via gel electrophoresis compared to positive and negative control samples

(discussed in 2.5). It is important to note that PCR was performed on DNA extracted from sponge samples collected from one "medium" aquarium (9 fish) and two "high" aquaria (12 and 15) for the 8-month time point, as beads were no longer available in the filters. PCR was also performed on sponge DNA in one replicate "high" aquarium at 11 months, as well as sponge samples collected at 16 and 20 months.

Table 3. AOB and AOA gene marker detection in DNA from aquarium filters in the variable fish biomass experiment.

AOB *amoA*
Primers: amoA-1F/amoA-2R

NH₃	Fish	1 wk	2 wk	1 mo	2 mo	4 mo	8 mo	11 mo	16 mo	20 mo
Low	1	+	+	+++	+++	++	+*	/	/	/
Low	3	++	+	+++	+++	+++	+++	/	/	/
Med	6	++	+++	+++	+++	+++	+	/	/	/
Med	9	+	+++	+++	+++	+++	+	/	/	/
High	12	+++	++	+++	+++	+++	+*	/	/	/
High	15	+++*	+++*	+++	+++	+++	+++*	+	-	-

AOA *amoA*
Primers: Arch-amoAR/Arch-amoAF

NH₃	Fish	1 wk	2 wk	1 mo	2 mo	4 mo	8 mo	11 mo	16 mo	20 mo
Low	1	-	-	-	+*	-	-	/	/	/
Low	3	-	-	-	-	-	-	/	/	/
Med	6	-	-	-	-	-	-	/	/	/
Med	9	+*	-	-	-	-	-	/	/	/
High	12	-	-	-	-	-	-	/	/	/
High	15	-	-	-	-	-	-	-	-	-

Thaumarchaeal 16S rRNA
Primers: 771F/957R

NH₃	Fish	1 wk	2 wk	1 mo	2 mo	4 mo	8 mo	11 mo	16 mo	20 mo
Low	1	-	+*	-	+/-	-	-	/	/	/
Low	3	-	+	+	-	-	++*	/	/	/
Med	6	-	+	+	-	+/-	+*	/	/	/
Med	9	-	+	+	-	-	-	/	/	/
High	12	-	+*	+	-	-	-	/	/	/
High	15	-	+	-	-	-	-	-	-	++

AOA *amoA*
Primers: CrenamoA23F/CrenamoA616R

NH₃	Fish	1 wk	2 wk	1 mo	2 mo	4 mo	8 mo	11 mo	16 mo	20 mo
Low	1	-	-	+*	+*	-	-	/	/	/
Low	3	-	++	+	-	-	++*	/	/	/
Med	6	-	+*	+*	-	-	+	/	/	/
Med	9	-	+*	+/-	-	-	+*	/	/	/
High	12	-	-	+*	-	-	-	/	/	/
High	15	-	-	+/-	-	-	-	-	-	-

Note: "+++" indicates strong, dark and clearly visible bands on an electrophoresis gel. "-" indicates no visible detection. "+/-" refers to difficult detection. Observations from replicate tanks have been combined; strongest detection between replicates was recorded. "/" indicates non-applicable samples from which genomic DNA was not extracted.* no detection for one of the replicates.

AOB *amoA* genes were detected consistently throughout most of the study, and were detected in the biofilters over the early aquarium establishment period (two months; Table 3). Visible PCR detection of AOB *amoA* genes began to decrease after eight months, and AOB *amoA* genes were not seen in post 1-year time points. Weak detection of thaumarchaeal 16S rRNA and *amoA* genes (CrenamoA23F/616R) was seen during the first month of the study, while low-ammonia conditions were stabilizing in aquaria (Table 3). AOA detection was minimal for the remainder of the study, and was associated with inconsistent trends in visual amplification. Some archaeal detection occurred at the 8-month time point (16S rRNA and *amoA)* and the 20-month time point (16S rRNA). Very minimal amplification was seen with the Arch-amoF/Arch-amoR primers, suggesting that the organisms in these aquaria were not well detected with those primers.

3.2   SUPPLEMENT EXPERIMENT RESULTS

3.2.1   WATER CHEMISTRY MEASUREMENTS AND ANALYSIS

Concentrations of ammonia and nitrite were measured in aquarium water over the first 50 days (approx. 7 weeks) of the study (Figure 4). Water chemistry was performed for the five supplement treatment groups, in addition to the aquaria fed with pellets. Measurements were taken weekly.
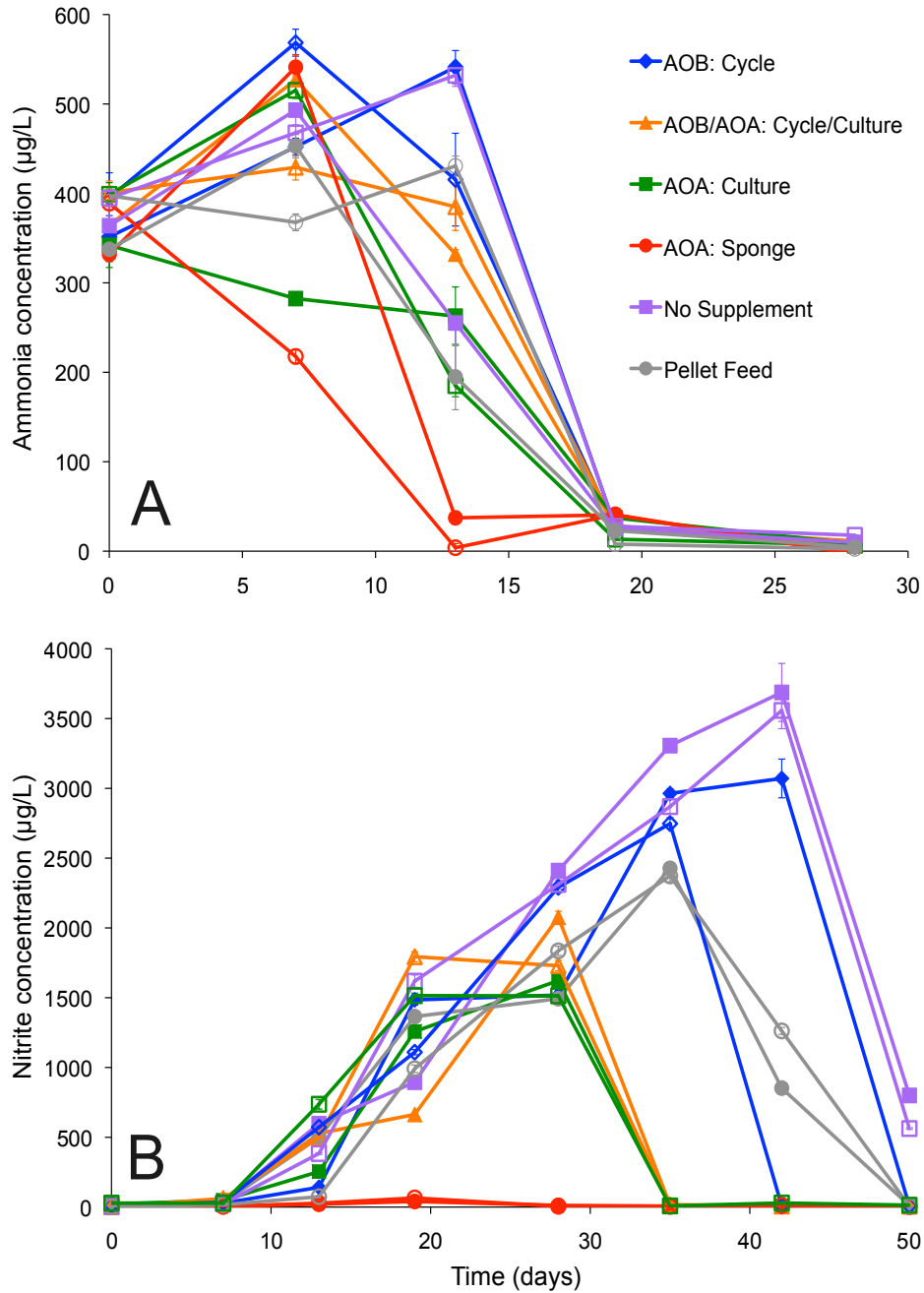
Figure 4. Ammonia and nitrite concentrations in water samples from aquaria in the supplement experiment. Triplicate readings for ammonia (A) and nitrite (B) were averaged for each time point. Error bars are standard deviation of triplicate measurements. Biological replicates are distinguished by filled and hollow markers. Day 0 refers to a sample set collected prior to adding fish to the newly set-up aquaria, but after water received conditioner.

In the supplement experiment, ammonia concentrations increased in most aquaria during early development, and were highest between the first and second weeks of the study (Figure 4A). Ammonia concentrations at time point zero were within 300-400 µg/L, which was likely due to the release of ammonia from chloramine (Kuhns 1987), as discussed in section 3.1.1. However, in this case, the water treatment may have been ineffective in binding ammonia, causing concentrations to increase initially in all tanks. Ammonia concentrations in all aquaria decreased to a level below 20 µg/L after 19 days of operation (Figure 4A). The Cycle treatment was associated with the highest ammonia concentrations, with one replicate at 570 µg/L, and the longest duration before ammonia began to decrease, followed by one replicate aquarium that did not receive supplements. One aquarium inoculated with an AOA-populated sponge, and another inoculated with an AOA enrichment culture, showed no increase in ammonia at any point, avoiding the peak phase; their replicates did show an increase by approximately 200 (sponge) and 100 (culture) µg/L from the starting ammonia concentration. Aquaria treated with pre-enriched sponge showed the earliest depletion in ammonia, as both replicates established a concentration below 50 µg/L by the second week of the experiment (Day 13), while all other groups had concentrations close to or above 200 µg/L by that time (Figure 4A). Analysis of variance of ammonia concentrations measured on Day 13 did not show statistical differences across the treatment groups (one-way ANOVA). However, ammonia concentrations were significantly different between aquaria that received archaeal supplements (AOA-enriched sponge, AOA laboratory culture, and AOA culture combined with Cycle) and aquaria that did not receive archaeal supplements (Cycle, no supplement, pellet-fed), $F_{(1,10)} = 5.073$, $p = 0.048$. Thus, it can be predicted that the addition of

archaeal supplements improved ammonia depletion. It is possible that significant differences in ammonia depletion across all treatment groups would be seen with an increase in replicate sample size (>2).

Nitrite concentrations increased after the first week of the experiment (Figure 4B). Aquaria with no supplement added showed the highest nitrite concentrations, at about 3,700 μg/L by 42 days, followed by aquaria treated with Cycle that showed peak concentrations at about 2,700 and 3,000 μg/L by 35 and 42 days, respectively. Although aquaria with pellet food had slightly lower nitrite concentrations, with peak concentrations just below 2,500 μg/L, high nitrite concentrations were not depleted until approximately 50 days, as seen with the aquaria lacking supplements and one aquarium treated with Cycle. Aquaria treated with an AOA enrichment culture, and with a combination of AOA culture and Cycle, were associated with lower nitrite concentrations, at or below 2,000 μg/L, and peak nitrite concentrations were fully depleted by 35 days into the experiment. Nitrite concentrations remained low for aquaria treated with AOA-populated sponge samples.

3.2.2   PCR ANALYSIS

PCR was performed on 1-10 ng of genomic DNA extracted from ceramic biofilter beads over an 8-month period, for thaumarchaeal 16S rRNA gene amplicons, as well as AOB and AOA *amoA* gene amplicons (Table 4). All data collection involved visual inspection of PCR products seen through gel electrophoresis, compared to positive and negative control samples (discussed in section 2.5).

Table 4. AOB and AOA gene marker detection in DNA from aquarium filters in the supplement experiment.

AOB *amoA* Primers: amoA-1F/amoA-2R								
Treatment	Rep. #	3 Days	2 wk	1 mo	2 mo	4 mo	6 mo	8 mo
AOB Cycle	1	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++
AOA Culture	1	-	++	+	+++	++	+/-	-
	2	+/-	-	++	+++	+	+	-
AOB/AOA Cycle/Culture	1	+++	+++	+++	+++	+++	++	+++
	2	+++	+++	++	+++	+++	++	+++
AOA Sponge	1	-	+/-	-	+	++	++	++
	2	-	+	-	-	+/-	+	+++
No Supplement	1	-	-	+	+++	+	+	-
	2	-	+++	+++	+++	+++	+	+
Pellet	1	+	++	++	+++	+	+++	++
	2	-	-	+	+++	++	+	+

AOA *amoA* Primers: Arch-amoAF/Arch-amoAR								
Treatment	Rep. #	3 Days	2 Wks	1 mo	2 mo	4 mo	6 mo	8 mo
AOB Cycle	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
AOA Culture	1	++	+/-	+	-	-	-	-
	2	+	-	+/-	-	-	-	-
AOB/AOA Cycle/Culture	1	++	-	-	-	-	-	-
	2	+	-	+/-	+/-	-	-	-
AOA Sponge	1	+++	++	++	+++	+	+++	++
	2	+++	++	++	++	+++	++	+
No Supplement	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
Pellet	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-

Thaumarchaeal 16S rRNA Primers: 771F/957R								
Treatment	Rep. #	3 Days	2 wk	1 mo	2 mo	4 mo	6 mo	8 mo
AOB Cycle	1	-	+/-	-	-	-	-	-
	2	-	-	-	-	+/-	+	-
AOA Culture	1	+	+	++	++	+	+	+
	2	+	+	+	++	+	+	+
AOB/AOA Cycle/Culture	1	++	-	-	+	-	+/-	-
	2	+	-	-	+	+	-	-
AOA Sponge	1	++	+	++	+++	+++	++	+++
	2	+++	+++	++	+++	+++	+++	+++
No Supplement	1	-	-	-	-	-	-	+/-
	2	-	-	-	-	-	-	+
Pellet	1	-	-	-	-	-	-	-
	2	-	-	-	-	+/-	+	+

AOA *amoA* Primers: CrenamoA23F/CrenamoA616R								
Treatment	Rep. #	3 Days	2 Wks	1 mo	2 mo	4 mo	6 mo	8 mo
AOB Cycle	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
AOA Culture	1	++	+	-	+	+	+/-	+
	2	++	-	+	+	+	+	+/-
AOB/AOA Cycle/Culture	1	++	+/-	-	-	-	-	-
	2	+/-	-	++	-	+	-	+
AOA Sponge	1	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	++	+++	+++	+++
No Supplement	1	-	-	-	-	-	-	-
	2	-	-	+	-	-	-	-
Pellet	1	/	-	-	/	-	-	-
	2	-	-	-	/	-	-	+/-

Note: "+++" indicates strong, dark and clearly visible bands on an electrophoresis gel. "-" indicates no visible detection. "+/-" refers to difficult detection. "/" indicates non-applicable samples from which genomic DNA was not extracted.

AOB *amoA* genes were observed in all aquaria in the supplement experiment (Table 4).

Replicate tanks that were treated with Cycle (AOB) supplement, both with and without the

additional AOA culture, showed the strongest visible detection of AOB *amoA* gene

amplicons, and strong detection was consistent over the 8-month period. Prominent AOB

amoA gene amplification was detected in aquaria treated with AOA culture only, with

pellet feed, and those lacking supplements; however, strong detection was not visible in

each replicate at all time points, especially towards the end of the experiment for the

culture-treated aquaria and control aquaria. On the contrary, AOB *amoA* genes were not

well detected in sponge-treated aquaria during the early months of the experiment, while

the aquaria were establishing stable water chemistry levels, but showed an increase in

detection towards the end of the experiment.

Strong thaumarchaeal 16S rRNA and *amoA* gene amplification was observed in the

sponge-inoculated aquaria throughout the entire experiment (Table 4), indicating that AOA

were consistently detected in those environments over the eight months. Aquaria

inoculated with an AOA enrichment culture also showed consistent thaumarchaeal

detection; however, gene amplicons were not strong and amplification was often difficult

to obtain. For sponge-treated aquaria, all three archaeal primers produced strong gene

amplification. For culture-treated aquaria, 771F/957R and CrenamoA23F/616R were

associated with consistent amplification, yet amplification was rarely obtained using the

Arch-amoAF/R primers, especially in time points later than one month. Thaumarchaeal

16S rRNA and *amoA* genes were detected in aquaria treated with Cycle and AOA culture,

particularly following early inoculation (Day 3); detection in later time points was

inconsistent. Minimal AOA detection with all three primers was seen in all other aquaria;

all gene amplicons appeared as faint bands on the gels and were not associated with any recognizable patterns in AOA detection.

3.2.3   MICROBIAL ACTIVITY

Ammonia-oxidizing activity of Archaea and Bacteria on biofilter beads were measured to help determine whether AOB, AOA, or both, were active in the filters. Ammonia oxidation rates were observed over a 5-day period (Figure 5) for beads sampled from aquaria inoculated with AOA-enriched sponge, showing strong AOA *amoA* gene detection (Table 4), and aquaria inoculated with Cycle, showing strong AOB *amoA* gene detection (Table 4). Bacterial and archaeal ammonia-oxidizing activities were distinguished using microbial inhibitors; ATU inhibited AOB and PTIO inhibited AOA. Activity rates for AOA and AOB were compared to samples lacking inhibitors.
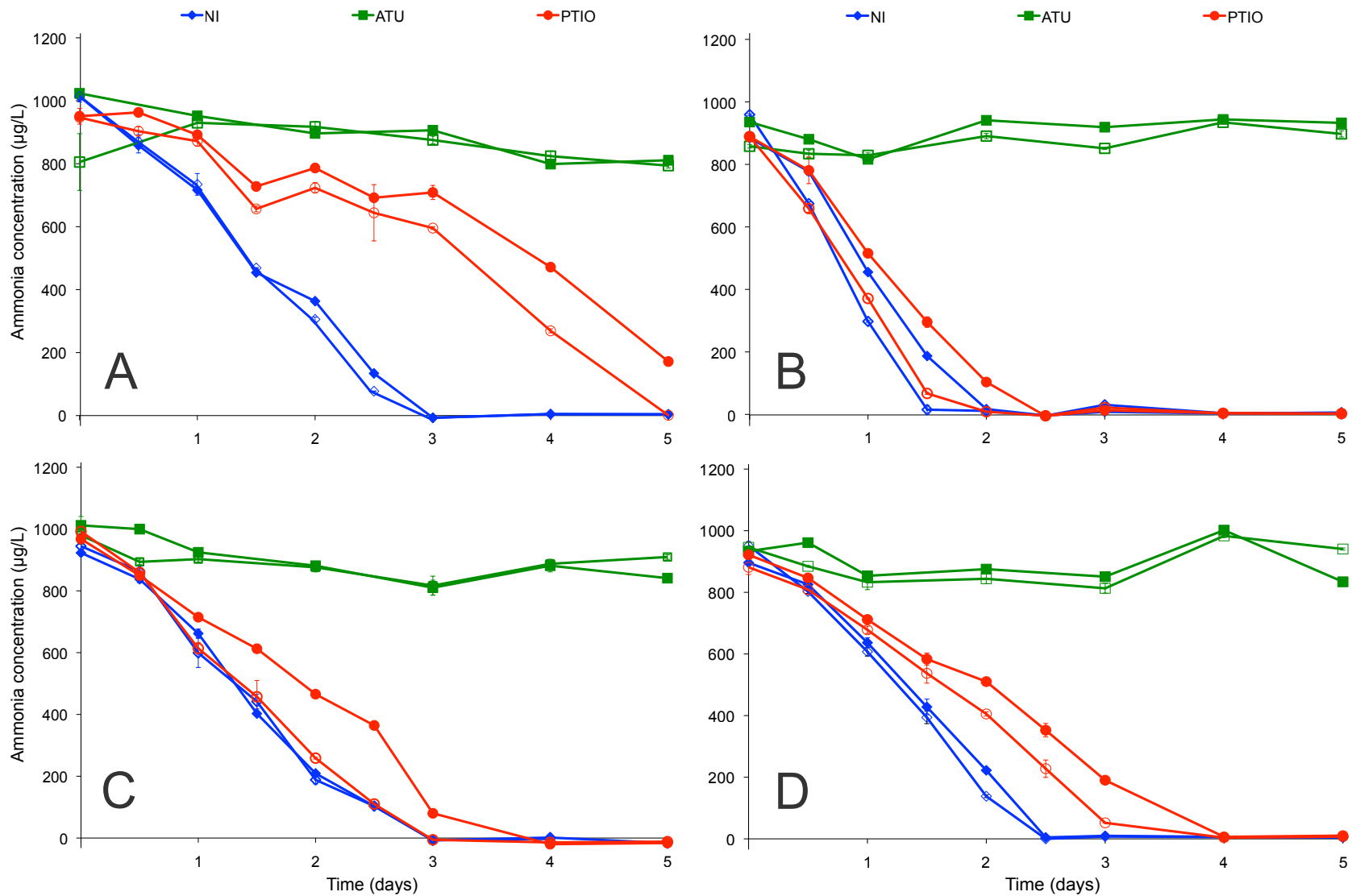
Figure 5. Ammonia oxidation activity of microorganisms in aquarium biofilters from the supplement experiment. Beads were sampled from aquaria inoculated with AOA-enriched sponge (A and B) and Cycle (C and D). Replicate trials were performed for each treatment. Replicates are distinguished by filled and hollow markers. A and B are replicate aquaria A6 and A10, respectively, both inoculated with AOA-enriched sponge. C and D are replicate aquaria A1 and A8, respectively, both inoculated with Cycle.

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Ammonia-oxidizing activity was observed in all samples lacking inhibitors (Figure 5). Rates in total ammonia oxidation differed between biofilter groups, and between replicate treatment groups. Aquaria A6 (A) and A1 (C) showed depletion of 1000 µg/L of NH_4Cl by 3 days. Aquarium A10 (B) depleted ammonia in 1.5 days, and aquarium A8 (D) depleted ammonia in 2.5 days. Bacterial ammonia-oxidizing activity was seen in all samples treated with PTIO (AOA inhibited; Figure 5). Samples from replicate aquaria inoculated with AOA-enriched sponge (A6 and A10) showed distinct rates of AOB ammonia oxidation. One replicate of A6 (A) beads showed depletion in ammonia by Day 5, while the other replicate did not even fully deplete ammonia over the five days. Meanwhile, ammonia was depleted by AOB in both replicates of A10 (B) beads between 1.5-2.5 days, demonstrating increased ammonia oxidation efficiency with these biofilm samples. Ammonia oxidation seen between replicate tanks, A1 and A8, were more similar to each other than that seen between A6 and A10 (Figure 5). For beads taken from both aquaria inoculated with Cycle, A1 and A8 (C and D), AOB in the biofilm depleted ammonia between 3-4 days. Ammonia depletion showed a faster trend in one replicate with A1 beads (C) than the other, with one replicate showing ammonia oxidation rates parallel to that of the uninhibited samples, indicating full AOB activity within the biofilm. Ammonia oxidation in both replicates with beads from A8 (D) was less efficient than the uninhibited samples. Control samples consisting of media with and without inhibitors showed minimal to no decrease in ammonia concentration (data not shown), indicating that contamination did not occur.

Very minimal activity was seen for all samples treated with ATU (Figure 5). While this would be indicative of strong AOB activity, it is predicted that ATU inhibited both AOA and AOB activity in these trials. In a later experiment performed by Laura Sauder,

activity of AOA in a freshwater laboratory enrichment culture (the culture used as a supplement in this experiment) was inhibited by 10 µM of ATU (unpublished observations). This laboratory culture was originally enriched from the source of AOA-dominated sponge that was used in the supplement experiment (aquaria A6 and A10). Even though ATU can inhibit both AOB and AOA activity at high concentrations, the concentration used in this experiment (10 µM) was chosen based on previous testing with *N. viennensis* and *N. multiformis*, suggesting maximal AOB inhibition and minimal AOA inhibition (Shen *et al.* 2013). However, *N. viennensis* is a soil microorganism, with the potential to respond differently to concentrations of inhibitors than AOA found in freshwater environments. Thus, the AOA in these samples may have been more sensitive to ATU than *N. viennensis*.

Quantitative PCR (qPCR) was used to quantify thaumarchaeal 16S rRNA genes in DNA extracted from beads in aquaria inoculated with AOA-populated sponge, one from each of A6 and A10 tanks. DNA was extracted from beads sampled at the 8-month time point. Primers 771F/957R were used for quantification. DNA extracted from A6 and A10 had 2560 and 1978 thaumarchaeal 16S rRNA gene copies per ng of DNA, respectively, indicating that biofilm from the two aquaria had similar concentrations of AOA. Thus, any differences in ammonia-oxidation rates between samples from the replicate aquaria are not likely due to large disparities in AOA concentration in the biofilm.

3.2.4 DENATURING GRADIENT GEL ELECTROPHORESIS

Denaturing gradient gel electrophoresis (DGGE) analysis was performed to observe changes in AOA communities in selected aquarium biofilters over an 8-month period. The *amoA* gene amplicons in DNA from aquaria supplemented with AOA-cultivated sponge

and an AOA enrichment culture were subject to fingerprint analysis (Figure 6). DGGE

analysis was not performed on samples from other supplement treatment groups because

they did not show consistent archaeal *amoA* gene detection by PCR over the study (Table

4).
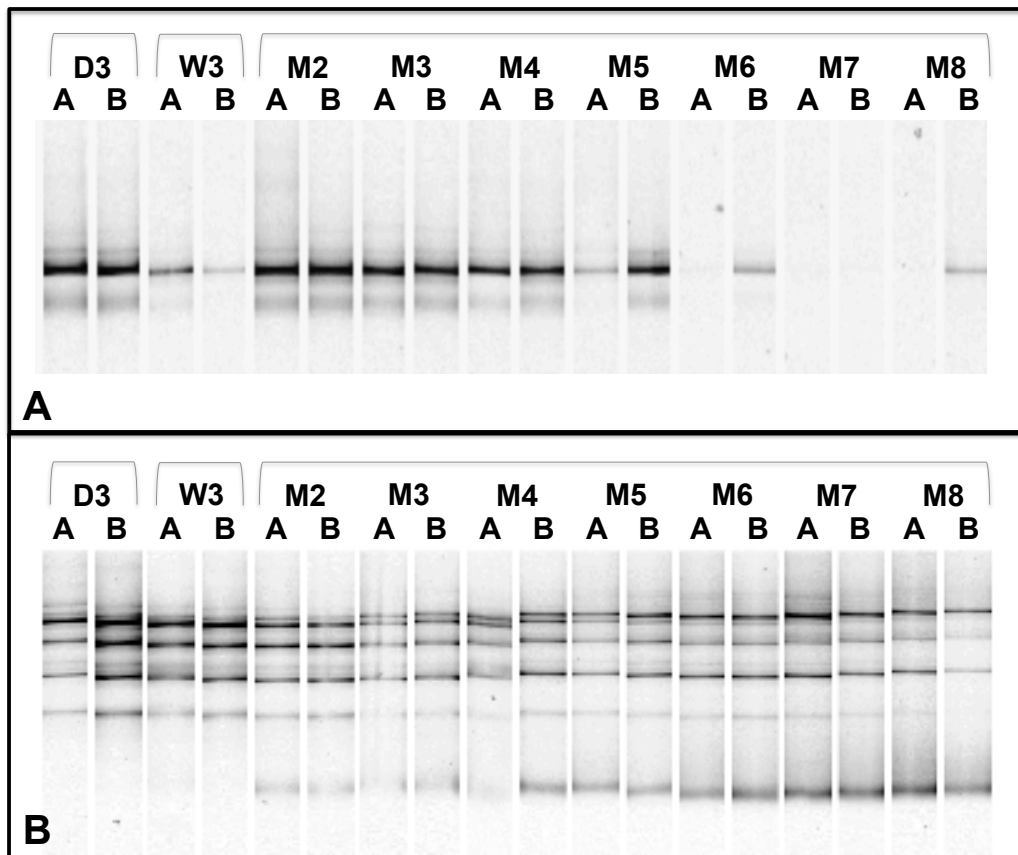
Figure 6. Denaturing gradient gel electrophoresis analysis of archaeal *amoA* gene amplicons. Fingerprints from AOA-culture inoculated aquarium samples (A) and sponge-treated aquarium samples (B) were aligned using Gelcompar II. D3,W3 and M2-8 indicate daily, weekly and monthly time points, respectively. A and B represent biological replicates of DNA yielded from similarly treated aquaria.

Samples from the two treatment groups showed distinct profiles from one another, with

samples from the sponge-treated aquaria (Figure 6B) demonstrating more complexity than

samples from the culture-inoculated aquaria (Figure 6A). DNA beads in sponge-inoculated

aquaria showed a potential shift in G+C content, and thus AOA community; four major bands were observed in all time points, while a fifth band appeared in the profile by the second month of the study, and became more intense over time (Figure 6B). Variability between samples from the culture-inoculated aquaria was low over time, suggesting no change in AOA community; one major band was seen in all samples, and a potential second band was seen in samples that had good amplification of *amoA* genes with PCR (Figure 6A). Fingerprints could not be produced for some samples collected at the later time points due to very low PCR amplification, which is why they are displayed without a visible band (Figure 6A).

All fingerprints were compared to a control pattern for *amoA* gene amplicons obtained from DNA of a mature AOA-enriched sponge (source of sponge and laboratory enrichment culture used in the supplement experiment; data not shown). The control *amoA* gene amplicons were run alongside the samples in this experiment for DGGE analysis. Through visual comparison of fingerprints from the sponge-inoculated and culture-inoculated samples in relation to the control *amoA* fingerprint, it was observed that the single band in the culture-inoculated samples (A) was the same as the third band from the top in the sponge-inoculated samples (B). Furthermore, the fingerprints for the sponge-inoculated samples extracted at Day 3 and Week 3 time points appeared similar to the control *amoA* fingerprint from the same aquarium sponge source (data not shown). The control fingerprint was produced with *amoA* amplicons in DNA extracted over one year prior to this experiment, indicating stability of the AOA community within this biofilter sponge.

3.3 HOME AQUARIUM EXPERIMENT

3.3.1 PCR DETECTION

PCR was performed on genomic DNA extracted from ceramic biofilter beads and biofilter sponge collected from filters of three participating home aquaria. Thaumarchaeal 16S rRNA and AOA *amoA* genes were amplified using primers 771F/957R, and both of Arch-amoAF/Arch-amoAR and CrenamoA23F/CrenamoA616R, respectively. AOB *amoA* genes were amplified using AmoA-1F/AmoA-2R. The 16S rRNA and *amoA* gene amplicons were visually observed using gel electrophoresis for samples collected during this study (Table 5).

Table 5. AOB and AOA gene marker detection in DNA from aquarium filters in the home aquarium experiment

AOB *amoA*
Primers: AmoA-1F/AmoA-2R

Aquarium #	Sample	Pre-Fish	Post-Fish	Week 1	Week 2	Week 3	Week 4
Home 1	Bead	-	-	-	-	-	+/-
	Sponge	-	-	+	-	-	+
Home 2	Bead	+	+	+++	+++	+++	+++
	Sponge	+	+/-	+++	+++	+++	+++
Home 3	Bead	-	+++	-	-	-	-
	Sponge	+	+++	-	-	-	-

AOA *amoA*
Primers: Arch-amoAF/Arch-amoAR

Aquarium #	Sample	Pre-Fish	Post-Fish	Week 1	Week 2	Week 3	Week 4
Home 1	Bead	-	-	+/-	+	++	+++
	Sponge	-	-	-	-	+/-	-
Home 2	Bead	-	-	+/-	+	+	+++
	Sponge	+/-	+	+	+/-	-	-
Home 3	Bead	-	-	-	-	-	+/-
	Sponge	-	-	-	-	-	-

Thaumarchaeal 16S rRNA
Primers: 771F/957R

Aquarium #	Sample	Pre-Fish	Post-Fish	Week 1	Week 2	Week 3	Week 4
Home 1	Bead	+/-	-	+	++	+++	+++
	Sponge	+	-	-	+	+	+
Home 2	Bead	+/-	+	+	+	+	++
	Sponge	-	+/-	+	-	+/-	-
Home 3	Bead	-	-	-	-	+	+
	Sponge	+	-	+/-	+	+/-	-

AOA *amoA*
Primers: CrenamoA23F/CrenamoA616R

Aquarium #	Sample	Pre-Fish	Post-Fish	Week 1	Week 2	Week 3	Week 4
Home 1	Bead	-	-	-	+/-	++	+++
	Sponge	-	-	-	-	-	-
Home 2	Bead	-	-	-	+	+	++
	Sponge	-	-	-	-	-	-
Home 3	Bead	-	-	-	-	-	-
	Sponge	-	-	-	-	-	-

Note: "Pre-Fish" refers to samples collected 24 hours before fish were added to aquaria. "Post-Fish" refers to samples collected 48 hours after fish were added to aquaria. "+++" indicates strong, dark and clearly visible bands on an electrophoresis gel. "-" indicates no visible detection. "+/-" refers to difficult detection.

The three participating aquaria showed distinct PCR results, as each aquarium demonstrated a unique variation in AOA and AOB gene marker detection (Table 5). In Home Aquarium 1, thaumarchaeal 16S rRNA genes were amplified in bead biofilm DNA by the first week of aquarium development, and signal detection increased over subsequent weeks. Weak amplification of thaumarchaeal 16S rRNA genes was seen in sponge DNA, and low detection was seen prior to adding fish. AOA *amoA* genes were successfully amplified in Home Aquarium 1 using both primers, but with detection only from the bead DNA samples. AOB *amoA* genes were not detected in DNA extracted from the beads in Home Aquarium 1, and low amplification occurred in only two DNA samples extracted from sponge.

On the contrary, AOB *amoA* detection was very strong in Home Aquarium 2, with "+++" detection as early as one week into the experiment (Table 5). Weak archaeal 16S rRNA and *amoA* genes were detected in DNA from this aquarium during the first three weeks, but detection increased with the use of all three archaeal primers by samples collected at the fourth week. Archaeal gene marker detection was more consistent in the bead DNA than the sponge DNA.

Home Aquarium 3 had very little ammonia-oxidizing microbial detection overall (Table 5). There was strong AOB *amoA* gene detection in both the beads and sponge in the post-fish sample, but that detection did not persist in the subsequent samples. Weak detection for thaumarchaeal 16S rRNA genes was seen by the third week of the study in DNA extracted from beads, and these genes were detected weakly in the sponge for other time points. Strong amplification of bacterial 16S rRNA genes was obtained from all DNA samples for Home Aquaria 1, 2 and 3 collected from all time points (data not shown).

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4 DISCUSSION

4.1 AOB DETECTED IN START-UP AQUARIA

The variable fish biomass experiment was designed to look at differences in ammonia concentration on biofilter biofilm development. Fish biomass correlated positively with peak ammonia concentrations that occurred during the first month of the experiment (Figure 3). Aquaria with low fish biomass (i.e., 1-3 fish) had relatively low peak ammonia concentrations in comparison to aquaria with high fish biomass (i.e., 12-15 fish), demonstrating that early high ammonia concentrations increased with increasing fish biomass. Nitrite concentrations showed the same correlation with biomass, as expected, because ammonia is converted to nitrite in aquaria. There was a higher accumulation of nitrite than ammonia in the aquaria. Increased accumulation of nitrite was expected because of a lag between the development of ammonia-oxidizing and nitrite-oxidizing microbial communities within the filter systems (Figure 2). In start-up aquaria, oxidation of ammonia is more efficient earlier on than nitrite oxidation, because ammonia accumulation in tanks, driving the growth of ammonia-oxidizers, precedes nitrite accumulation.

Based on niche separations previously reported for AOB and AOA in aquarium environments with stable levels of high and low ammonia (Sauder *et al.* 2011), respectively, I hypothesized a strong detection of AOB in the filters during early aquarium development, specifically following initial high-ammonia concentrations. I predicted that AOB would be more abundant in biofilters of aquaria showing high peak ammonia concentrations, as seen in the high fish biomass tanks, than those with low peak ammonia

concentrations, as seen in the low fish biomass tanks. I further predicted that strong detection of AOA would be seen in filters following phases of ammonia and nitrite accumulation, once low ammonia conditions had been established. The duration prior to AOA detection was expected to be less in aquaria with lower peaks in early ammonia concentrations than those with higher peaks in ammonia concentrations. PCR detection of ammonia-oxidizing microorganisms (Table 3) demonstrated that AOB are likely the dominant ammonia-oxidizers in all aquarium filters tested throughout and following aquarium establishment under the conditions of this experiment, regardless of fish biomass. AOB were detected in nearly all filters by the first week of the study. They persisted in the filters throughout initial phases of high ammonia and nitrite, and continued to thrive beyond stabilization of ammonia and nitrite at low concentrations. Meanwhile, AOA were only detected consistently within the first month of the study during early ammonia oxidation, and their detection was observed to be much less than AOB. By eight months, consistency in AOB detection decreased, as AOB were not detected in all aquaria at this time point, nor were they detected in the aquarium remaining alongside the supplement experiment at the 16-month time point. Archaeal gene abundance increased as AOB became less prominent; thaumarchaeal 16S rRNA genes were detected in DNA extracted at a 20-month time point. However, AOA *amoA* genes were not amplified alongside the 16S rRNA genes at this time, resulting in an unclear determination as to whether or not AOA were beginning to establish in filter biofilm. This final time point indicates that AOA may maintain nitrification far beyond establishment of low-ammonia conditions, but the reason for such a long duration prior to AOA detection is unclear.

For some time points, neither AOB nor AOA genes were detected by PCR in the DNA samples (Table 3). DNA extractions were considered successful, as a screen for bacterial 16S rRNA genes showed strong amplification in a subset of samples (2-week and 16-month time points) that lacked AOB and AOA gene marker detection (bacterial 16S rRNA PCR data not shown). The primers used to detect AOB and AOA gene markers may not have been well suited for amplification of these genes, especially if they were in low abundance in the DNA samples from this experiment. Particularly for AOA, few genomes have been fully sequenced, and thus, there are limited available primers for AOA *amoA* gene amplification. The primers used in this experiment may have been designed for genes with low sequence homology to the genes in the DNA from this experiment, possibly causing inconsistent amplification.

For those filter samples collected late in the experiment that showed no detection of ammonia-oxidizing microorganisms (i.e., 8 and 16-month time points; Table 3), it is important to note that these aquaria were maintained long-term with minimal fish death. Thus, ammonia oxidizers must have been in the filters for nitrification to have continued successfully. Major disruptions to nitrogen cycling, such as death to ammonia-oxidizing microbial populations in the biofilters, would have resulted in high ammonia accumulation. While imperfect primers may have contributed to poor amplification, it is suspected that Cyanobacteria dominated the aquarium systems, due to the fact that these aquaria were maintained for several months without changing the water. In some tanks, red slime algae, suspected to be Cyanobacteria (Weber 2010), coated the aquarium walls and biofilter media. Cyanobacteria thrive in high nitrate aquatic systems with ample lighting (Weber 2010). A water maintenance check for the aquarium facility showed high nitrate

concentrations (6.5 mg/L for the remaining "high" aquarium after 19 months) late in the study as expected, since nitrate accumulates over time (data not shown). While the fish stocking densities were not high enough to produce unsafe nitrate concentrations, nitrate levels could have been high enough to promote cyanobacterial growth. If the population of these bacteria were more dominant in the filters than the ammonia-oxidizers, it may have been difficult to detect genes for AOB or AOA using PCR. While algal growth was not as prolific in the tanks at 8 months, as opposed to 16 months, high cyanobacterial concentrations in the filters may still have been the cause for a lack of detection of ammonia-oxidizers at this time. Further investigation using primers specific for cyanobacterial organisms common to aquaria, and qPCR for ammonia-oxidizing microbial quantification, would support this possibility.

Because no previous study has examined AOA in start-up aquarium biofilters, it cannot be determined if AOA typically begin to thrive in the filters closer to the establishment of low ammonia and nitrite concentrations than observed in this experiment. Since AOA have been previously discovered dominating mature aquarium biofilter systems (Sauder *et al.* 2011), it is known that AOB are typically not the prominent ammonia oxidizers in established low-ammonia aquaria. Thus, perhaps an extension of the variable fish biomass experiment would have led to a clear dominance of AOA in the filters. The home aquarium experiment (discussed below) was aimed to further investigate the possibility of AOA establishment in biofilters during aquarium development, as there are additional factors that may not have enabled AOA to populate the filters earlier on in this study. Firstly, AOA may not have been introduced into the aquarium system early on or in high abundance; aquaria were kept in a clean, controlled facility, and researchers

wore gloves while performing feeding, sampling, and maintenance procedures. AOA have been found in a broad range of environments, many of which could be an original source of Archaea in household and commercial aquaria, such as those aquaria analyzed in previous studies (Sauder *et al.* 2011). Live aquarium plants, aquarium gravel and rocks, and fish themselves could potentially introduce Archaea into aquaria. Furthermore, Archaea have recently been identified as part of the human skin microbiome; Thaumarchaea were isolated from surfaces in hospital rooms and on human skin (Probst *et al.* 2013), as detected by 16S rRNA and *amoA* gene amplification. Thus, interactions between skin and the aquarium environment through regular maintenance may be an important source of Archaea. Cleanliness in the aquatics facility housing these aquaria during the variable fish biomass experiment, in addition to precautions taken to avoid cross-contamination between filters, may have reduced introduction of Archaea into the aquaria earlier on.

It is important to note that the aquarium showing archaeal detection at the 20-month time point was maintained alongside the aquaria that were part of the supplement experiment. AOA thrived in aquaria in the supplement experiment (Table 4), as they were initially inoculated into multiple aquarium filters. If AOA are indeed introduced into filters by regular interaction with the researcher and the surrounding environment, it is likely that this would have occurred for the remaining aquarium during this stage in the experiment. The aquarium was in close proximity to other tanks in the supplement experiment containing AOA, and more contact inevitably occurred between the researcher and AOA from other aquaria in the supplement experiment. Even with precautionary measures taken to avoid cross-contamination, incidental introduction of AOA into this filter was more

likely to occur with AOA in the immediate surrounding environment. During the majority of the variable fish biomass experiment, AOA in the surrounding environment were presumably rarer, since all tanks set-up during the first ten months of the experiment contained little to no Archaea.

Another potential reason for a lack of early AOA detection in the filters is the possibility of insufficient organic carbon in the aquaria, due to minimal food decay in the tanks. While most studies have indicated the ability for AOA to fix inorganic carbon, including that seen with *N. maritimus* (Könneke *et al.* 2005), some have suggested an ability for AOA to use organic carbon, as previously discussed (Ingalls *et al.* 2006; Tourna *et al.* 2011; Wessén *et al.* 2010). During the variable fish biomass experiment, fish were fed with a pre-weighed amount of food, and the tanks contained few accessories that could trap uneaten food. Thus, these tanks did not have excess decaying food found commonly in unregulated tanks. The fish food may have contained organic compounds that AOA use as sources of energy or carbon, which these systems would have lacked. In addition, the type of food used in this experiment may have not had the appropriate carbon ingredients that AOA utilize. Alongside these factors, temperature, pH, and dissolved gas concentration could also all impact archaeal growth throughout the study, as different organisms have shown variations in preferences to these components (Stahl and de la Torre 2012 and references therein).

4.2 AMMONIA REDUCTION WITH AOA SUPPLEMENTS

The supplement experiment was conducted as a follow-up to the variable fish biomass experiment, because conclusions about the key players in early nitrification were

not definitive, due to a lack of AOA populations in the filters during the main portion of the study. The supplement experiment aimed to look at differences in early ammonia oxidation between newly set up aquaria that were inoculated with AOB and AOA supplements. Based on speculations from the variable fish biomass experiment, I hypothesized that the introduction of AOA via supplements into new aquarium environments would improve initial ammonia-oxidation efficiency and reduce early peak ammonia concentrations. My results confirm this hypothesis; aquaria that received AOA supplements showed significant improvements in ammonia depletion over aquaria that did not receive AOA supplements. Filters that were inoculated with AOA-enriched sponge were associated with the earliest reduction in ammonia in comparison to other treatment groups (Figure 4). Furthermore, one replicate from each of the AOA sponge-treated and AOA culture-treated aquaria did not show any peak in ammonia concentrations above the initial amounts, suggesting that AOA present in these aquaria helped to prevent ammonia accumulation. On the contrary, samples that were inoculated with Cycle (AOB) showed the highest accumulations in ammonia, alongside tanks that did not receive supplements, suggesting that AOB had low impact on ammonia oxidation efficiency. Although the differences in ammonia depletion were not significant between the individual treatment groups, an increase in the number of replicates within the experimental design of future research would help to validate this outcome in larger sample sets.

Aquaria that were inoculated with established sponge introduced a fully developed microbial consortium into the new biofilter systems. Essentially, AOA, AOB and nitrite oxidizers were supplemented together. Although AOB were present in the sponge supplement, it is likely that AOA were contributors to ammonia oxidation, since consistent

AOA detection was seen throughout the eight months of the experiment, using all three archaeal primers (Table 4). This indicates that AOA continued to thrive in the tanks beyond establishment, when inoculated early on. Even though small AOB populations were likely present in the sponge, they were poorly detected over the early aquarium development period (two months; Table 4). Increasing AOB detection towards the later months of the study did not appear to impact AOA detection, as strong AOA detection remained consistent. These trends were also seen in the filters that received AOA-culture inoculations (Table 4). Even though AOA detection in culture-treated tanks was not as strong as in the sponge-treated tanks, and AOA were only detected consistently using two out of three primer sets, AOA did persist in these tanks. Lower detection was likely due to low AOA abundance in the initial inoculum, in comparison to the heavily AOA-populated sponge inoculation.

The results seen with ammonia oxidation extended through to the nitrite oxidation phase. Sponge-inoculated tanks were associated with essentially no nitrite accumulation. This was expected because the sponge was cultivated with a fully established microbial consortium, containing nitrite-oxidizing bacteria. Aquaria inoculated with Cycle, as well as those that did not receive supplements, showed nitrite accumulations over 5 fold those for ammonia oxidation. This is expected with the no supplement control, but Cycle supposedly contains *Nitrobacter* (Nowak *et al.* 2008), and thus should have shown increased efficiency in nitrite oxidation. Despite this, it was interesting to see that aquaria treated with pellet food (showing AOA in later time points), a combination of AOA and AOB, as well as the AOA culture alone, were all associated with nitrite concentrations below those

seen in aquaria with Cycle (Figure 4). This could suggest that AOA have an impact on the establishment of a more effective biofilter community as a whole.

Although water chemistry analysis for this experiment supports the concept that AOA may improve early aquarium nitrification, arguments challenging this idea could be made based on PCR detection of ammonia-oxidizers. If AOA were indeed improving ammonia-oxidation efficiency during early aquarium phases, a combination of AOA and AOB should show greater improvements (Figure 4), similar to those seen with the AOA sponge treatments. However, it is likely that AOB were more abundant than AOA in these filters. AOA were only supplemented with a small volume of an enrichment culture, similar to the filters treated with AOA culture only, while AOB were taken from a commercial supply of *Nitrosomonas* in Cycle (Nowak *et al.* 2008). PCR detection helps to demonstrate this, as AOB were strongly detected in aquarium filters treated with both microorganisms; AOA detection was less prominent and less consistent (Table 4). It is likely that AOB outcompeted AOA simply due to higher concentrations in initial inocula.

Although the results from the supplement experiment helped guide understanding of the roles of AOA and AOB in early aquarium nitrification, the results are still unclear. Batches of Cycle comprised of *Nitrosomonas* and *Nitrobacter* have been found to contain additional bacterial species (Nowak *et al.* 2008) that are not typically expected in aquarium environments. These additional bacteria could have an impact on the nitrification ability of AOB, AOA, or the biofiter community as a whole. In addition, AOA may be relying on other components of a pre-established biofilm that are not available in new aquaria. Experimentation with pure cultures of AOA and AOB in newly set-up aquaria could help to identify some of these factors and provide a more definitive understanding.

To address a question proposed in the variable fish biomass experiment about a correlation between food source and archaeal establishment in the filters, food type did not appear to have impact on archaeal establishment in these aquaria. Both types of food were associated with typical ammonia trends (Figure 5). With either food, AOB appeared to dominate filters, but lessened towards the eight-month time point (Table 4). Archaea were present in the pellet-fed tanks toward the end of the study (Table 4), suggesting that they could become more dominant over time, but they were not the prominent ammonia-oxidizers in the filter system at this stage.

4.3   DUAL ACTIVITY: AOB AND AOA

Ammonia-oxidizing activity was analyzed for microorganisms in the biofilm of beads from aquaria in the supplement experiment. Ammonia-oxidizing microbial activity was tested in biofilm of aquaria supplemented with AOA-enriched sponge (A6 and A10; Figure 5A and 5B) and Cycle (A1 and A8; Figure 5C and 5D). The aim was to observe differences in microbial activity between filter biofilm with strong AOA detection and strong AOB detection (Table 4). PTIO and ATU were used to inhibit AOA and AOB ammonia-oxidizing activity, respectively. It was expected that biofilm from aquaria supplemented with Cycle would show only AOB activity (Figure 5C and 5D), because AOB were the only ammonia-oxidizers detected with PCR (Table 4). Thus, ammonia-oxidizing activity would be seen in samples treated with PTIO (AOA-inhibited), as well as uninhibited controls. Biofilm from beads in aquaria supplemented with established sponge were expected to show ammonia oxidation when samples were treated with either PTIO or ATU, as well as uninhibited controls (Figure 5A and 5B), as both AOB and AOA were detected in the bead biofilm prior to performing activity experiments (Table 4). It was

predicted that less activity would be seen in flasks with added inhibitors compared to uninhibited controls, demonstrating a loss in contributing activity from the inhibited ammonia-oxidizing microbial counterpart. For all experiments, direct detection of archaeal activity was not obtained for any of the biofilm samples, since ammonia concentrations did not decrease. A lack of observed archaeal activity was likely due to the potential inhibition of both AOB and AOA by ATU, as discussed in section 2.7.

Even though archaeal ammonia-oxidizing activity could not be directly measured by inhibiting AOB, it could be indirectly observed by looking at differences in ammonia oxidation rates between uninhibited samples and PTIO-treated samples (AOA inhibition). It can be inferred that any decrease in ammonia oxidation efficiency in PTIO samples results from a lack of AOA contribution to the combined rate. Ammonia oxidation was seen in biofilm from aquaria treated with Cycle (A1/A8) in all uninhibited and PTIO-treated trials (Figure 5). This indicates that AOB were actively oxidizing ammonia in these environments. There was some lag in ammonia decline observed with samples treated with PTIO compared to uninhibited controls, more so in one replicate tank (A8; Figure 5D) than the other (A1; Figure 5C). However, it is predicted that there was some AOB inhibition with PTIO, because AOA were not detected with PCR in these aquaria (Table 4). Although the concentrations of PTIO used in this study were well below published concentrations for AOB inhibition (Shen *et al.* 2013), AOB populations from a heterogeneous aquarium filter system may be affected differently by PTIO than organisms in pure culture. Furthermore, if AOB populations in bead biofilm studied here were less abundant than AOB concentrations used in published studies, a small impact of PTIO may have been more noticeable with fewer bacteria.

Ammonia oxidation was seen with biofilm on beads sampled from aquaria supplemented with AOA-enriched sponge (A6/A10), specifically in uninhibited and PTIO treated incubations (Figure 5A and B). This indicates that AOB were actively oxidizing ammonia in these samples. In biofilm tested from one replicate aquarium (A6; Figure 5A), replicate activity trials showed a decrease in ammonia oxidation efficiency with PTIO inhibition in comparison to uninhibited samples. Microorganisms in the uninhibited samples depleted 1000 μg/L of ammonia within three days of the experiment, while ammonia had not been fully depleted until five or more days after PTIO was added. This shows that AOA were likely active in the uninhibited samples, and that their activity was represented by the differences seen between oxidation rates in samples with and without AOA inhibition. Beads from the replicate aquarium treated with AOA-sponge (A10; Figure 5B) showed contrasting results, demonstrating that AOB were fully responsible for all ammonia oxidation; a difference in oxidation efficiency between uninhibited and PTIO-treated samples was not clearly observed in tests with biofilm from A10 beads. Certain factors could have contributed to an inability to detect AOA activity, or could have caused an inhibition of AOA activity all together. Firstly, the rate of ammonia oxidation was higher with samples from A10 than all other samples tested (A6, A1 and A8). Ammonia was fully oxidized in both uninhibited tests and one PTIO-treated test between 1.5-2 days, as opposed to 2.5-3 days as seen with samples from the other aquaria. Higher concentrations of AOB in the biofilm could have contributed to an increased overall oxidation efficiency, masking any potential resolution between AOB and AOA ammonia oxidizing activity. qPCR performed on DNA previously extracted from A6 and A10 biofilter beads showed more archaeal 16S rRNA gene OTUs in DNA from A6; however,

there was a small difference in thaumarchaeal 16S rRNA gene abundance between the two samples. This verifies that a lack in archaeal activity in biofilm from A10 was not because of low archaeal abundance in comparison to the biofilm of A6, which showed archaeal activity. Previous troubleshooting activity trials involving the source of sponge used in this experiment demonstrated differences between uninhibited and PTIO-treated samples, for both bead and sponge biofilm (data not shown). This indicates that AOA in this sponge biofilm have previously demonstrated ammonia-oxidizing activity.

Although aquariums A6 and A10 were treated with similar sponge inoculations for the supplement experiment, they were placed on separate racks in the aquatics facility. Aquarium A10 was on a top shelf, close to a fluorescent light, while A6 was two rows down. Additional light stimulated algal growth in A10 towards the end of the experiment, which could have altered dynamics in the biofilter biofilm affecting AOA growth. Furthermore, studies have demonstrated that some strains of AOA, including ones enriched from freshwater sediment (French *et al.* 2012), are sensitive to light, more so than their bacterial counterparts (French *et al.* 2012; Merbt *et al.* 2012). AOA have also shown sensitivity to prolonged light cycles, such as those used for aquarium settings, and have little recovery from photoinhibition when transferred to dark (Merbt *et al.* 2012). It is unknown whether photoinhibition of AOA occurred in A10, but this could possibly have contributed to activity differences between biofilm from the two replicate aquaria. AOA may have been active when initially inoculated into aquarium A10, but could have lost activity with prolonged exposure to a close light source. DNA from non-viable organisms could have been what was detected in the filters with PCR.

4.4 AOA COMMUNITY SHIFTS OVER TIME

AOA communities in the aquarium biofilters were analyzed throughout the supplement experiment. DNA fingerprints were analyzed specifically for the aquaria that were inoculated with AOA-populated sponge (Figure 6B) and an AOA enrichment culture (Figure 6A), as those aquaria showed consistent archaeal detection throughout the experiment with PCR (Table 4). The AOA population sourced from the laboratory enrichment culture did not appear to change over the course of the study; one major band was seen in DNA from all time points, with the possibility of a second minor band (Figure 6A). A lack of one or both bands in later time points was likely due to an insufficient amount of DNA loaded into the gel; PCR amplification of archaeal *amoA* genes became increasingly more difficult to detect in DNA extracted from the later time points (6-8 months). Thus, archaeal communities did not appear to change in these systems.

In the aquaria inoculated with established sponge, a possible shift in *amoA* G+C content was detected over time (Figure 6B). As visually inspected on the fingerprint images, four major bands were seen early in the study (Day 3 and Week 3); however, a fifth band was recognized in DNA extracted from the second month, which persisted in the profiles for the remainder of the study. It appeared that patterns from the AOA-enriched culture (Figure 6A) and AOA-enriched sponge (Figure 6B) shared a band (third band from the top in AOA sponge pattern), suggesting that the AOA phylotypes were the same in these two samples. This was expected because AOA in the laboratory culture (*Ca.* N. aquariensis, unpublished) was enriched from the same source of AOA-populated sponge used in this experiment, further demonstrating the stability of this archaeal organism over time and between different environments.

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Fingerprints from the early time points for the sponge-inoculated samples (Figure 6B) appeared similar to those previously created for *amoA* genes from the same sponge source, FW27 in Sauder *et al.* (2011), displaying four possible bands. Sequenced DGGE bands from the original sponge source (FW27 in Sauder *et al.* 2011) fell into a freshwater cluster, sharing bands with AOA from other engineered aquatic environments, as well as *Ca.* Nitrosoarchaeum limnia (Blainey *et al.* 2011; Sauder *et al.* 2011). While the ecological implications are unknown for this potential shift in community, similarities with AOA in other freshwater aquarium environments, including the source biofilter sponge, provide insight as to the types of AOA (in addition to *Ca.* N. aquariensis) that inhabit the biofilter communities in this experiment.

4.5   EARLY DETECTION OF AOB AND AOA IN HOME AQUARIA

The home aquarium experiment was performed to detect the establishment of ammonia-oxidizing microbial populations within biofilter communities of start-up aquaria in independent, unregulated home environments. The aim was to observe whether AOA, AOB, or both, dominate aquarium biofilters during early start-up phases in aquarium environments lacking controlled conditions. I hypothesized that AOA would dominate start-up freshwater aquarium biofilters in home aquaria during or shortly following an early aquarium development period (one month). PCR detection of AOA and AOB gene markers showed variable results between home aquarium filters (Table 5). PCR for one aquarium agreed with the hypothesis. Thaumarchaeal 16S rRNA and AOA *amoA* genes were detected in the biofilter of Home Aquarium 1 (Table 5), and archaeal gene detection persisted over the experiment. Meanwhile, weak AOB detection was seen in DNA

extracted from beads and sponge from this aquarium, strongly indicating that AOA were the primary ammonia-oxidizers in the filter of Home Aquarium 1 over aquarium development.

While Archaea were detected in Home Aquarium 2 and Home Aquarium 3 (Table 5), they were not the only ammonia-oxidizers present in these filters. Samples collected from these two filters demonstrated patterns distinct from Home Aquarium 1, and from each other. Both AOB and AOA were detected in Home Aquarium 2 (Table 5); however, AOB appeared to be more abundant than AOA in the filters. This indicates that AOB were likely dominant in the filters during early aquarium development, but AOA populations began to establish in the filters following predicted peaks in ammonia concentration.

Both AOB and AOA were also seen in Home Aquarium 3 (Table 5); however, detection of these ammonia-oxidizers did not occur simultaneously, as in Home Aquarium 2. Strong AOB *amoA* gene amplification was observed after the addition of fish, which was likely due to the addition of Cycle to this aquarium immediately following the introduction of fish. However, strong AOB signals did not persist in the filter biofilm of Home Aquarium 3, different from the results seen in aquaria from the supplement experiment that were inoculated with Cycle (Table 4). This suggests that other environmental factors may have inhibited further establishment of AOB in the biofilm, or that bacterial organisms in the Cycle product were not viable. There was weak AOA detection in filter biofilm from Home Aquarium 3 towards the end of the first month of the experiment, indicating that AOA may have begun to populate the filters. The use of Cycle in this tank may have prevented AOA from thriving earlier in the filters, as previously suggested for the supplement experiment (section 4.2). Cycle has been shown to contain a

wide variety of non-nitrifying microorganisms (Nowak *et al.* 2008), which could impact biofilm community establishment during early stages in aquarium development.

For all three home aquaria in this experiment, gene amplification was generally more successful in DNA extracted from biofilter beads rather than sponge, except for strong AOB detection in sponge of Home Aquaria 2 and 3 (Table 5). DNA was successfully extracted from all samples; a screen for bacterial 16S rRNA genes showed strong amplification in all DNA extracts from this experiment (data not shown). However, perhaps microbial populations in the sponge were not as dense as those on beads during this early stage in aquarium development (one month), as the sponge has a larger surface area than beads. Alternatively, primers used for amplification of AOB and AOA gene markers may have had low sequence homology with genes in the DNA from these samples, as discussed in section 4.1 for the variable fish biomass experiment. This could have caused inconsistent amplification in samples with low ammonia-oxidizing microbial gene abundance.

Detection of ammonia-oxidizing microorganisms in the home aquarium experiment further demonstrated that microbial inoculation from external sources may have a major impact on whether or not ammonia-oxidizers establish early on in the filters. Low detection of thaumarchaeal 16S rRNA genes was seen in sponge DNA from Home Aquarium 1 prior to adding fish, while AOB *amoA* genes were detected in both sponge and bead DNA from Home Aquarium 2 (Table 5); AOA and AOB thrived thereafter as the dominating ammonia-oxidizers in filters from Home Aquaria 1 and 2, respectively. A link between natural inoculation of microorganisms from the environment and filter establishment is not as clear with Home Aquarium 3, due to the known addition of Cycle.

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The fact that biofilm communities differed between all three aquaria demonstrates that microorganisms were inoculated with sources from the surrounding environment, and inoculation could have been associated with variations in plants/accessories, fish, fish food, or the volunteer participants themselves, as discussed in section 4.1.

The variability in detection of ammonia-oxidizing microorganisms in the filters of the three participating aquaria means it is difficult to pinpoint the key ammonia-oxidizers in early aquarium nitrification, and the specific contributions of AOA and AOB. Water chemistry analysis may improve this understanding, as differences in early ammonia and nitrite oxidation rates can be compared between the three aquaria analyzed in this study, as well as in relation to the microorganisms that thrive in the filters. This experiment does demonstrate that AOA are able to establish in aquarium filters, both with and without AOB, during early aquarium development, suggesting that AOA could have a major role in early aquarium nitrification. Further, it indicates that external factors in unregulated aquarium environments can have a large impact on biofilm development, and that the controlled conditions used for the variable fish biomass and supplement experiments may have prevented the establishment of AOA in new aquarium biofilter communities.

5 CONCLUSIONS

Prior to this research, little was known about the contributions of AOA to nitrification in engineered aquatic environments, such as aquarium biofilter systems. AOA were found dominating over AOB in freshwater aquarium biofilters and other engineered systems, particularly in low-ammonia conditions (Sauder *et al.* 2011; Sauder *et al.* 2012), suggesting that AOA are responsible for nitrification in low-ammonia environments. However, it was unknown when AOA begin to dominate aquarium biofilters over the course of aquarium development, and if they contribute to early nitrification in new aquaria, when efficient ammonia oxidation is vital for aquatic health. This research provides insight into factors influencing establishment of early aquarium biofilter communities, and the potential role that AOA may play in nitrification in new aquaria. In controlled aquarium environments, where introduction of AOA into an aquarium biofilter system is limited, AOB are the dominant ammonia-oxidizers in biofilter biofilm. However, when AOA are inoculated into new aquaria by the means of controlled supplement inoculations, or via unregulated environmental sources (i.e., fish, human skin, aquarium accessories), they may contribute to improved rates in initial ammonia oxidation, less accumulation of ammonia in aquarium water, and faster stabilization of low-ammonia aquarium conditions. Although it remains unclear whether the contributions of AOA towards early aquarium nitrification surpass those of AOB in aquarium filters, AOA can dominate and establish in the biofilters over early aquarium development, as either the sole ammonia-oxidizers, or within a community alongside their bacterial counterparts. This suggests that AOA in aquarium biofilters are likely key contributors to early aquarium

nitrification, helping to improve aquatic health in aquarium systems, and may benefit

nitrogen cycling processes in other engineered and freshwater environments beyond home

aquaria.

As the roles of AOA within early aquarium nitrification become better defined,

particularly in relation to the contributions of AOB, development of microbial consortia

containing AOA for potential application to improve aquatic ecosystem health will be

conceivable. The future may see a change in focus of the aquarium industry from AOB

alone, to consideration of both AOB and AOA, to improve aquarium establishment

methods that increase nitrification in new aquaria. Furthermore, this focus on AOA

application may extend to large-scale biofiltration systems, such as wastewater and

drinking water treatment systems, and may shape future practices and methods for

obtaining efficient nitrification within these systems.

Although all AOA were assumed chemolithoautotrophic (Könneke *et al.* 2005;

Stahl and de la Torre 2012), additional research over the recent years has suggested

mixotrophic and heterotrophic metabolic processes for AOA (Tourna *et al.* 2011; Wessén

et al. 2010), including AOA in aquatic engineered environments (Mußmann *et al.* 2011).

This indicates that ammonia may not be the only source of energy for these organisms.

Further distinctions in growth preference between AOA and AOB have been revealed,

such as differences in sensitivity to light and pH (Bengtson *et al.* 2012; Merbt *et al.* 2012).

Observations from my research concur with these recent findings, and all together may

indicate that the contributions of AOA within aquarium biofilters, and other engineered

aquatic environments, may exceed nitrification alone. As more AOA are able to be

cultivated, especially those from freshwater aquatic environments, there will be

opportunities to expand our understanding of the contributions of AOA within aquarium

nitrification, and how AOA fit into the big picture of biofiltration in aquatic engineered

environments.

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