AMMONIA OXIDATION BY A NITRIFYING COMMUNITY CONTAINING NOVEL AMMONIA OXIDIZING ARCHAEA

by

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ABSTRACT

The disinfection properties of chlorine have long been known. These properties have been leveraged in the disinfection of drinking water. However, in the presence of organic matter, chlorine can form potentially carcinogenic disinfection by-products (DBPs). As a result, the U.S. Environmental Protection Agency promulgated the Stage I and Stage II Disinfectants/Disinfection By-Products Rules, limiting the amount of DBPs that can be present in a distribution system. An economical solution for many drinking water utilities to meet these new regulations was to use to chloramine as a secondary disinfectant. However, chloramines are not without their own disadvantages: free ammonia is added during chloramine formation and released by chloramine decay. This free ammonia can then be used as an energy source by indigenous microoganisms during nitrification. Nitrification can have deleterious effects on drinking water such as decreased disinfectant residual, pH, dissolved oxygen, and alkalinity, as well as an increase in nitrite, nitrate, and heterotrophic bacteria. This study uses effluent from a nitrifying reactor simulating premise plumbing to quickly establish a nitrifying community in glass bead packed bed reactors. Importantly, ammonia oxidizing archaea have been identified in both systems while no known ammonia oxidizing bacteria have been found. Once this nitrifying community was established, the reactors were used as batch reactors with effluent recycle to measure ammonia oxidation during a two hour batch phase. A least squares regression analysis was performed to generate the kinetic constants ν_{max} and K_m for the nitrifying community in the packed bed reactors. ν_{max} was calculated to be 2.23 hr^{-1}. K_m was calculated to be 2.35 mg L^{-1}. This work will aid in the effort to characterize the nitrifying population in a premise plumbing system and mitigate nitrification in drinking water.

CHAPTER 1

INTRODUCTION

Secondary Disinfection in Drinking Water Distribution Systems

The disinfection properties of chlorine have long been known and are advantageous to applications such as secondary disinfection in drinking water. Yet in the presence of organic matter chlorine can form potentially carcinogenic disinfection by-products (DBPs). The discovery of these DBPs gave way to increasing public health and regulatory concern (Rahman et al., 2011). As a result, the U.S. Environmental Protection Agency (EPA) promulgated the Stage I and Stage II Disinfectants/Disinfection By-Product Rules (D/DBPR) (USEPA, 2002). Upon the D/DBPRs' implementation, many drinking water utilities found they were no longer in compliance with EPA regulations (USEPA, 2002). The resulting lowest cost solution for many utilities was to implement chlorine and ammonia mixtures or chloramines into their operations for secondary treatment (Wilczak et al., 1996). In 2009, more than 30 percent of drinking water utilities previously using chlorine switched to chloramines. Upwards of 60 percent were expected to be using chloramines by the end of the D/DBPR grace period in 2012 for utilities serving more than 50,000 customers and 2013 for utilities serving less than 50,000 customers (Wilczak et al., 1996; Zhang et al., 2009).

The application of chloramines is not without consequences. Free ammonia is released through chloramine formation or decay. This free ammonia can then be used as an energy source for nitrification (Zhang et al., 2009). An estimated two-thirds of

drinking water utilities using chloramines experience nitrification in their distribution systems to some degree (*Nitrification*, 2002; USEPA, 2002; Wilczak et al., 1996); this number was expected to double by the end of the D/DBPR grace period (Zhang et al., 2009).

Nitrification can have many deleterious effects on drinking water. Table 1 from the EPA's report on nitrification summarizes the issues coupled with nitrification. The maximum contaminant limit (MCL) for nitrite is 1.0 mg/L as nitrogen, a concentration easily exceeded stoichiometrically based on the concentrations of chloramines added for secondary disinfection. Nitrite ions can also form nitrosamines in the presence of ammonia, which are known carcinogens (Wilczak et al., 1996). The MCL for nitrate is 10 mg/L as nitrogen, a level more difficult to reach through stoichiometric conversion of ammonia but realistically achievable, especially when combined with background levels of nitrate present in some waters. High levels of nitrates can cause methemoglobinemia or blue baby syndrome in infants (Zhang et al., 2009). In addition, the organic compounds resulting from nitrification can result in taste and odor issues (Bouwer & Crowe, 1988; van der Wielen et al., 2009).

Table 1: A summary of water quality issues associated with nitrification (USEPA, 2002).

Chemical Issues	Biological Issues
Disinfectant Depletion	HPC Increase
Nitrite/Nitrate Formation	Ammonia Oxidizing Bacteria (AOB) Increase
Dissolved Oxygen Depletion	Nitrite Oxidizing Bacteria (NOB) Increase
Reduction in pH and Alkalinity	
DBP Formation due to Mitigation Techniques	

Nitrification

Nitrification is a microbial process by which reduced nitrogen, primarily ammonia, is oxidized to nitrite and subsequently to nitrate. Typically, the oxidation of ammonia to the intermediate hydroxylamine and then to nitrite is thought to be primarily a bacterial process. An additional bacterial population further oxidizes nitrite to nitrate. Nitrite oxidizing bacteria (NOB) are almost universally found with ammonia oxidizing bacteria (AOB). Though ammonia oxidation has traditionally been viewed as a bacterial process (Francis et al., 2007; French et al., 2012; Hatzenpichler et al., 2008), recent discoveries have credited archaea with ammonia oxidation in environments with low ammonia concentrations (Encarnacion, 2012; Konneke et al., 2005).

Ammonia Oxidizing Archaea

Archaea were initially discovered in extreme environments and, thus, were traditionally thought to be extremophiles, (Francis et al., 2007). However, ammonia oxidizing archaea (AOA) have been identified in a wide variety of habitats. AOA were first discovered in marine environments by Fuhrman and DeLong in 1992 (DeLong, 1992; Fuhrman et al., 1992). Subsequently, 20-40 percent of the bacterioplankton in the ocean were estimated to be AOA (Konneke et al., 2005). AOA were also identified in soils (Tourna et al., 2011) where Leininger et al. (2006) suggests AOA are dominant among prokaryotes. Hatzenpichler et al. (2008) identified an AOA in a hot spring in moderately thermophilic conditions. AOA have been identified in temperatures ranging from 0.2 - 97 ˚C and pH values as low as 2.5 (Lehtovirta-Morley et al., 2011). Recent

studies have also identified AOA in freshwater (French et al., 2012; Hugoni et al., 2013) and in drinking water (Encarnacion, 2012). Thus, the discovery of AOA has fundamentally reshaped our knowledge of archaea and prompted a reassessment of the drivers of the nitrogen cycle.

Ammonium Oxidation.

In the global nitrogen cycle, nitrification is the rate limiting aerobic step (Canfield et al., 2006). Furthermore, the first step in the process, the oxidation of ammonia to nitrite is often the limiting step in nitrification (French et al., 2012). As noted previously, current estimates suggest that AOA comprise a large portion of organisms in environments with low ammonia concentrations and AOA have been identified in a wide range of habitats. Thus, it is increasingly important to characterize AOA and their relative contributions to their environments and the nitrogen cycle.

Since the first description of AOA, only two isolates have been obtained. Konneke et al. (2005) first isolated *Nitrosopumilus maritimus* from gravel in a tropical marine fish tank. Though this was not a true ocean isolate, the ensuing studies revealed some of the first growth characteristics of marine AOA. The maximum growth rate for this isolate was reported by Konneke et al. (2005) to be 0.78 d^{-1} at an optimal ammonia concentration of 0.5 mM. Further work by Martens-Habbena et al. (2009) determined a half saturation constant for the organism to be 0.132 μM $NH_3 + NH_4^+$. In contrast, the half saturation constant for the well-studied ammonia oxidizing bacterium (AOB) *Nitrosomonas europaea* ranges from 30-61 μM NH_3. Furthermore, the half saturation constant for *Nitrosomonas oligotropha*, an AOB found in low nutrient, neutral pH

environments, is 1.9-4.2 μM NH_3 (Koops & Pommerening-Röser, 2001). Martens-Habbena et al. (2009) also determined the isolate's substrate affinity (calculated from kinetic constants: specific affinity = $\nu_{max} \times K_m^{-1}$) for reduced nitrogen to be 68,700 L/g cells/h at pH = 7.5, one of the highest known substrate affinities for any organism. These findings resemble nitrification kinetics of *in-situ* marine systems (Prosser & Nicol, 2008) and support the relevance of this organism to natural environments.

The second isolate was obtained by Tourna et al. (2011); *Nitrososphaera viennensis* was isolated by applying bacterial antibiotics to AOA enrichments from a garden soil in Vienna. Growth studies yielded a higher cell concentration (Tourna, et al., 2011) than that of the marine isolate. In addition, comparable growth rates for the soil isolate were observed in 10 and 15 mM NH_3 concentrations, but no growth occurred in 20 mM NH_3. However, this is much higher than the inhibitory concentration of 2 mM NH_3 for *N. maritimus* at pH 7.5 which led to a decrease in cellular activity (Konneke et al., 2005; Martens-Habbena et al., 2009). Interestingly, this soil isolate was also able to utilize urea as its energy source in the absence of ammonia, further expanding the metabolic spectrum of AOA (Tourna et al., 2011).

In addition to the isolates, some recent enrichment studies have also shed light on the oxidation potential of AOA. An enrichment of an AOA from an agricultural soil parallels the findings of Tourna and coworkers. Jung and coworkers (2011) found optimal strain growth at 10 mM NH_3, with inhibition at 15 mM NH_3. In addition they found a half saturation constant of 0.69 μM NH_3, much higher than for *N. maritimus*. Interestingly, they also reported a half saturation constant for oxygen of 10.38 μM

compared to 3.09 μM for *N. maritimus* (Martens-Habbena et al., 2009) and 186 μM for *N. europaea* (Jung et al., 2011).

Furthermore, Hatzenpichler and coworkers (2008) successfully enriched a moderately thermophilic (46°C) AOA from a hot spring. They found that this AOA is highly active between concentrations of 0.14 and 0.74 mM NH_4^+ at pH = 7.4. Additionally, partial inhibition was observed at 3.08 mM NH_4^+ with pH = 7.4.

A more recent study by French et al. (2012) was the first of its kind to enrich AOA and AOB cultures from freshwater environments and directly compare their growth. Enrichments were subjected to varying pH, light exposure, ammonia and oxygen concentrations. The highest AOA growth rates (approximately 0.48 d^{-1}) were observed at 1 mM NH_4^+ with greater concentrations yielding a slightly lower growth rate. In contrast, higher NH_4^+ concentrations yielded increasingly higher AOB growth rates. Interestingly, higher oxygen concentrations (0.8 mg L^{-1} O_2) seemed to be slightly inhibitory, as indicated by a lower growth rate when compared the growth rates at lower oxygen concentrations. Highest growth rates occurred between pH values of 7.0 and 7.5. Finally, overall AOA metabolism was inhibited by light (French et al., 2012) in a similar manner to AOB (Merbt et al., 2012).

Carbon Assimilation.

Ammonia oxidizing organisms are known to be autotrophic. AOB use the Calvin cycle to fix carbon dioxide (Arp et al., 2007). Yet, there is evidence that supports the idea that AOA use the 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle (Erguder et al., 2009; Hatzenpichler, 2012; Tourna et al., 2011). Tourna et al. (2011) identified the

genetic precursors for the 3HP/4HB cycle while Erguder et al. (2009) were able to demonstrate carbon from bicarbonate was indeed incorporated into the cell. These findings correlate with the neutral to slightly alkaline pH ranges in which AOA are typically found.

Tourna et al. (2011) also observed cell yields 12 times higher on pyruvate than those in purely autotrophic conditions. Yet, less than 10% of cellular carbon seemed to be derived from pyruvate. In addition, genetic analysis conducted by Hallam et al. (2006a, 2006b) suggested a potential for mixotrophic growth but there has been no experimental support of a mixotrophic ability (Hatzenpichler, 2012). However, these notions may aid in ongoing research of AOA and provide areas for further studies.

Thesis Goal

As noted by Hatzenpichler (2012), there is a lack of knowledge of AOA in ground- and freshwater environments, despite their importance to humans. Horak et al. (2013) also noted the lack of *in-situ* kinetic studies of AOA. Thus, this work aims to contribute to the current knowledge in regards to both of these needs. Samples collected from a drinking water distribution system were positive for genes encoding archaeal *amoA*, further expanding the environments in which AOA have been discovered. This study determines the ammonia oxidation capacity of a nitrifying biofilm in oligotrophic freshwater. In addition, this work supplements previous work of Rahman et al. (2011) and Encarnacion (2012) intended to characterize and mitigate nitrification in premise plumbing. It is important to emphasize that no known bacterial nitrifiers have been identified in these systems, only ammonia monooxygase sequences related to AOA

(Encarnacion, 2012). In order to effectively study population kinetics and establish a nitrifying biofilm community, glass bead packed bed reactors were used. Once a nitrifying community was established, effluent from the packed bed reactors was used as inocula for a planktonic batch study to determine growth requirements. The packed bed reactors were then used as biofilm batch reactors with an effluent recycle to conduct rate experiments of ammonia oxidation. Ammonia measurements were taken during the batch phase to determine the rate of ammonia oxidation. Ultimately, these experiments also allowed for the determination of the populations' capacity for nitrification. These results contribute to the effort to characterize the nitrifying population in a premise plumbing system. As our understanding of nitrification in chloraminated systems increases, mitigation techniques can be developed, better decisions can be made on disinfection strategies and superior plumbing materials can be chosen. Furthermore, this research aids in the effort to understand AOA and their importance in the nitrogen cycle on a broader scale.

CHAPTER 2

MATERIALS AND METHODS

Reactor Systems

Packed Bed Biofilm Reactor

A packed bed reactor (Figure 1) was used to establish a nitrifying biofilm community. Each reactor was constructed of a polycarbonate cylinder 6 cm in diameter and 6 cm tall. The cylinder was filled with approximately 220 g of 3 mm borosilicate beads (Chemglass Vineland, NJ, USA, CAT No. CG-1101-02), yielding a fluid volume of approximately 85 mL. To ensure the system was well mixed and minimize preferential flow paths, flow was directed tangentially to a conical recession in the polycarbonate base of the reactor that contained a stir bar spun at 150 rpm opposite the flow direction. The reactor was capped with a polycarbonate top. Stainless steel screens were placed on top and bottom of the cylinder to contain the borosilicate beads. A peristaltic pump delivered flow such that the residence time in the reactor was one hour. Aluminum foil was used to shield the reactor contents from the light.

Figure 1: An overview of the packed bed reactor set up is shown on the left. A close up of a single packed bed reactor is shown on the right.

Feed Solution

Reactors were fed a combination of mineral amended reverse osmosis water (RO), biologically treated Bozeman tap water (BAC), and effluent from reactors designed to replicate premise plumbing with an established nitrifying population (Rahman et al., 2011). Constituents of the RO water plus mineral feed were $MgSO_4$ (396 mg L^{-1}) and $KHCO_3$ (710.3 mg L^{-1}). Ammonium sulfate was added to provide a final concentration in the reactors of 0.71 mg L^{-1} NH_3-N which is equivalent to the amount of ammonia that would result from the decay of 4 mg L^{-1} of chloramine. Bozeman tap water (surface source, no background ammonia, chlorinated) was passed through a granular activated carbon filter for dechlorination followed by flow through a biologically active carbon column. This water served as a continuous source of indigenous organisms. Effluent from established nitrifying reactors was collected and not altered.

An Erlenmeyer side arm flask received 1 mL min^{-1} mineral amended RO water, 1 mL min^{-1} effluent from established nitrifying reactors, and 8 mL min^{-1} BAC water. The

flask was stirred to ensure complete mixing. This mixture was then pumped from the flask to each reactor at a flow rate as noted previously. Influent was periodically checked to ensure proper ammonia concentration.

Once reactors were able to completely nitrify 0.71 mg L^{-1} NH_3-N (no NH_3 was detected in reactor effluent in approximately six weeks), the feed of effluent from the existing nitrifying reactors was discontinued. The BAC feed was increased to 9 mL min^{-1} while the RO feed containing $MgSO_4$, $KHCO_3$, and $(NH_4)_2SO_4$ remained at 1 mL min^{-1}.

Batch Study with Packed
Bed Reactor Effluent

A planktonic batch study was developed in order to determine nutrient requirements of the nitrifying community in the packed bed reactors. Effluent was collected for 24 hours from packed bed reactors containing a microbial population capable of nitrifying 0.71 mg L^{-1} NH_3-N yielding a volume of approximately 2100 mL. Baseline data was collected from 100 mL. The remaining volume was divided into ten 200 mL batch cultures. The batches were paired and subjected to five different conditions in 500 mL bottles. A batch pair control was amended with ammonium sulfate to obtain a starting concentration of 0.71 mg L^{-1} NH_3-N. A second pair was amended with ammonium sulfate to approximately 1.42 mg L^{-1} NH_3- N. Effluent from an existing set of reactors designed to replicate premise plumbing (Rahman et al., 2011) was added in one part effluent to 10 parts batch culture to a third pair to determine if the feed solution to these reactors contained a required micronutrient. Soil derived humic substances were added to a fourth set of batches to attain an organic carbon concentration of 4 mg L^{-1} to

verify if the population requires additional carbon. Starting ammonia concentrations for the third and fourth batches were adjusted to 0.71 mg L^{-1} NH_3-N . All batches were kept in the dark at room temperature on a shake table. A final pair of batches was amended to a starting ammonia concentration of 0.71 mg L^{-1} NH_3-N, kept in the dark at 4˚C, and not shaken. Batches were aseptically sampled daily from days 2-10; three consecutive samples combined to generate enough volume for ion chromatography analysis. Samples were also taken on day 17 and on day 25. Prior to analysis, samples were stored at 4˚C.

Reactor Experiments

A number of operating conditions were used with the established nitrifying packed bed reactors, including a study with continuous flow through the reactor, and in-place batch experiments with stagnant and recycle conditions. A flow-through study was designed to determine an observed ammonia oxidation rate in the reactor when exposed to an increased ammonia concentration. The packed bed reactors were then used as batch reactors with and without flow in order to more tightly control experimental conditions and increase kinetic parameter calculation accuracy. Because the reactor populations had a decreased ammonia oxidation capacity when operated in stagnant batch mode, the set up was modified to a batch system with recycle. This batch system with recycle system produced predictable and reproducible results and was used to determine population kinetics. To avoid minimize conditioning, reactors were allowed to rest for at least two weeks between experiments.

Flow-Through Study. To conduct a flow-through study, the ammonium sulfate concentration was increased to a single packed bed reactor to deliver a set concentration of 1.42 mg L^{-1} NH_3-N. Effluent samples of approximately 14 mL were collected every ten minutes for the first six hours, followed by one sample per hour for five hours. Subsequent samples were taken daily until the ammonia concentration in the reactor effluent reached a quasi-steady state. At the conclusion of the experiment, the ammonia concentration was returned to 0.71 mg L^{-1} NH_3-N.

Packed Bed Reactor to Batch Reactor. To conduct a batch study with the packed bed reactor, the ammonium sulfate concentration was increased for one hour. After one hour of increased ammonia feed, flow was stopped and the reactor was subjected to a one hour batch stagnant phase. Samples were collected with a 6 inch 16 gauge needle inserted through a septum in a "Y" in the effluent port of the packed bed reactor as shown in Figure 2. Samples were collected for nitrogen species analysis in 2 mL volumes and drawn from the reactor with a sterile 3 mL syringe every 15 minutes for one hour. The first sample was taken immediately after flow was turned off.

Packed Bed Reactor to Batch Reactor with Recycle. To conduct a recycle batch study with the packed bed reactor, the ammonium sulfate concentration was increased to duplicate packed bead reactors for a period of one hour during which samples of reactor content were taken every 15 minutes. Approximately 10 mL of effluent from one reactor was collected in a 50 mL filter flask just before the batch phase. After one hour of

increased ammonia feed, feed flow was stopped and the reactors were subjected to a two hour batch phase with recycle. During the batch phase, the effluent was collected,

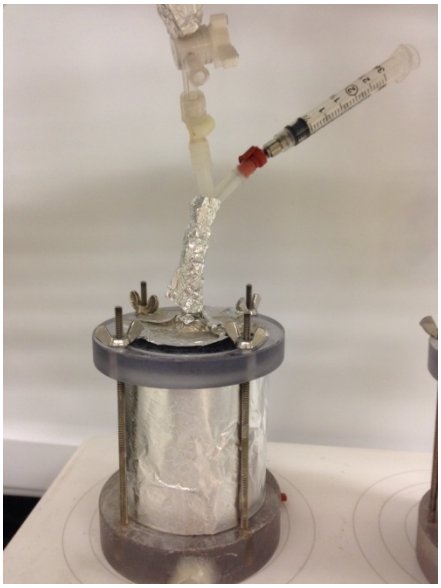

Figure 2: Packed bed reactor with "Y" in effluent line for sampling.

sparged with laboratory air, and pumped back into the same reactor from which it was collected. Effluent from a second reactor was recycled directly back into that reactor. Flow rates were such that the residence time in the reactors was one hour. A six-inch 16 gauge needle was inserted through a septum in a “Y” in the effluent port of the packed bed reactor as shown in Figure 2. Samples were collected in 2 mL volumes and drawn from the reactor through the needle with a sterile 3 mL syringe. Four samples were taken at equal time intervals during the batch phase for nitrogen species analysis.

Analytical Methods

Analysis of Water Samples from Florida Drinking Water Distribution System

Water samples were collected from 31 locations in the Fort DeSoto, Florida drinking water distribution system. A sample volume of 2.5 L was filtered through 0.22-μm-pore-size mixed cellulose ester filters (Millipore, Billerica, MA) that were shredded using sterilized tweezers and placed in 2 mL Lysing Matrix A tubes (MP Biomedicals, www.mpbio.com). The tubes from each sample were frozen at -70˚C and shipped to Montana State University upon completion of sample collection. DNA extraction was completed on the samples using the same protocol as for laboratory kinetic studies. A 1:10 aliquot of the resulting extraction product from each sample was then used as template for PCR. Undiluted extraction product was kept at -20˚C.

Sample Collection for Molecular Analysis of Reactor Systems

For molecular analysis of reactor systems and batch studies, 100 mL of effluent sample was collected and filtered through a 47 mm 0.2 μm polycarbonate membrane filter (Millipore, Billerica, MA, USA, CAT No. GTTO04700DNA). Filters were then shredded with sterile tweezers and placed in lysing tubes for DNA extraction.

DNA Extraction

DNA was extracted using the FastDNA® Spin Kit for Soil (MP Biomedicals, www. mpbio.com) according to the manufacturer's protocol. DNA extraction products were checked for DNA by mixing 1:1 with Blue/Orange 6X Loading Dye (Promega,

www.promega.com). Extraction product was then run on a 1.0% agarose gel with ethidium bromide in 1X TBE buffer for 45 minutes at 40 V. Gels were viewed using a FluorChem™ 880 fluorescence imager (www.alphainnotech.com).

Polymerase Chain Reaction (PCR)

PCR amplifications were carried out in 25 μL volumes: Go Taq® Green Master Mix (Promega, www. promega.com), 0.2 μM forward and reverse primers, Ultrapure BSA (Ambion www.lifetechnologies.com), DEPC treated water, and 1 μL of template DNA. Reactions were conducted in an Eppendorf Mastercycler® ep (Eppendorf North America, www.eppendorfna.com). All oligonucleotide primers were synthesized by IDT (Integrated DNA Technologies, www.idtdna.com). Table 2 lists the primer sequences and Table 3 lists the cycling conditions. PCR products were visualized on a 1.0% agarose gel with ethidium bromide in 1X TBE buffer for 45 minutes at 40 V. Gels were viewed using a FluorChem™ 880 fluorescence imager (www.alphainnotech.com).

Cloning and Sequencing

PCR products were extracted from gels and purified using the QIAquick® Gel Extraction Kit (QIAGEN, www.qiagen.com). Purified products were then cloned using the manufacturer's protocol for the TOPO® TA Cloning Kit and the plasmid vector pCR™4-TOPO® (Invitrogen, www.invitrogen.com). Transformants were scraped from Luria-Bertani (LB) plates and inoculated in 10 mL of LB broth plus 50 mg mL^{-1} ampicillin.

Table 2: PCR primer sequences and corresponding references.

Target Gene	Application	Primer	Sequence(5'-3')	Amplicon Length	Reference
Bacterial 16S rRNA	qPCR	1070 F	ATG GCT GTC GTC AGC T	322	(Ferris et al., 1996)
		1392+GC R	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CAC GGG CGG TGT GTA C		
Bacterial *amoA*	PCR	*amoA*1-F	GGG GTT TCT ACT GGT GGT	491	(Rotthauwe et al., 1997)
		*amoA*2-R	CCC CTC KGS AAA GCC TTC TTC		
Archaeal 16S rRNA	PCR/qPCR	931F	AGG AAT TGG CGG GGG AGC A	169	(Einen et al., 2008)
		m110R	BGG GTC TCG CTC GTT RCC		
Archaeal *amoA*	PCR	arch-*amoA*F	STA ATG GTC TGG CTT AGA CG	617	(Francis et al., 2005))
		arch-*amoA*R	GCG GCC ATC CAT CTG TAT GT		
Archaeal *amoA*	PCR/qPCR	Arch-*amoA*-for	CTG AYT GGG CYT GGA CAT C	256	(Wuchter et al., 2007)
		Arch-*amoA*-rev	TTC TTC TTT GTT GCC CAG TA		

Table 3: PCR cycling conditions

Target Gene	Primer	Cycling Conditions
Bacterial 16S rRNA	1070 F/1392+GC R	Initial denaturation at 94˚C for 15 min, followed by 30 cycles of: 94˚C for 45 sec, 55˚C for 45 sec, 72˚C for 45 sec, final elongation at 72˚C for 7 min
Bacterial *amoA*	*amoA*-1F/*amoA*-2R	Initial denaturation at 94˚C for 5 min, followed by 30 cycles of: 94˚C for 20 sec, 53˚C for 45 sec, 72˚C for 60 sec, final elongation at 72˚C for 7 min
Archaeal 16S rRNA	931F/m1100R	Initial denaturation at 94˚C for 5 min, followed by 30 cycles of: 94˚C for 2 min, 50˚C for 1 min, 72˚C for 1.5 min, final elongation at 72˚C for 10 min
Archaeal *amoA*	arch-*amoA*F/ arch-*amoA*R	Initial denaturation at 95˚C for 10 min, followed by 30 cycles of: 95˚C for 20 sec, 53˚C for 45 sec, 72˚C for 60 sec, final elongation at 72˚C for 10 min
Archaeal *amoA*	Arch-*amoA*-for/ Arch-*amoA*-rev	Initial denaturation at 95˚C for 10 min, followed by 30 cycles of: 95˚C for 20 sec, 53˚C for 45 sec, 72˚C for 60 sec, final elongation at 72˚C for 10 min

Cultures were incubated overnight in a shaking incubator at 37˚C. The Wizard Plus SV Minipreps DNA Purification System (Promega, www.promega.com) was used to purify the plasmid DNA from the cultures. DNA was quantified using the NanoDrop ND-1000 spectrophotometer (Nano Drop, Wilmington USA). Plasmids were sent to the Research Technology Support Facility (RTSF) at Michigan State University and were sequenced using the M13F (5'-TGTAAAACGACGGCCAGT-3') or M13R (5'-CAGGAAACAGCTATGAC-3') primers. Sequences were analyzed using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and compared with known

sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Quantitative PCR (qPCR)

Archaeal *amoA* and nitrite oxidizing bacteria 16S rDNA functional genes abundances were determined at the beginning and end time points of the planktonic batch experiment with the packed bed reactor effluent. The archaeal *amoA* gene was detected using arch-*amoA*-for (5'- CTG AYT GGG CYT GGA CAT C -3') and arch-*amoA*-rev (5'- TTC TTC TTT GTT GCC CAG TA -3') primers (Wuchter et al., 2007). Nitrite oxidizing bacteria 16S rDNA was detected using Nspra-675f (5'- GCG GTG AAA TGC GTA GAK ATC G -3') and Nspra-746r (5'- TCA GCG TCA GRW AYG TTC CAG AG -3') (Graham et al., 2007). qPCR reactions were carried out in 25 µL volumes comprised of Power SYBR® Green PCR Master Mix (Applied Biosystems, www.appliedbiosystems.com), 0.2 µM of both forward and reverse primers, DEPC treated water, and 8 µL of template DNA.

qPCR Standards. Archaeal *amoA* and nitrite oxidizing bacteria 16S rDNA gene amplicons were obtained using PCR and the conditions described above. PCR products were extracted from gels and purified using QIAquick® Gel Extraction Kit (QIAGEN, www.qiagen.com). Purified products were then cloned using the manufacturer's protocol for the TOPO® TA Cloning Kit and the plasmid vector pCR™4-TOPO® (Invitrogen, www.invitrogen.com). Transformants were extracted from Luria-Bertani (LB) plates and inoculated in 10 mL of LB broth plus 50 mg mL^{-1} ampicillin. Cultures were incubated

overnight in shaking incubator at 37˚C. The Wizard Plus SV Minipreps DNA Purification System (Promega, www.promega.com) was used to purify the plasmid DNA from the cultures. DNA was quantified using the NanoDrop ND-1000 spectrophotometer (Nano Drop, Wilmington USA) and by using a SybrGold assay (Invitrogen, www.invitrogen.com) with known concentrations of double stranded Lambda DNA (Promega, www.promega.com) as a standard for DNA quantification using a ND-3300 fluorospectrometer (Nano Drop, Wilmington, USA). Final copy number was calculated by assuming a molecular mass of 660 daltons (Da) for each basepair (bp) and considering the size of the plasmid (3890 bp) plus the insert length. Plasmids were sent to the Research Technology Support Facility (RTSF) at Michigan State University and were sequenced using the M13F (5'- TGT AAA ACG ACG GCC AGT -3') or M13R (5'- CAG GAA ACA GCT ATG AC -3') primers to confirm that the standard sequence was correct.

qPCR Cycling Conditions. All qPCR was conducted in a Rotor-Gene 3000 real time PCR cycler (Corbet Life Sciences, Mortlake, NSW, Australia) in a 72 well rotor. Archaeal *amoA* genes were amplified under the following conditions: 95 ˚C (15 min), 45 cycles of 94 ˚C (15 sec), 61 ˚C (30 sec), and 72 ˚C (30 sec) (Encarnacion, 2012). Nitrite oxidizing bacterial 16S rDNA genes were amplified under the following conditions: 95 ^{o}C (10 min), and 40 cycles of 94 ^{o}C (20 sec), 60 ˚C (1 min), and 72 ^{o}C (40 sec) (Huang et al., 2010). Standards and samples were prepared in triplicate and negative controls containing no template DNA were also run to ensure reactions were not contaminated. The FAM/Sybr detection channel was used to acquire data during the run. To verify

amplification of the correct PCR product, melt curve analysis was performed. The analysis was completed from 60-95 ˚C in 0.1 ˚C increments held for 5 sec following an initial pre-melt hold for 90 sec at the first step.

Heterotrophic Plate Count (HPC)

Heterotrophic organisms were monitored as a baseline microbial population during the planktonic batch study with packed bed reactor effluent by spread plating triplicate samples. Samples were serially diluted in sterile, reverse osmosis water such that 100 µL spread on an agar plate produced 30-300 colonies. All samples were plated on Difco™ R2A agar (Becton, Dickinson and Company, Cat # 218261) and incubated at room temperature for 5 days. Colony forming units (cfu) were counted on a Leica Colony Counter (Leica Inc., Buffalo, NY, USA) at the conclusion of the incubation period.

Ammonia Measurements

Free NH_3-N was measured using the salicylate HACH Method 10023. A HACH 2000 (HACH Company, Loveland, CO, USA) spectrophotometer was set to 655 nm to measure sample absorbance. Readings were recorded as mg L^{-1} NH_3-N.

Where necessary due to sample volume limitations, Method 10023 was scaled down 1:4 such that 0.5 mL of sample was required. A standard curve was generated using 0.5, 1.0, and 1.5 mg L^{-1} NH_4NO_3. All duplicate samples were then read on a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 655 nm.

To obtain ammonia measurements in the batch study with packed bed reactor effluent, unfiltered samples were measured using a Metrohm® IC (Metrohm USA, Riverview, FL, USA) ion chromatography system with two 819 conductivity detectors. A

Metrohm® C4 fast 4x150 mm column was used with 1.00 mL min^{-1} flow rate and a 20 µL injection loop. Eluent used contained 2.5 mM nitric acid and 0.5 mM oxalic acid. To calibrate the ion chromatography system for the, a standard solution containing NH_4NO_3, KNO_3, KH_2PO_4, and $MgSO_4$ was made resulting in concentrations of 100 mg L^{-1} of NH_4^+ as N, NO_2^- as N, NO_3^- as N, PO_4^{3-} as P, and SO_4^{2-} as S. The standard solution was diluted to provide five concentrations (0.10, 0.5, 1.0, 2.0, 5.0 mg L^{-1}). Data was processed using the program ICNet.

Nitrite and Nitrate Measurements

To obtain nitrite and nitrate measurements in the batch study with packed bed reactor effluent, unfiltered samples were measured using a Metrohm® IC (Metrohm USA, Riverview, FL, USA) ion chromatography system with two 819 conductivity detectors. A Metrohm® C4 fast 4x150 mm column was used with 1.00 mL min^{-1} flow rate and a 20 µL injection loop. Eluent used contained 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate. To calibrate the ion chromatography system for the flow-through study, a standard solution containing NH_4NO_3, KNO_3, KH_2PO_4, and $MgSO_4$ was made resulting in concentrations of 100 mg L^{-1} of NH_4^+ as N, NO_2^- as N, NO_3^- as N, PO_4^{3-} as P, and SO_4^{2-} as S. The standard solution was diluted to five concentrations (0.10, 0.5, 1.0, 2.0, 5.0 mg L^{-1}). Data was processed using the program ICNet.

Nitrite and nitrate in unfiltered samples for all other experiments were measured using Dionex® ICS-1100 (Thermo Scientific, Waltham, MA, USA) ion chromatography system with a microprocessor-controlled DS6 digital signal processor conductivity detector and a serial dual-reciprocating piston, microprocessor controlled constant stroke,

variable speed pump unit. A Dionex® IonPac™ AS22 4x250 mmm column was used with Dionex® AS22 eluent. The flow through the column was 1.20 mL min^{-1} with a 25 µL injection loop. To calibrate the Dionex® ion chromatography system for the flow-through study, Dionex® Standard was diluted to six concentrations (0.05, 0.5, 1.0, 3.0, 5.0, 7.0 mg L^{-1} NO_2^-/NO_3^- as N). For the packed bed reactor to batch reactor study, a standard solution containing NH_4NO_3, KNO_3, KH_2PO_4, and $MgSO_4$ was made resulting in concentrations of 100 mg L^{-1} of NH_4^+ as N, NO_2^- as N, NO_3^- as N, PO_4^{3-} as P, and SO_4^{2-} as S. The standard solution was diluted to six concentrations (0.1, 0.25, 0.5, 1.0, 1.5, 2.0, mg L^{-1}). The packed bed reactor to batch with recycle also used the same standard solutions diluted to six concentrations (0.5, 1.0, 2.0, 4.0, 8.0, 16.0 mg L^{-1}). Data was processed using Chromeleon 7 software. A standard solution was run after every eight sample measurements to check the accuracy of the measurement. If the obtained measurement of the standard was outside 90-110% of the actual standard value, then the calibration was repeated and the sample was measured again.

CHAPTER 3

RESULTS

Analysis of Water Samples from Florida Drinking Water Distribution System

Water samples were taken from a drinking water distribution system (DWDS) in Pinellas County, Florida experiencing a severe nitrification outbreak. The DWDS had switched from chlorination to chloramination in 2002 to address disinfectant by-product issues. Since 2004, the DWDS has experienced nitrification and has been performing chlorine burns for mitigation. As part of a large study to determine the microbial response to the chlorine burn, PCR was used to determine if ammonia oxidizing archaea are present in the DWDS.

PCR

PCR was used to determine the presence of bacterial 16S rRNA, bacterial *amoA*, archaeal 16S rRNA, and archaeal *amoA*. Of the 31 samples run, eight samples were positive for archaeal *amoA*. Bacterial *amoA* was not identified in any of the 31 samples. Initial PCR experiments revealed the presence of archaeal 16S rRNA in two of the 31 samples. The archaeal 16S positive samples were then tested for the presence of archaeal *amoA* genes using the primer set from Francis et al. (2006). Initial results from this PCR were negative. However, the resulting PCR product was then used as template for subsequent PCR using primers for a shorter *amoA* sequence developed by Wuchter et al. (2007) in a 'nested' approach. This PCR indicated the presence of archaeal *amoA*. As a

result, the remaining 29 samples were analyzed in the same 'nested' manner. PCR indicated 12 of the 31 samples contained archaeal *amoA* genes. These 12 samples were then cloned for sequencing. Sequencing was successful for eight of the 12 samples. Table 4 shows the taxonomic identity of the sequences obtained. All samples had strong similarities to the archaeal ammonia monooxygenase subunit A.

Table 4: Sequences from each sample were entered into the BLAST query. The closest relative and corresponding GenBank Accession number for each sample and corresponding BLAST search are shown.

Sample	Closest Relative and GenBank Accession Number	Similarity (%)
2	Uncultured archaeon clone F79 ammonia monooxygenase subunit A (*amoA*) gene, partial cds. JN183700	98
10	Uncultured ammonia-oxidizing archaeon clone JS4-AOA-36 ammonia monooxygenase subunit A (*amoA*) gene, partial cds. KF976045	99
11	Uncultured crenarchaeote clone E1_24 m ammonia monooxygenase subunit A gene, partial cds. JX990404	99
13	Uncultured archaeon *amoA* gene for ammonia monooxygenase alpha subunit, partial cds, clone: GAC_A_A2-OTU2. AB910275	99
14	Uncultured archaeon clone BU21 ammonia monooxygenase (*amoA*) gene, partial cds. KF 618758	99
15	Uncultured crenarchaeote clone E1_24 m ammonia monooxygenase subunit A gene, partial cds. JX990404	99
19	Uncultured archaeon ammonia monooxygenase subunit A (*amoA*) gene, partial cds. JQ717298	99
31	Uncultured archaeon ammonia monoxygenase subunit A (*amoA*) gene, partial cds. JQ406519	97

Batch Study with Packed Bed Reactor Effluent

A batch study was conducted with effluent from the packed bed biofilm reactors to obtain a picture of the major genes involved in ammonia and nitrite oxidation. Batch cultures were also subjected to varying environmental factors and inoculum amendments to see if the packed bed reactors were experiencing nutrient limitation. Amendments included 1.42 mg L^{-1} NH_3 –N, adding effluent from existing nitrifying reactors (Rahman et al., 2011) one part effluent to ten parts batch, and 4 mg L^{-1} organic carbon from humics.

PCR

PCR was used to confirm the presence of the archaeal *amoA* gene in the packed bed reactor effluent collected for the batch study. It is assumed that the effluent primarily contains cells detached from the biofilm since the influent contains only a small quantity of BAC water. The PCR run on the samples from the reactors produced bands that aligned with a previously sequenced positive control from existing nitrifying reactors (Rahman et al., 2011); the effluent from these reactors was used as the inoculum for the packed bed reactors. This result indicated the presence of ammonia oxidizing archaea.

qPCR

qPCR was used to quantify archaeal *amoA* and nitrite oxidizing bacteria 16S rDNA functional genes at the beginning and end of the batch studies.

Archaeal *amoA*. Archaeal *amoA* genes were approximately 10^6 copies μL^{-1} at the beginning of the batch experiment. At the end of the experiment, all samples contained

approximately 10^5 copies μL^{-1}. A summary of the data is presented in Figure 3. Despite efforts to wash the samples, copy numbers were not obtained for the batches with humics added. It is likely that humics were inhibiting the PCR (Albers et al., 2013). qPCR attempts with the addition of bovine serum albumen (BSA) added to the reaction were unsuccessful.

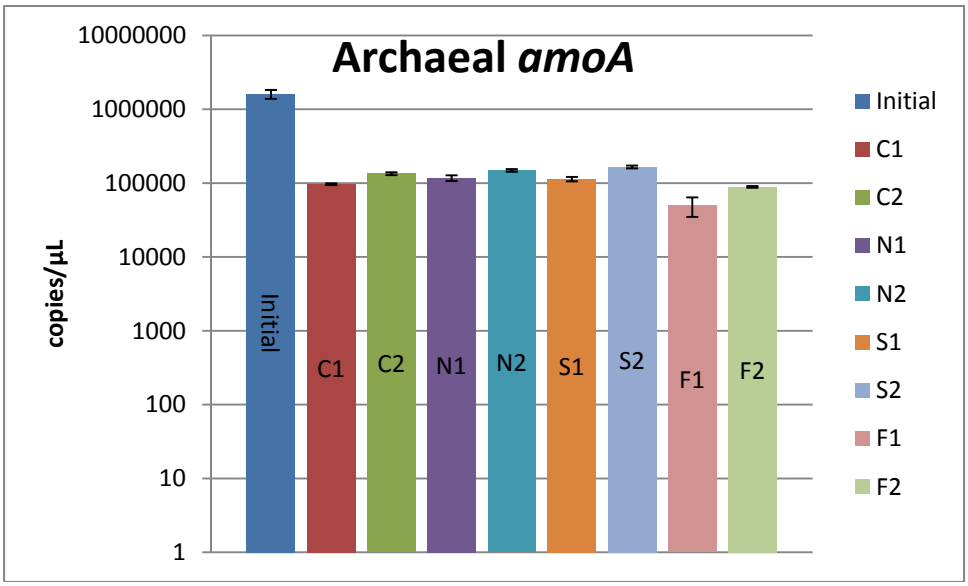

Figure 3: Archaeal *amoA* copies uL^{-1} measured in the initial batch inoculum and at day 25 for batch controls (C), increased ammonia (N), 1:10 effluent from existing reactor (S), incubated at 4 °C (F), and 4 mg organic carbon L^{-1} (H). All batch cultures had a starting ammonia concentration of 0.71 mg L^{-1} NH_3 –N, except those with increased ammonia which had a starting concentration of 1.42 mg L^{-1} NH_3 –N.

Nitrite Oxidizing Bacterial 16S rDNA. Nitrite oxidizing bacterial 16S rDNA was 3.2×10^2 copies μL^{-1} at the beginning of the experiment. The samples incubated at 4° C decreased slightly to 1.8×10^2 copies μL^{-1} and 1.9×10^2 copies μL^{-1}. All other samples increased to approximately 10^4 copies μL^{-1}, as shown in Figure 4. Despite efforts to wash the samples, copy numbers were not obtained for the batches with humics added. It is

likely that humics were inhibiting the PCR (Albers et al., 2013). qPCR attempts with the addition of BSA were unsuccessful.

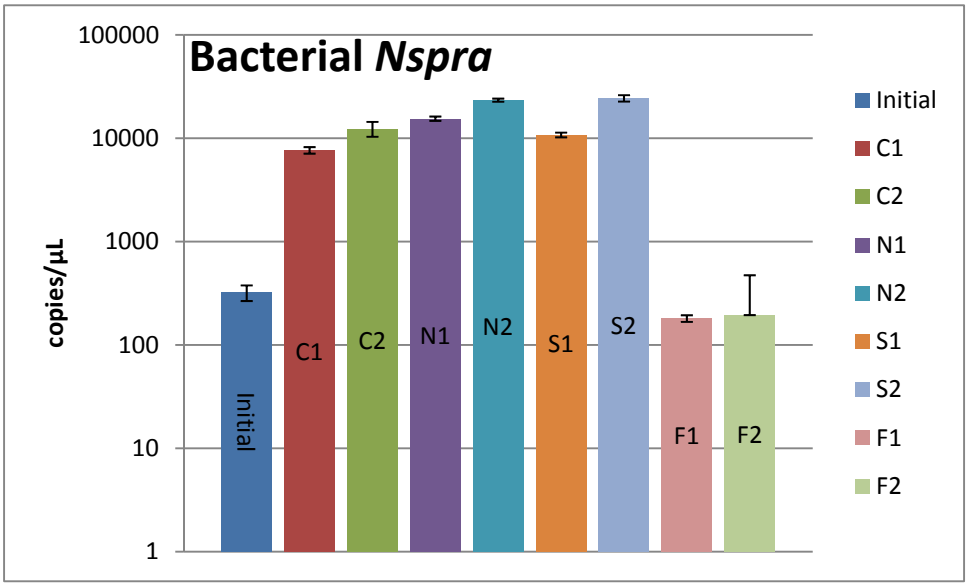

Figure 4: Bacterial *Nspra* copies μL^{-1} measured in the initial batch inoculum and at day 25 for batch controls (C), increased ammonia (N), 1:10 effluent from existing reactors (S), incubated at 4 °C (F), and 4 mg organic carbon L^{-1} (H). All batches had a starting ammonia concentration of 0.71 mg L^{-1} NH_3 –N, except those with increased ammonia which had a starting concentration of 1.42 mg L^{-1} NH_3 –N.

HPC

Heterotrophic organisms were monitored throughout the experiment by culturing. Initial CFUs varied, however all samples increased approximately one $\log_{10}$ from their initial CFU value. The exception was the batch cultures incubated at 4˚ C, in which HPCs decreased slightly.

Nitrogen Measurements

Ammonia. Ammonia in all batches decreased slightly from the initial concentration within the first three days (Figure 5). Subsequently, the ammonia concentration remained relatively constant up to day 10. There was another slight decrease between day 10 and 18 before day 25 when no ammonia was measured in any batch culture except those incubated at 4 °C and S1 that cultures displayed little decrease in ammonia. Figure 5 displays the ammonia data collected for all batch cultures.

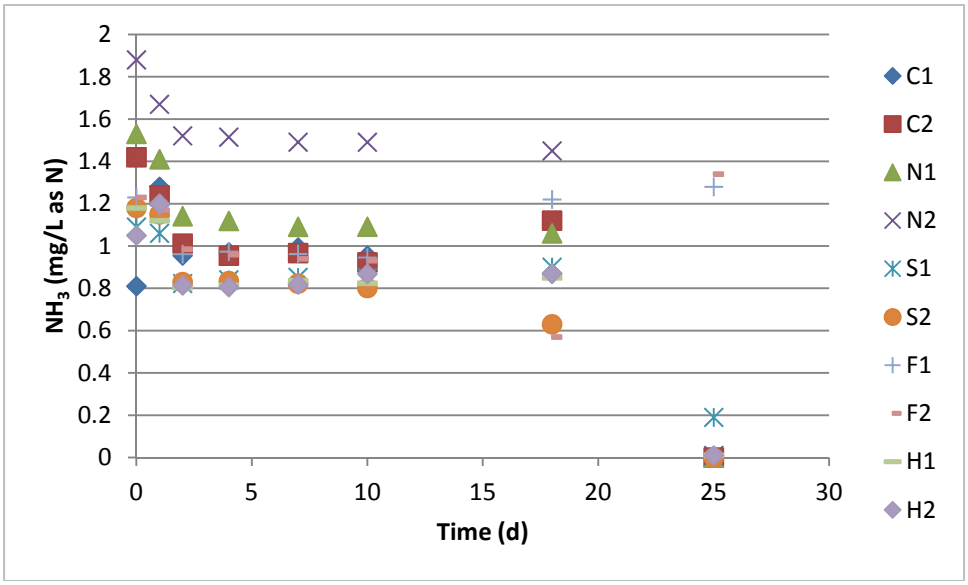

Figure 5: Ammonia measurements from batch controls (C), increased ammonia (N), 1:10 effluent from existing reactors (S), incubated at 4 °C (F), and 4 mg organic carbon L^{-1} (H). All batch cultures had a starting ammonia concentration of 0.71 mg L^{-1} NH_3–N, except those with increased ammonia which had a starting concentration of 1.42 mg L^{-1} NH_3–N.

Nitrite. Nitrite was not measured within the detection limits (< 0.1 mg $L^{-1}NO_2^-$-N) of the method in any batch during the experiment.

Nitrate. Nitrate production mirrored the decrease in ammonia as can be seen in Figure 6. There was a slight increase in nitrate compared to initial values by day three in all batch cultures. Nitrate then remained constant up to day ten. Nitrite increased slightly between day 10 and 18. On day 25, nitrate reached a maximum in all batches. At least 90% of the nitrogen as ammonia was converted to nitrate-nitrogen for all batch cultures except those incubated at 4 ˚C.

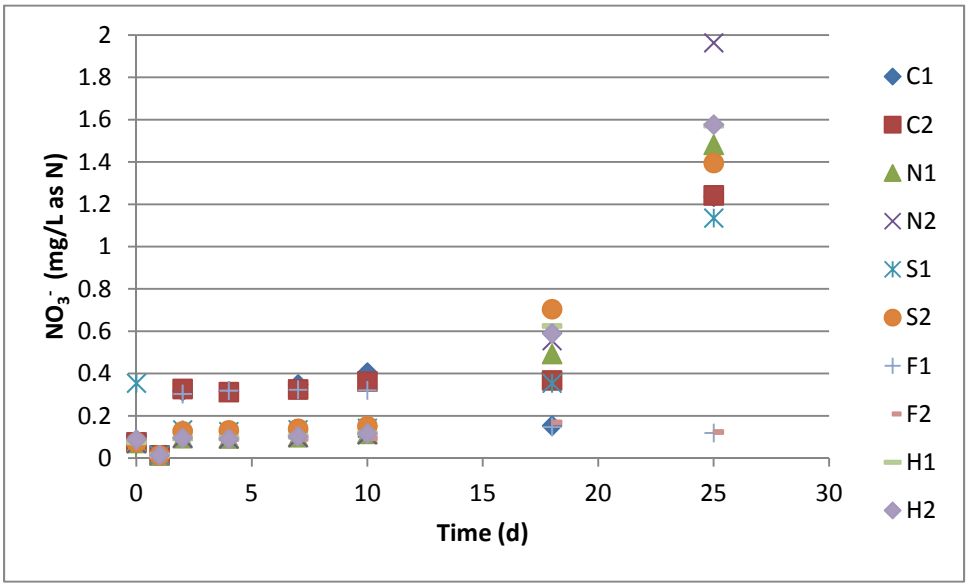

Figure 6: Nitrate measurements from batch controls (C), increased ammonia (N), 1:10 effluent from existing reactors (S), incubated at 4 °C (F), and 4 mg organic carbon L^{-1} (H). All batch cultures had a starting ammonia concentration of 0.71 mg L^{-1} NH_3–N, except those with increased ammonia which had a starting concentration of 1.42 mg L^{-1} NH_3–N.

Ammonia Oxidation Kinetics Studies in Packed Bed Reactors

Upon establishment of a nitrifying community able to oxidize 0.71 mg L^{-1} NH_3–N as demonstrated by complete removal, a series of experiments were performed to determine the maximum rate of oxidation and the half saturation constant for the biofilm

population within the packed bed reactors. A flow-through study was the first experiment followed by batch and batch-with-recycle studies. The succession of these experiments led to a plot of observed reaction rate versus initial ammonia concentration.

Flow-Through Study using Packed Bed Reactor

A flow-through study was designed to determine an observed ammonia oxidation rate for the nitrifying biofilm population. A single packed bed reactor was operated with continuous flow while subjected to a feed solution containing 1.42 mg L^{-1} NH_3 –N. Samples of the effluent were collected from the packed bed reactor were taken every ten minutes for the first day of the experiment followed by daily sampling for approximately 60 days.

PCR. PCR was used to confirm the presence of the archaeal *amoA* gene in the packed bed reactors. PCR run on reactor effluent samples from the packed bed reactors produced bands that aligned with a previously sequenced positive control (data not shown). This result indicated the presence of ammonia oxidizing archaea. Bacterial *amoA* genes were not detected.

Ammonia. A packed bed reactor containing a nitrifying community capable of oxidizing 0.71 mg L^{-1} NH_3 –N was subjected to an increased target ammonia concentration of 1.42 mg L^{-1} NH_3-N (exact concentration was 1.54 mg L^{-1} NH_3-N). Figure 7 shows the nitrogen species in the effluent from the reactor. Ammonia increased from zero to 1.1 mg L^{-1} NH_3-N during the first day and then decreased between days two

and 20 to approximately 0.3 mg L^{-1} NH_3-N followed by a slight increase in NH_3-N. Since complete ammonia disappearance was not observed, it was suspected that there was a phosphorous limitation as there was no phosphorous added to the packed bed reactor. To investigate, 0.01 mmol L^{-1} KH_2PO_4 was added on day 32. However, no change in the ammonia concentration in the packed bed reactor effluent was observed. The observed rate of ammonia oxidation was 1.2 mg NH_3-N L^{-1} min^{-1} when the reactor reached a final quasi-steady state by approximately day 20. The observed reaction rate was calculated by simplifying a mass balance on the reactor. In this case, $r_{obs}=(F/V)(\Delta NH_3)$, where F = flow rate, V = volume of the reactor, and ΔNH_3 is the change in ammonia concentration in the reactor. A plot of the observed reaction rate over time is shown in Figure 8.

Nitrite. Nitrite was consistently reported below 0.08 mg L^{-1} NO_2^--N, indicating complete conversion of ammonia to nitrate and that nitrite was not accumulating in the packed bed reactor. These values were below the detection limits (< 0.1 mg $L^{-1}NO_2^-N$) of the instrument.

Nitrate. Nitrate in the reactor effluent was approximately 0.60 mg L^{-1} NO_3^--N before increasing the ammonia concentration. Nitrate remained at this concentration until day two when it began to increase. From day 10 to the end of the experiment nitrate values were between 0.90 and 1.1 mg L^{-1} NO_3^--N as shown in Figure 7. Conversion of ammonia nitrogen to nitrate nitrogen varied between experiment. Approximately 40-60% of the nitrogen measured at the beginning of the batch phase was recovered as nitrate at the end of the batch phase.

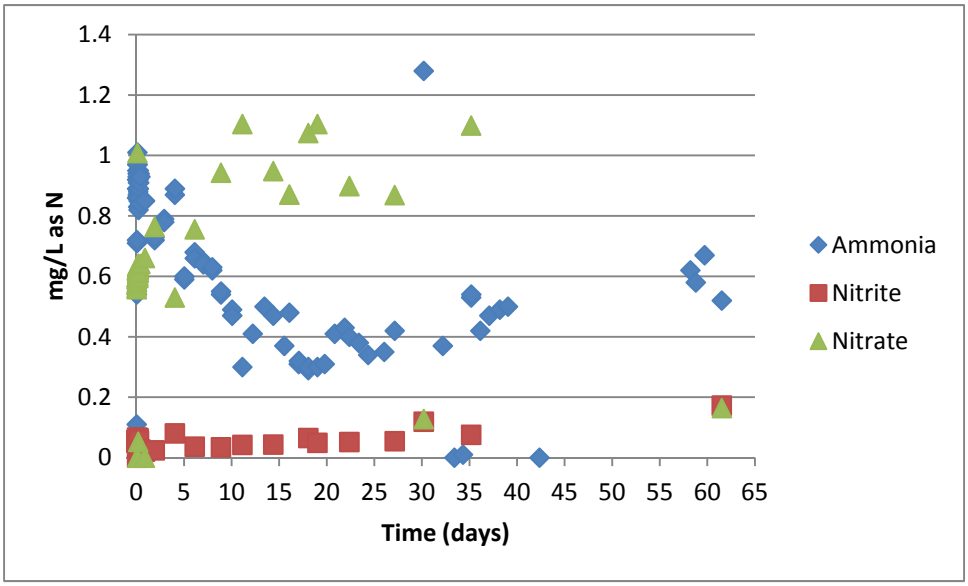

Figure 7: Nitrogen species measurements in flow-through study.

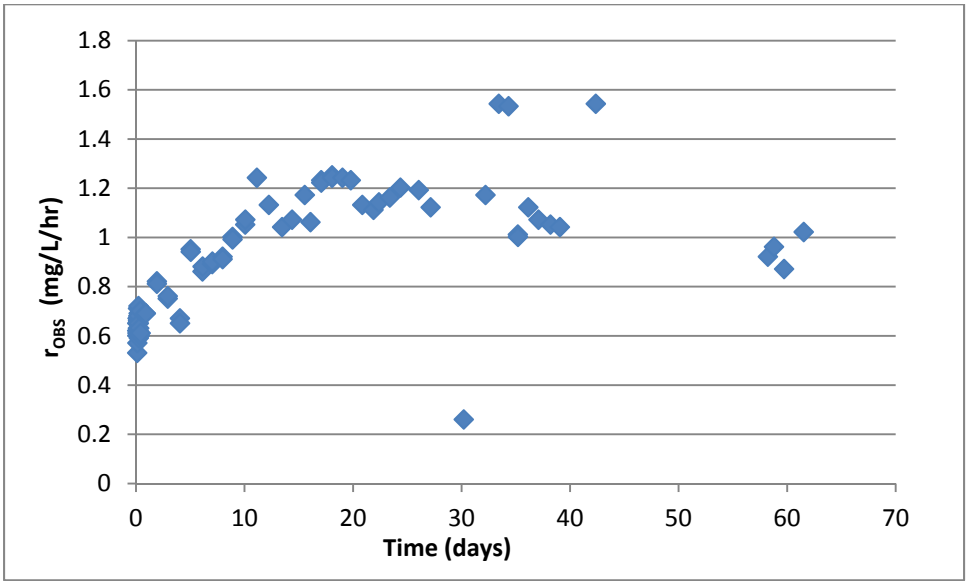

Figure 8: Flow through study observed ammonia oxidation reaction rate versus time.

Packed Bed Reactor to Batch Reactor

To increase the concentration in the packed bed reactors to proceed with a subsequent batch experiment, the ammonia in the influent was increased in the feed solution for one residence time. At the conclusion of this hour, influent flow was terminated and a one hour batch experiment was carried out.

Ammonia. Target ammonia values in the batch phase were 0.35, 0.71, and 1.42 mg L^{-1} NH_3-N. At initial values of approximately 0.35 mg L^{-1} NH_3-N, ammonia concentrations went to zero by the end of a one hour batch experiment. However, when the reactors had higher initial ammonia concentrations such as 0.71 and 1.0 mg L^{-1} NH_3-N, little to no ammonia oxidation was observed. Experiments with two hour batch experiments also showed almost no ammonia oxidation at initial concentrations of 0.71 mg L^{-1} NH_3-N or greater. Figure 9 shows typical results of two separate experiments, one with an initial ammonia concentration of 0.35 mg L^{-1} NH_3-N and another with an initial ammonia concentration of 0.71 mg L^{-1} NH_3-N.

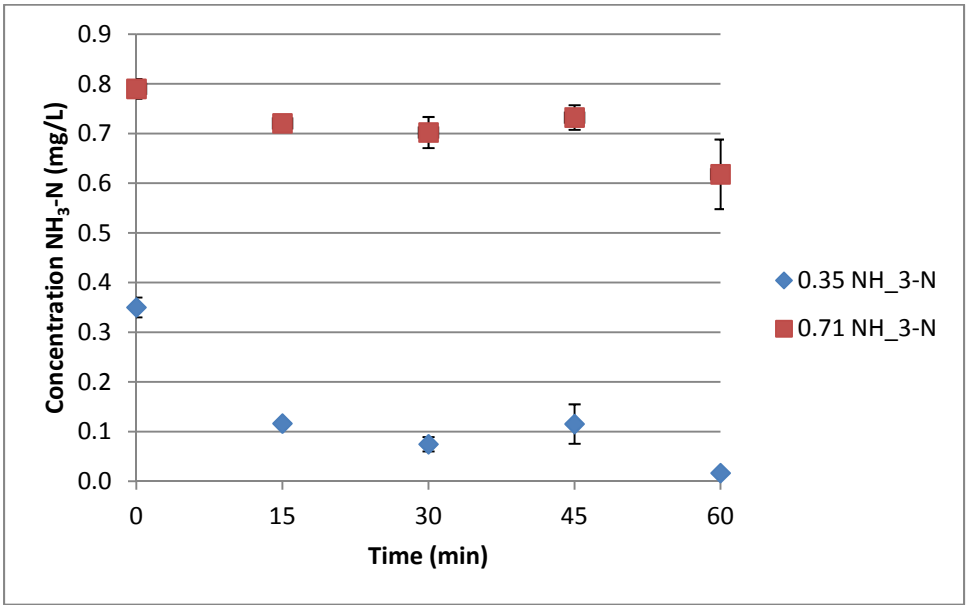

Figure 9: Ammonia measurements from one hour batch experiment with packed bed reactor. Error bars represent standard error of each sample measurement (n=2). Complete ammonia oxidation was observed with an initial concentration of 0.35 mg L^{-1} NH_3-N. Little ammonia oxidation was observed with an initial concentration of 0.71 mg L^{-1} NH_3-N.

Nitrite. Nitrite was not measured within the detection limits of the method during any batch experiment, indicating complete conversion of ammonia to nitrate and that nitrite was not accumulating in the packed bed reactor.

Nitrate. Slight increases were observed in nitrate concentrations of those batch experiments where ammonia oxidation occurred. In batch experiments with initial ammonia concentrations greater than 0.35 mg L^{-1} NH_3-N, nitrate increases appeared to be slight. Conclusions are difficult to draw as initial nitrate measurements were not obtained.

Packed Bed Reactor to
Batch Reactor with Recycle

To increase the ammonia concentration in the packed bed reactors, the ammonia in the influent was increased in two reactors for one residence time. At the conclusion of this period, a two hour batch experiment was carried out with one reactor in batch with recycle and one reactor in batch with recycle containing an air sparge. Initial target concentrations were approximately 0.71 mg L^{-1} NH_3 –N. Once complete ammonia oxidation was observed, the initial ammonia concentration was continuously increased in separate experiments to generate a Michaelis-Menten plot. Because some conditioning was observed in the reactors when experiments were conducted back to back, reactors were undisturbed for at least two weeks as a flow through system before being used again. In addition, the air sparge was alternated between reactor pairs for successive experiments.

Ammonia. Initial experiments demonstrated that complete ammonia removal at concentrations of greater than 0.71 mg L^{-1} NH_3-N occurred in a one hour batch phase when the recycle was air sparged as shown in Figure 10. Similar results were obtained with reactors that did not have an air sparge in the recycle, but ammonia removal was not as complete.

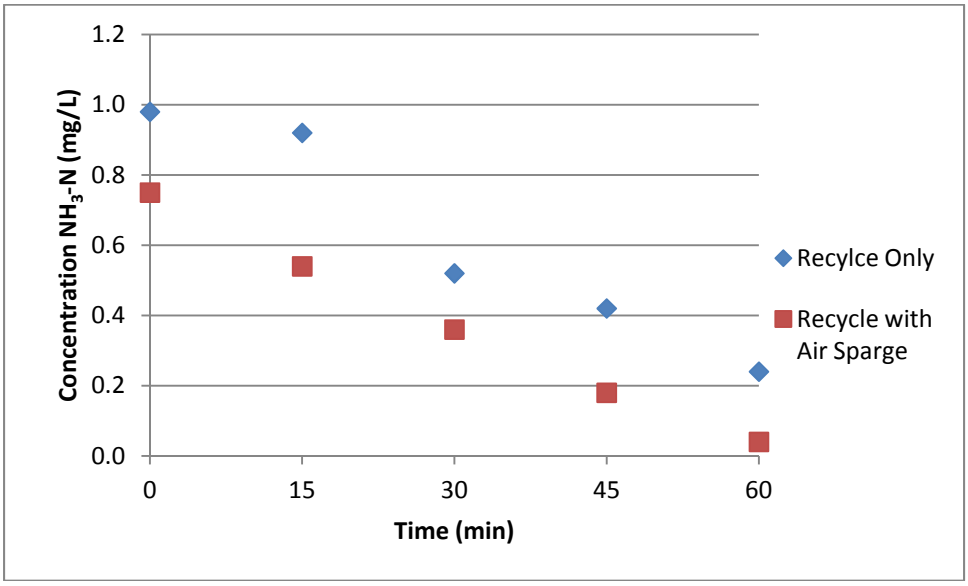

Figure 10: Ammonia measurements from a one hour batch with recycle experiment. Two reactors were used. Effluent from the first reactor was recycled directly back into the reactor. Effluent from the second reactor was air sparged before re-entering the reactor.

Based on the results shown above, initial batch ammonia concentrations were continually increased to determine a saturation concentration. The batch phase of the experiments was increased to two hours and data was collected during the one hour pre-batch feed phase. Figure 11 shows a typical result of the ammonia measurements collected during the experiments. Initial batch concentrations were difficult to predict and varied between reactors and experiments. Still, the ammonia concentration was continually increased in the experiments to generate a plot of observed reaction rates versus initial ammonia concentrations with sufficient points to determine the desired kinetic constants.

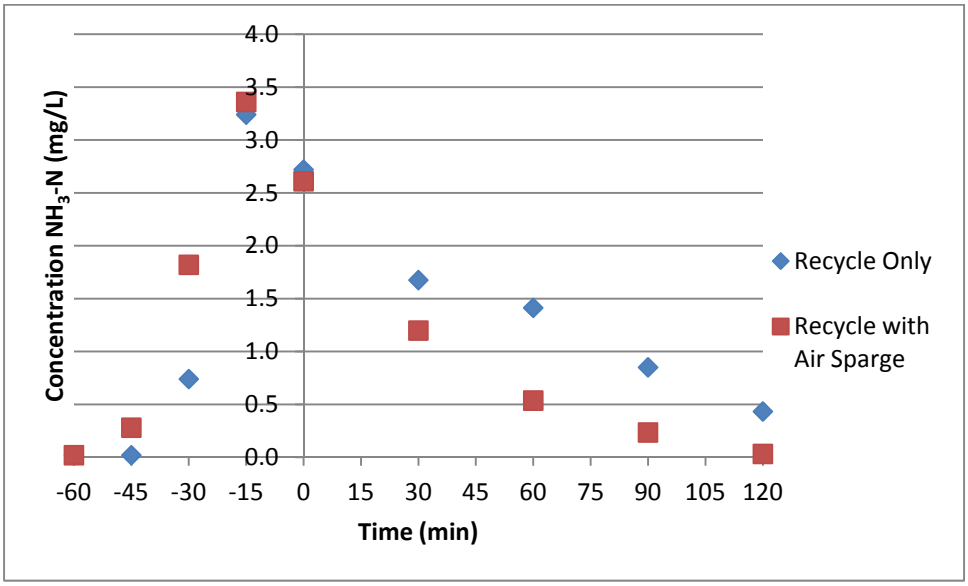

Figure 11: A graph of ammonia concentration versus time typical of batch with recycle experiments. Negative time values represent the one hour prior to the batch experiment of increased ammonia feed.

The results of all experiments (batch with recycle and batch with recycle with air sparge) are shown in Figure 12. A least squares regression analysis was performed to generate the kinetic constants ν_{max} and K_m for the nitrifying biofilm community in the packed bed reactors. ν_{max} was calculated to be 2.23 hr^{-1}. K_m was calculated to be 2.35 mg L^{-1}. A Student's t-test was used to calculate the 95% confidence interval for the data set.

Nitrite. Nitrite values were on the edge of detection limits of the method, indicating complete ammonia conversion to nitrate and that nitrite was not accumulating in the reactor.

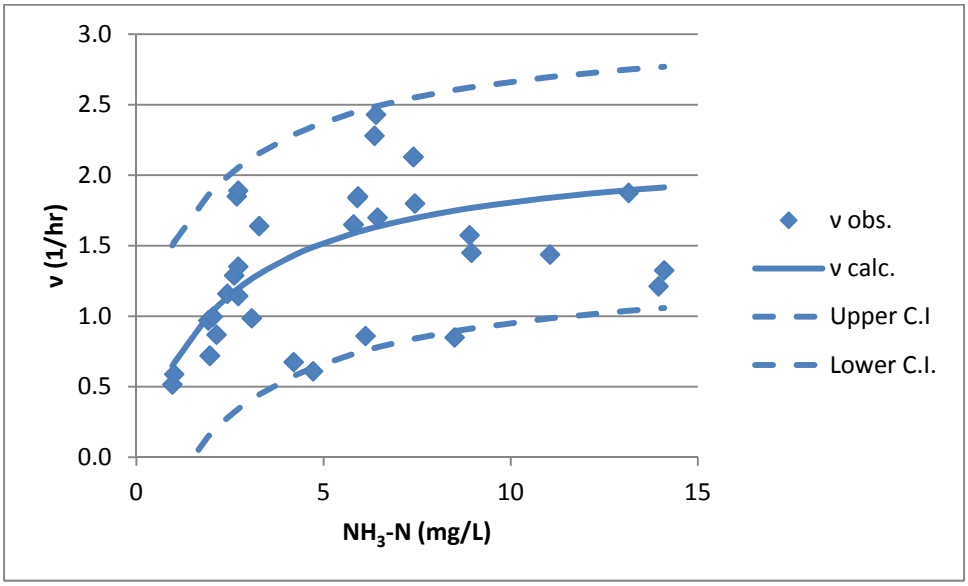

Figure 12: Michelis-Menton plot of nitrifying population. Calculated ν was obtained by using ν_{max}=2.23 hr^{-1} and K_m=2.35 mg L^{-1} which were determined by a least squares analysis of the observed data. Upper and Lower C.I. values are the bounds of the 95% confidence interval determined by a Student's t-test.

Nitrate. Near linear nitrate production was observed in all experiments. Figure 13 is a typical example of nitrate production (initial ammonia concentration was 3.90 mg L^{-1} NH_3-N). However, production rates were not consistent enough across all experiments to draw conclusive statements on the rate of nitrate production, as Figure 14 shows.

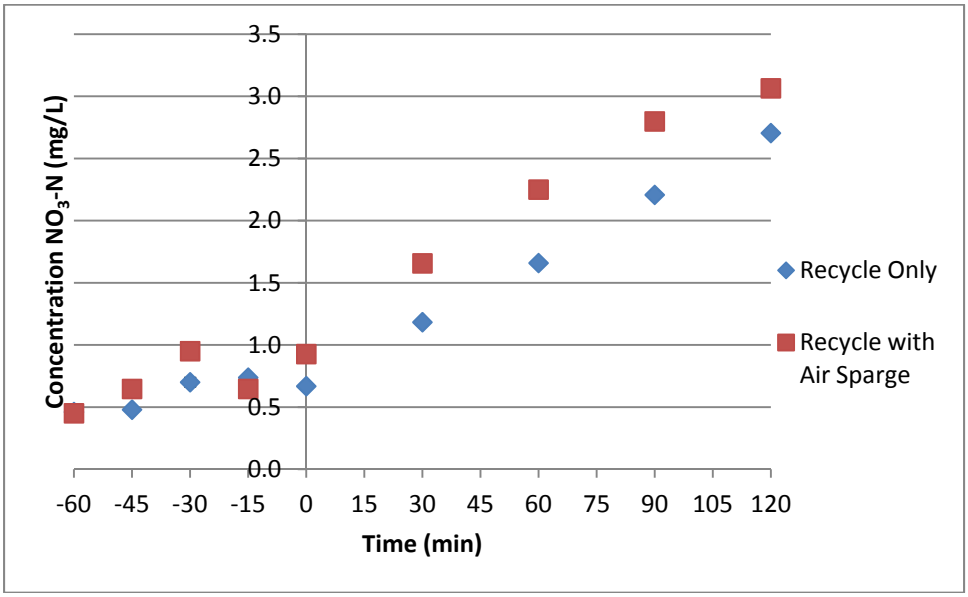

Figure 13: Nitrate production was nearly linear for all experiments. This figure depicts a typical result. Negative time values represent the one hour prior to the batch experiment of increased ammonia.

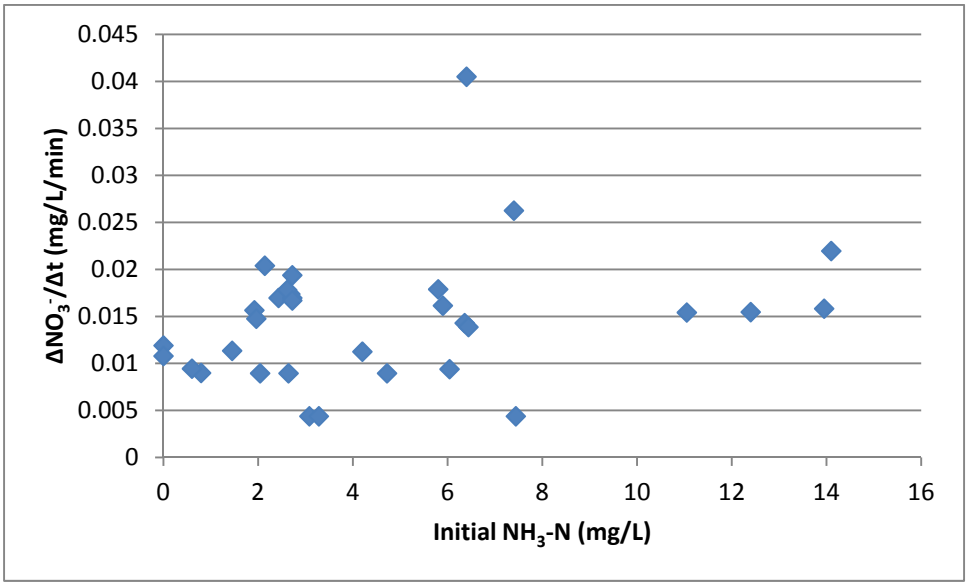

Figure 14: The change in nitrate during batch with recycle experiments was calculated and plotted versus initial ammonia nitrogen concentration for all experiments.

CHAPTER 4

DISCUSSION

Analysis of Water Samples from a Florida Drinking Water Distribution System

Drinking water utilities continue to implement the use of chloramine as a solution to meet the more stringent regulations resulting from the Stage I and Stage II Disinfectants and Disinfection By-Products Rule (D/DBP). An estimated two-thirds of medium and large drinking water distribution systems (DWDS) using chloramines as a secondary disinfectant experience nitrification problems (Stark & Firestone, 1996; Wilczak et al., 1996). Still, there has been little effort to identify the drivers behind this nitrification problem. A comprehensive review by Zhang et al. (2007) credits ammonia oxidizing bacteria (AOB) despite only citing two studies linking AOB to drinking water. The identification of ammonia oxidizing archaea (AOA) presented in this work suggests the answer is not that simple. Kasuga et al. (2010) found that AOA present in raw water can settle and oxidize ammonia on granular activated carbon (GAC) filter in an advanced treatment system. Along the same lines, Van der Wielen (2009) showed that AOA can be present in distribution systems. Encarnacion (2012) identified a novel archaeon, present in planktonic and biofilm forms, which was likely the sole party responsible for ammonia oxidation as no other know ammonia oxidizers were identified in a reactor system designed to simulate premise plumbing. Thus, this work contributes to the small but growing evidence that AOA are a player in nitrification in DWDS.

Water samples collected from the Pinellas County, Florida drinking water distribution system were analyzed using PCR and primer sets to target the genes encoding for the alpha subunit of the ammonia monooxygenase enzyme found in AOA. Of the 31 samples collected, eight were positive for AOA. When the sequences of the containing AOA were searched using the National Center for Biotechnology's (NCBI) Basic Local Alignment Search Tool (BLAST), all had at least 97 percent similarity to sequences of uncultured AOA deposited in GeneBank. These results are evidence for the case that AOA may be present on DWDSs experiencing nitrification. Further work is necessary to determine the diversity of the AOA present. Initial deductions suggest that there may be significant diversity as no two samples positive for archaeal *amoA* were the same. Also, the contribution to ammonia oxidation of AOA versus AOB in this system is not known. Nonetheless, these results demonstrate the need to expand current thoughts as to the drivers of nitrification in DWDS. As a result, new paths leading to the prevention and control of nitrifying communities in DWDSs can be explored.

Batch Studies with Packed Bed Reactor Effluent

A batch study was conducted with packed bed reactor effluent to quickly compare the results from AOA subjected to various conditions and feed parameters. This was done because initial experiments with the packed bed reactors yielded lower ammonia oxidation capacities than those of the existing modified CDC reactors established by Rahman et al. (2011). They demonstrated the disappearance of ammonia concentrations greater than 1.2 mg L^{-1} NH_3-N within a four hour stagnation period. However, the nitrifying population in the packed bed reactors was only able to oxidize 1.2 mg L^{-1}

NH_3-N after nearly 20 days despite the much larger surface area to volume ratio. In this study, control batches with 0.71 mg L^{-1} NH_3-N provided baseline data. Batches with initial ammonia concentrations of 1.42 mg L^{-1} NH_3-N were used to confirm AOA present had the capacity to oxidize at least this amount of ammonia. Effluent from existing reactors established by Rahman et al. (2011) was added to another set of batch cultures to determine if nitrification required a unique combination of nutrients. Another set of batches containing humics was used to determine if the nitrifying population required additional organic carbon as suggested by Hallam et al. (2006a, 2006b). Finally, a set of batches with feed constituents identical to the control batches were incubated at 4° C to determine if temperature was a factor in ammonia oxidation.

Interestingly, very little ammonia oxidation occurred over the first two weeks of the batch experiment followed by all ammonia being oxidized by day 25. The exception was in the batches incubated at 4° C which showed little ammonia oxidation. Still, the decrease in all other batches at room temperature is congruent with the findings of Encarnacion (2012) with enrichment cultures. Current thought suggests there are organisms within the consortia of a nitrifying population that AOA depend on for growth (Encarnacion, 2012; French et al., 2012; Zhang et al., 2009). Thus, this lag time may be the result of growth of this population; the increase in nitrite oxidizing bacteria (NOB) 16S rDNA gene copy numbers may be evidence indicating they are part of this supporting population. Furthermore, from work done by Encarnacion (2012) with modified CDC reactors established by Rahman et al. (2011), it is known that there are fewer AOA in the planktonic phase than present in a biofilm. As a result, the ammonia

oxidation of a planktonic culture would be much slower than that of biofilm. Encarnacion (2012) also suggested that a decrease in pH below 6.0 may have inhibited growth, though significant pH changes were not observed in the batch cultures in this study.

Though some resolution may have been lost when samples from the intermediate phase of the batch experiment were combined as described in the Batch Study with Packed Bed Reactor Effluent section in Materials and Methods, some important conclusions can be drawn based on the oxidation of all the ammonia in the batches. First, the feed solution for the packed bed reactors was sufficient for the AOA to oxidize at least 1.42 mg L^{-1} NH_3-N, two times the baseline ammonia concentration of 0.71 mg L^{-1} NH_3-N. This further supports work by Martens-Habbena et al. (2009), Erguder et al. (2009), French et al. (2012) and Encarnacion (2012) that AOA thrive in low ammonia, low nutrient environments. Furthermore, the batch cultures amended with effluent from existing nitrifying reactors were not different from the other conditions, providing further evidence that AOA are not restricted to unique water chemistry. Along the same lines, the batch cultures amended with organic carbon did not oxidize ammonia at a higher rate than the controls. This implies that AOA are not relying on organic carbon for growth as suggested by Hallam et al. (2006). These results may also may indicate that AOA are not relying on a specific heterotrophic organism as part of the support community proposed to aid in growth.

This batch study using packed bed reactor effluent demonstrated that the AOA in the biofilm established in packed bed reactors are capable of completely oxidizing 1.42 mg L^{-1} NH_3-N without additional amendments and given enough time. The results

implied the need to re-design experiments using the packed bed reactors for kinetics studies.

Reactor Studies

Packed bed porous media reactors were used in this study to determine the kinetic parameters of ammonia oxidation by a nitrifying biofilm community containing a novel ammonia oxidizing archaeon. These reactors were chosen because of the large surface area to volume ratio making it possible to quickly establish a robust biofilm. The population was established and able to completely nitrify 0.71 mg L^{-1} NH_3-N in the one hour residence time in approximately six weeks. A flow through study was the first experiment conducted. The reactor reached a quasi-steady state, utilizing 1.2 mg L^{-1} NH_3-N in one hour, therefore yielding an observed rate of 1.2 mg NH_3-N L^{-1} hr^{-1}. Next the flow to the reactors was terminated to produce a batch mode to better control the experimental conditions and increase the accuracy of the kinetic parameters. Interestingly, in strictly batch mode, the nitrifying biofilm population in the packed bed reactor oxidized only 0.35 mg L^{-1} NH_3-N. However, once a recycle was added to the batch mode, the nitrifying population could oxidize much greater ammonia concentrations. This recycle set-up created a transport plus diffusion scenario versus strictly diffusion in the stagnant batch studies, reducing niche nutrient and substrate limitations. As a result, a least squares analysis of all of the batch data with varying initial ammonia concentrations determined the ν_{max} for the population to be 2.23 hr^{-1} and K_m was 2.35 mg L^{-1}.

Flow-Through Study
with Packed Bed Reactor

A single packed bed reactor with a population capable of nitrifying 0.71 mg L^{-1} NH_3-N was subjected to 1.42 mg L^{-1} NH_3-N with no other feed amendments. It took nearly ten hours for the population to recover to oxidize 0.71 mg L^{-1} NH_3-N and nearly 20 days to reach a new, quasi-steady state. Preferential flow paths may explain the incomplete ammonia oxidation. Feasibly, the ammonia oxidizing enzymes exposed to ammonia in these paths were at capacity while others saw little or no ammonia outside the paths, allowing some ammonia to pass through un-oxidized; a tracer study could aid in confirming this theory.

In addition, growth rates for isolated or enriched AOA indicate they are very slow growing (French et al., 2012; Hatzenpichler et al., 2008; Konneke et al., 2005; Martens-Habbena et al., 2009; Tourna et al., 2011). However, even at these slow growth rates (0.78 d^{-1}), 20 days should have been ample time to allow for an increase in AOA. Another possible reason for incomplete oxidation of the ammonia is cell detachment. AOA may have been detaching from the biofilm as fast as new ones were growing. This may also explain the slight increase in remaining ammonia towards the end of the experiment (Figure 5).

Inhibition may have been another cause of the incomplete nitrification. Known potential inhibitors include light (Merbt et al., 2012), pH (Encarnacion, 2012; French et al., 2012; Gubry-Rangin et al., 2010), and nitrite accumulation. The packed bed reactor was shielded from light using aluminum foil. Consequently, light inhibition is unlikely. Significant pH decreases were not measured which would lead to inhibition. Finally,

nitrite concentrations were not detected in concentrations greater than 0.10 mg L^{-1} NO_2^--N.

Nutrient limitation, particularly phosphate, was also suspected as a reason for incomplete ammonia oxidation. This was considered despite some evidence that AOA prevail when bio-available phosphorous is low (Herfort et al., 2007). Adding 0.10 mM phosphorous resulted in two days of complete ammonia oxidation before a return to the previous quasi-steady state.

The above results were not expected; Rahman et al. (2011) showed that the nitrifying population present in fed-batch reactors was capable of oxidizing over 2 mg L^{-1} NH_3-N within 8 hours in a fed-batch system. This suggested that the nitrifying population in the flow-through packed bed reactors should be able to oxidize 1.42 mg L^{-1} NH_3-N within the one hour residence time. The packed bed reactor was effective in providing surface area to quickly establish a nitrifying population. However, it proved to be difficult to conduct studies to determine kinetics. For example, it was difficult to control the feed conditions for the prolonged duration of the flow-through study. Furthermore, this duration also allowed for fluctuations in influent indigenous organisms and water temperature.

Packed Bed Reactor to Batch Reactor

For the packed bed reactor where influent was stopped to create a batch system, the reactors were subjected to a feed solution with an increased ammonia concentration (all other amendment concentrations were unchanged) for one hour prior. After this initial feed phase, all flow to the reactor was stopped, while the stir bar in the bottom of

the reactor continued to operate. Though it was more difficult to achieve specific initial ammonia concentrations, this experimental set up did not require precise flow rate and feed concentration maintenance for extended time periods. Prior experiments determined that the populations in the reactors could nitrify 0.35 mg L^{-1} NH_3-N. However, when an initial concentration of approximately 0.71 mg L^{-1} NH_3-N was attained, very little ammonia oxidation was observed. Based on the batch studies with packed bed reactor effluent and the initial flow-through study, it was unlikely that nutrient limitation was inhibiting ammonia oxidation. Specifically, the batch with packed bed reactor effluent study demonstrated the ammonia oxidation capacity of the planktonic culture alone was at least twice 0.71 mg L^{-1} NH_3-N without any additional feed amendments, given enough time. Batch experiments with the packed bed reactors that were carried out for two hours did not yield greater ammonia oxidation than one hour experiments. Furthermore, there was no nitrite accumulation observed in any of the experiments. The reduction in ammonia corresponded to an increase in nitrate, indicating the nitrite oxidizing bacteria were able to oxidize nitrite as quickly as it was produced. Given these observations, oxygen may have been the inconsistency compared to previous observations. In the batch study with packed bed reactor effluent, the batches were placed on a shaker table to help ensure oxygen was near saturation. Encarnacion (2012) found there was less ammonia oxidation in enrichment cultures that were not shaken compared to cultures placed on a shaker table, despite findings by Martens-Habbena et al. (2009) who found shaking cultures to be inhibitory to growth. Along the same lines, oxygen was likely near saturation in the flow-through study due to mixing in the feed flask and the packed bed

reactor itself, except in niche locations of the consortia. In contrast, the only source of agitation during the batch study with the packed bed reactor was from the stir bar in the bottom. This may have prevented sufficient oxygen delivery to enough of the nitrifying population to allow significant ammonia oxidation. To test this theory, it was proposed to use packed bed reactor as a batch reactor and add a recycle stream.

Packed Bed Reactor to Batch Reactor with Recycle

Packed bed reactors containing a nitrifying population capable of oxidizing 0.71 mg L^{-1} NH_3-N were subjected to a feed phase of increased ammonia concentrations. The reactor set up was then modified to allow the operation of a recycle with and without air sparge. The recycle was intended to reduce suspected oxygen limitations in niche locations. Experiments were carried out with two packed bed reactors. Effluent from one reactor recycled directly back into the reactor using a peristaltic pump. A 50 mL flask containing an air sparge collected effluent from a second reactor which was then pumped back into the same reactor. The sparged set up assured near saturation conditions which could then be compared to the unsparged system. The reactor fluid recycling altered the batch set up to a transport and diffusion scenario versus strictly diffusion in the batch with no recycle. Furthermore, the batch with recycle system produced predictable and reproducible results. The nitrifying population was able to oxidize 0.71 mg L^{-1} NH_3-N within the one hour residence time in both the unsparged and sparged systems. It is likely the air sparge in the recycle stream created near saturation conditions in the packed bed reactor. It is important to note that the system without the air sparge in the recycle was not a closed system; fluid volume reductions from sampling were replaced with air.

Data were collected from 19 experiments using both sparged and unspared systems. Initial ammonia concentrations ranged from 0.96 mg L^{-1} NH_3-N to 14.1 mg L^{-1} NH_3-N. When the recycle only and recycle with air sparge data were analyzed separately, the same $\nu_{max} = 2.19\ hr^{-1}$ was determined for both cases. For the recycle only trials, $K_m =$ 2.24 mg L^{-1} NH_3-N while $K_m = 2.67$ mg L^{-1} NH_3-N for those in which the recycle was sparged. Furthermore, the 95% confidence interval was slightly smaller for the sparged recycle cases at 0.79 mg L^{-1} NH_3-N versus 1.05 mg L^{-1} NH_3-N for the recycle only. It was observed that it was possible to condition the nitrifying community in the packed bed reactors if the batch with recycle experiments were conducted without allowing ample rested time at baseline flow-through conditions. After an increased ammonia oxidation rate was observed (noted by points significantly above the final calculated curve), the reactors were returned to baseline conditions for a period of at least two weeks. Also of note, the initial batch duration of one hour used to determine the viability of the recycle set up was sufficient for complete oxidation of lower concentrations of ammonia. Once the initial ammonia concentrations were increased, the bulk of the batch experiments were carried out for two hours. A final exception was four batches with some of the highest initial ammonia concentrations that ran for four hours. These longer durations provided assurance that no important information was missed beyond the two hour batches as the observed ammonia oxidation rate was well within the 95% confidence interval of the final Michaelis-Menten curve.

As noted above, a least squares analysis was used to determine ν_{max} and K_m in the Michaelis-Menten equation, as shown below.

$$\nu = \frac{\nu_{max} \cdot [S]}{\nu_{max} + [S]}$$

The results for all batch with recycle experiments yielded ν_{max} = 2.23 hr^{-1} and K_m = 2.35 mg L^{-1} NH_3-N (168 µM NH_3). These values were much higher than those determined by previous enrichment studies with AOA. Martens-Habbena et al. (2011) determined a half saturation constant for *N. maritimus* to be 0.132 µM NH_3. Stark et al. (1996) found K_m = 0.036 µM NH_3 for AOA found in soil. In addition, the values in this study were also higher than those obtained from growth studies of successfully isolated AOA (Konneke et al., 2005; Martens-Habbena et al., 2011; Tourna et al., 2011). On the other hand, ν_{max} from this study was much lower than 110 mg N L^{-1} determined under optimum conditions of an enrichment containing AOB (Wong-Chong & Loehr, 1975). The K value for activity determined in this study was also below the values for AOB. Loveless and Painter (1968) determined a K value of 14 mM NH_4^+ for *Nitrosomonas* sp. while Koops and Pommerening-Roser (2001) reported a range of K = 30-61 µM NH_3 for the well-studied *N. europaea*. In a more direct comparison, the K value determined by Stehr et al. (1995) for *N. oligotropha*, an AOB found in oligotrophic waters, was 0.075 mM NH_4^++NH_3 and Koops and Pommerening-Roser (2001) reported a range of K = 1.9-4.2 µM NH_3. A summary of the aforementioned K_m values is presented in Table 5.

Table 5: Summary of relevant K_m values for ammonia oxidizing bacteria and ammonia oxidizing archaea.

Species	K_m	Units	Reference
N. europaea	110	mg L^{-1} NH_3	(Wong-Chong & Loehr, 1975)
N. europaea	30-61	μM NH_3	(Koops & Pommerening-Röser, 2001)
N. oligotropha	0.075	mM NH_3+NH_4^+	(Stehr et al., 1995)
N. oligotropha	1.9-4.2	μM NH_3	(Koops & Pommerening-Röser, 2001)
N. maritimus	0.132	μM NH_3	(Martens-Habbena et al., 2011)
Soil AOA	0.036	μM NH_3	(Stark & Firestone, 1996)
Freshwater AOA (this study)	168	μM NH_3	

This research was unique in that AOA were analyzed in-situ with both planktonic and biofilm components, whereas the aforementioned studies all used planktonic AOA. The results provided evidence that the biofilm component significantly increases the capacity for ammonia oxidation. The magnitude of the ν_{max} and K_m values suggest the nitrifying population has a modest capacity for ammonia oxidation in oligotrophic environments. In addition, the identification of AOA in a drinking water distribution system in Pinellas County, Florida further supports existing evidence that AOA are a driver of nitrification in drinking water systems. It is important to note that no known AOB were identified in the reactors systems used. The absence of AOB is important because, until recently, AOB were thought to be solely responsible for nitrification. However, Konneke et al. (2005) and Leininger et al. (2006) suggested that AOA comprise a signification percentage of their marine and terrestrial environments, respectively. Thus, on a broader scale, the findings of this work further advocate that

AOA have the capability to be significant players in the global nitrogen cycle, not only in numbers, but in ammonia oxidation capacity as well. This work also suggests the importance of further in-situ studies of nitrifying populations, particularly those containing AOA. It would be of value to correlate AOA *amoA* gene copy numbers to ammonia oxidation activity allowing for direct comparison to AOB specific activity. Also, the reactor design used for this study may allow further work to determine potential nitrification control mechanisms in drinking water distribution systems. As a result, these pieces would continue to add to the current understanding of the nitrification puzzle in engineered and natural systems.

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APPENDIX A

TABLE OF SAMPLES FOR PINELLAS COUNTY, FLORIDA

Appendix A: Table of Samples for Pinellas County, Florida

Sample #	Sample Date	Sample Time	Sample ID
1	6/19/2012	1010	Beach 1 of 2
2	6/19/2012	11:15	Ft. Desoto Main 1 of 2
3	6/19/2012	1215	100 Pinellas Bay Cucoy 1 of 2
4	6/19/2012	1315	9980 Gulf Blvd. 1 of 2
5	6/20/2012	1000	0 Feather Sound Dr. 2 of 2
6	6/20/2012	1150	12600 Roosevelt
7	6/20/2012	1345	16055 Fairchild Drive 1 of 2
8	6/28/2012	1150	Wall Springs Park 2" BF 1 of 2
9	6/28/2012	1000	3963 Desoto Blvd. 4" BF 1 of 2
10	6/28/2012	1430	Sutherland Crossing 6" Master Meter 1 of 2
11	6/28/2012	1200	301Crosswinds Blvd. LS 311 Back 1 of 2
12	6/28/2012	1430	Sutherland Crossing 6" Master Meter 1 of 2
13	7/5/2012	930	3340 Hibiscos Field 1' RP 1 of 2
14	7/5/2012	1050	117 6th st. 1 1/2" DCVM 1 of 2
15	7/5/2012	1140	LB097 Belle Isle 3/4" RP 1 of 2
16	6/19/2012	1010	N. Back Flow
17	6/192012	1115	FD Maint.
18	6/19/2012	1215	60 Pinellas Way
19	6/19/2012	1315	9980 Gulf Blvd.
20	6/20/2012	1000	0 Feather Sound Dr.
21	6/20/2012	1115	Marriott
22	6/20/2012	1345	16055 Fairchild
23	6/28/2012	915	Wall Springs 2" RP
24	6/28/2012		Wall Springs 4" 3963 Desoto
25	6/28/2012	1055	LS 3/4 Wailani
26	6/28/2012	1200	301 Crosswind LS 3"
27	6/28/2012	1430	Southerland Master Meter
28	6/28/2012	1430	Southerland Crossing Master Meter
29	7/5/2012	930	3340 Hibiscus 3/4" RP
30	7/5/2012	1050	11716…
31	7/5/2012	1140	LS097 Belle Isle