AMMONIA OXIDIZING ARCHAEA IN ENGINEERED WATER AND WASTEWATER

SYSTEMS: PRESENCE, ACTIVITY, RELATIONSHIP TO HEAVY METAL INHIBITION

AND DISINFECTANTS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota

State University's regulations and meets the accepted standards for the degree of

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ABSTRACT

Ammonia oxidizing archaea (AOA) have been found as a key player in ammonia oxidation. Over the past decade, AOA have been shown in some cases to outnumber ammonia oxidizing bacteria (AOB) in marine, terrestrial and aquatic environments, and to participate in nitrification. In this dissertation research, AOA along with AOB were examined for their presence and activities in wastewater treatment plants (WWTPs), and water treatment plants (WTPs) employing different disinfectants and their distribution systems (DSs).

Firstly, this research investigated the abundance and seasonal variation of AOA and AOB by quantitative polymerase chain reaction targeting the ammonia monooxygenase subunit A gene (*amoA*) in the biofilm from trickling filters (TFs) and a moving bed bioreactor (MBBR) employed separately at two WWTPs. AOA and AOB dominated nitrifying cultures from the same WWTPs were selected using specific inhibitors, and investigated for nitrification activity. Secondly, this research examined copper inhibition of nitrification by attached and suspended growth ammonia-oxidizing cultures containing different fractions of AOA. The third and final research part investigated AOA and AOB abundance and nitrification activity in different treatment steps of WTPs practicing chloramination and chlorination and distal and proximal zones of their DSs. A laboratory experiment simulating DS conditions with chorine and chloramine was conducted to understand the effects of disinfectant concentration and type, and water temperature on AOA and AOB populations and activity.

AOA were dominant in the nitrifying TF (NTF) and MBBR, while AOB dominated in the biochemical oxygen demand TF. AOA and AOB were more abundant during warm months. In-situ nitrification activity showed the oxidation of ammonia to nitrite decreased when AOA populations from the NTF and MBBR were inhibited, demonstrating that AOA contributes to

nitrification. Nitrification by the cultures heavily dominated by AOA were less inhibited by copper than the cultures with less AOA dominance. In WTP systems studied, more AOA were observed in chloraminated DS compared to chlorinated DSs. More AOA and AOB were detected in the distal zones than the proximal zones of DSs suggesting that AOA and AOB grew in the distribution systems. AOA had longer inactivation time under cold water temperature compared to AOB.

ACKNOWLEDGMENTS

DEDICATION

I would like to dedicate this dissertation to my beloved husband, Mayukh, my father

Surendranath Roy, and my mother Bula Roy for their support and encouragement to achieve my

goal.

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LIST OF ABBREVIATIONS

AMO ....Ammonia monooxygenase

amoA ....Ammonia monooxygenase subunit A

ANOVA ....Analysis of variance

AOA...Ammonia oxidizing archaea

AOB ....Ammonia oxidizing bacteria

AOM ....Ammonia oxidizing microorganisms

AOP..Ammonia oxidizing prokaryotes

AOR ....Ammonia oxidation rate

ATU ....Allylthiourea

AUR ....Ammonia uptake rate

BAC ....Biological activated carbon

BOD ....Biochemical oxygen demand

BTF ....BOD trickling filter

CDC ....Center for Disease Control

DBPs ....Disinfection by-products

DNA..Deoxyribonucleic acid

DO...Dissolved oxygen

DS ....Distribution system

FE..Filter effluent

GAC ....Granular activated carbon

HNO_3...Nitric acid

ICP-MS ....Inductively coupled plasma mass spectrometry

MBBR ....Moving bed biofilm reactor

NH_2Cl...Monochloramine

NJ ....Neighbor-Joining

NO_2 ....Nitrite

NO_3 ....Nitrate

NOB ....Nitrite oxidizing bacteria

NTF..Nitrifying trickling filter

OUR...Oxygen uptake rate

PBS ....Phosphate buffered saline

PCR..Polymerase chain reaction

PTIO..2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-Oxide

PVC..Polyvinyl chloride

qPCR...Quantitative polymerase chain reaction

RBC..Rotating biological contactor

sAOR...Specific ammonia oxidation rate

sOUR...Specific oxygen uptake rate

TF..Trickling filters

THMs...Trihalomethanes

VSS ....Volatile suspended solids

WTP..Water treatment plant

WWTP ....Wastewater treatment plant

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1. GENERAL INTRODUCTION

1.1. Background

As an important process for global N cycling, nitrification involves two distinct steps: (1) oxidation of ammonia by ammonia-oxidizers to nitrite (NO_2-N), and (2) oxidation of NO_2-N to nitrate (NO_3-N) by nitrite-oxidizing bacteria. Microbial ammonia oxidation is believed to be solely carried out by ammonia oxidizing bacteria (AOB) before ammonia-oxidizing archaea (AOA) were reported (Könneke et al., 2005). Autotrophic ammonia-oxidizing archaea are recently considered responsible for the majority of ammonia oxidation in marine waters (Beman et al., 2008; Mincer et al., 2007; Wuchter et al., 2006), marine sediments (Park et al., 2006) and soils (Chen et al., 2008; Leininger et al., 2006). AOA in suspended growth wastewater treatment plants (activated sludge) were first reported by (Park et al., 2006). It is not uncommon for AOA to outnumber AOB in activated sludge plants (Leininger et al., 2006; Zhang et al., 2011). The abundance of AOB and AOA is affected by temperature (Avrahami and Conrad, 2003; Tourna et al., 2008), ammonia availability (Kowalchuk and Stephen, 2001), pH (Nicol et al., 2008), and salinity (Bernhard et al., 2010).

Although there have been a number of studies available on AOA in suspended growth systems, there has been only one study (Sauder et al., 2012) that reported AOA in a fixed-film municipal wastewater treatment plant (WWTP) using a rotating biological contactor (RBC). The relative abundance of AOA increased as ammonia concentration decreased along the flow path of the RBC. Also, the relative abundance of AOA increased with the ambient temperature. In most of the samples, AOA outnumbered AOB, particularly at low ammonia concentrations.

Inhibition of nitrification in WWTP by heavy metals is a common concern. A loss in viability and changes in community structure in activated sludge, loss of floc structure (Neufeld,

1976), and/or decreases in treatment efficiency (Beyenal et al., 1997) may occur at high concentrations of heavy metals. Heavy metals such as copper have been found as a constant threat to the nitrification efficiency of WWTPs (Chandran and Love, 2008, Chandran and Smets, 2000, Hu et al., 2004). The effect of heavy metals such as copper on nitrification rate is related to the species and concentrations, and the species of nitrifiers (Mertoglu et al., 2008). Nitrification is typically not affected by low concentrations of metals; as concentrations increase heavy metals can inhibit the activity of nitrifiers (Hu et al., 2004). However, a study showed that a copper concentration of only 0.5 mg L^{-1} causes significant reduction (50%) in nitrification rates for *Nitrosomonas* (Sato et. al., 1988). Studies (Hu et al., 2002, 2004) showed that oxygen consumption rate for nitrification by activated sludge decreases when the wastewater contains copper.

Nitrification in drinking water treatment plant (WTP) and distribution system (DS) is common. Chloramines have been commonly used as a disinfectant in water treatment, and their use is strictly regulated. Chloramines are formed when ammonia is added along with chlorine in water treatment to increase residual disinfection effect, particularly in the DS. Although chloramination is common, many water utilities still practice chlorination. Ammonia oxidation which can be performed by AOA and/or AOB can occur in WTP that have naturally occurring ammonia or added ammonia for chloramination. Chloramine can also break down in water and release ammonia. Free ammonia is available to AOB and AOA. Based on the relative dosing of chlorine and ammonia, more free ammonia is available as the chloramine residual undergoes decay. The mechanisms of monochloramine decay include auto-decomposition (Vikesland et al., 2001), reaction with nitrite, and reactions with organic matter, including the cells of microorganisms and their metabolic products (Yang et al., 2008).

The ammonia monooxygenase subunit A *(amoA),* for AOA and AOB, has been detected in water treatment plants (Bai et al., 2013; van der Wielen et al., 2009). AOA were detected in three groundwater treatment and distribution systems in the Netherlands (van der Wielen et al., 2009). They were found in source water and throughout the treatment train up to the finished water without any disinfectant residuals. The ammonia concentration removed in each step of the treatment showed a correlation with the copy number of *amoA* (AOA and AOB combined). Ammonia, which is removed during biological filtration in the treatment plant, might play a key role in growth of AOA in the filter. A few studies found AOA on the surface or in the biofilm of anthracite/sand media of biologically active filtration systems (Bai et al., 2013; Pinto et al., 2012; van der Wielen et al., 2009).

In chloraminated distribution systems, AOB increased from the proximal to the distal parts of the DS due to an increase in ammonia (Lipponen et al., 2004; Lipponen et al., 2002). On the other hand, in a system without a disinfectant residual, there was no signinficant difference in the number of AOB in the proximal and distal areas of the DS. AOA were found in greater numbers in the distal portion compared to the proximal portion (van der Wielen et al., 2009). In comparison to chloramine, chlorine is more effective as a disinfectant. Biological instability was observed in a chlorinated DS where concomitant loss of disinfection residual and bacterial growth occurred (Nescerecka et al., 2014).

Sediment and biofilms can harbor nitrifying bacteria and reduce disinfectant efficiency, resulting in an increased likelihood of nitrification occurrence. Higher chloramine residual levels appear to prevent nitrification. However, once nitrification occurs, chloramine residual is quickly degraded. A study showed the detection of AOA in a distal zone of the DS that involves raw water with natural ammonia (van der Wielen et al., 2009). Free bacteria including nitrifiers in

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bulk water have been shown to dominate in portions of a DS that has no disinfection residuals (Regan et al., 2002; Srinivasan et al., 2008).

1.2. Research Problem Statement

Moving bed biofilm reactor (MBBR) and trickling filters (TF) are commonly used attached growth processes for municipal and industrial wastewater treatments. Yet, in contrast to activated sludge (AS) systems, nothing is known about the abundance of AOA in TF and MBBR. It is important to understand the presence and activity of AOA in TF and MBBR because AOA showed a substantial effect on nitrification in WWTPs. The effect of temperature on AOA for TF and MBBR biofilm systems has not been elucidated. No study has so far investigated seasonal effects on ammonia-oxidizers particularly AOA in trickling filter and MBBR biofilms.

Nitrification inhibition by heavy metals in wastewater has been widely studied in the context of AOB. A similar study on AOA does not exist because pure cultures of AOA are not widely available. Studying effect of heavy metals on the nitrification ability is worthwhile as AOA might play a crucial role in nitrification. Effects of heavy metals on AOB are different for attached growth and suspended growth systems. AOB such as *Nitrosomonas europea* in a suspended growth system experienced more inhibitory effect by heavy metals than a biofilm condition (Stehr et al., 1995). Under biofilm conditions, bacteria had reduced contact with heavy metals due to the presence of extracellular polymeric substances (Vu et al., 2009). Studies on these aspects for AOA are not available. There is a need to examine heavy metal inhibition on suspended growth and attached growth AOA.

Studies on AOA abundance and activity in WTP and DS have been limited. There is a knowledge gap in understanding the chlorine versus chloramine disinfection in relation to several

aspects of AOA. AOA have been detected in DSs, but it is unclear whether their presence has any relationships with the type and level of disinfection particularly chloramine versus chlorine. It is not known if there is any effect of chloramination, ambient temperature, and retention time and/or distance of the DS on the number of AOA. Understanding if the abundance and activity of AOA correlate with a loss of disinfectant residuals is also important. From the perspective of regulatory compliance, a loss of the disinfectant residual is the most critical consequence of nitrification in the DS. On the contrary, the presence of AOA in the treatment train (particularly in filtration units) may be beneficial as AOA may nitrify ammonia.

1.3. Research Objectives and Hypotheses

The following objectives and the corresponding hypotheses were developed:

Objective: To investigate the presence, activities and seasonal variation of AOA and AOB in WWTP biofilm.

Hypothesis: The abundance of AOA and AOB in biofilm correlates with the ambient temperature at WWTP

Objective: To investigate heavy metal (copper) inhibition on AOA in mixed cultures of suspended and attached growth based on specific oxygen uptake rate (sOUR), specific ammonia oxidation rate (sAOR), and AOA specific *amoA* gene abundance.

Hypotheses: sOUR, sAOR, and AOA specific *amoA* gene copy number decrease in the presence of copper. Suspended mixed culture experiences more inhibition than attached mixed culture. Activities of the culture with higher fraction of AOA is less inhibited by copper.

Objective: To investigate the presence and activities of AOA and AOB in WTPs and DSs associated with chloramination and chlorination practices.

Hypotheses: The presence and activities of AOA and AOB are different for DSs practicing chlorination and chloramination. Chloramination systems have more abundance and activities of AOA and AOB. Chloramine residual, chlorine residual, and ammonia concentration correlate with the numbers and activities of AOA and AOB. Temperature correlates with the number of AOA and AOB in WTPs and DSs.

1.4. Dissertation Organization

This dissertation is divided into 6 chapters. This chapter includes background, research problem statement, objectives, hypotheses, and dissertation organization. Chapter 2 provides a literature review on prevalence of AOA and AOB in the wastewater and drinking water environment. Chapter 3 describes the abundance and activity of AOA in nitrifying biofilm in two WWTPs, one with two-stage TFs and the other with MBBR. The work described in Chapter 3 is based on a manuscript titled "Seasonal Variation and Ex-Situ Nitrification Activity of AOA in Biofilm-based Wastewater Treatment Processes." This manuscript has been submitted to a peer reviewed journal. Chapter 4 was derived from a manuscript titled "Copper Inhibition of Wastewater Nitrification by Attached and Suspended Growth Ammonia-Oxidizing Cultures with Different Archaeal Populations." This manuscript will be submitted to a peer reviewed journal. Chapter 5 is based on a manuscript entitled "Abundance, Seasonal Variation and Activity of Ammonia Oxidizing Archaea and Bacteria in Bulk Water and Biofilm Associated with Water Supply Systems: Influence of Chlorination and Chloramination Practices." This manuscript will be submitted to a peer reviewed journal. Conclusions and recommendations for future work are presented in Chapter 6.

2. LITERATURE REVIEW

2.1. Ammonia Oxidizing Microorganisms (AOM)

Two types of ammonia oxidizing microorganisms (AOM), AOA and AOB, are discussed in this section.

2.1.1. AOA

The archaeal domain is divided into two major phyla, the Euryarchaeota and Crenarchaeota. AOA is a group of Crenarchaeota, an important participant in the global carbon and nitrogen cycles (Leininger et al., 2006; Schleper, 2010; Schleper et al., 2005; Wuchter et al., 2006). Autotrophic AOA are recently considered to be responsible for the majority of ammonia oxidation in marine waters (Beman et al., 2008; Mincer et al., 2007; Wuchter et al., 2006) marine sediments (Könneke et al., 2005) and soils (Chen et al., 2008; Leininger et al., 2006). The first evidence of autotrophic ammonia oxidation by AOA was obtained after characterizations of mesophilic crenarchaeon (group I.1a), *Candidatus Nitrosopumilus maritimus* SCM1 strain from an aquarium (Könneke et al., 2005), and later a related archaeon (Wuchter et al., 2006). Evidence was further supported by subsequent culturing of members of group I.1b, a moderately thermophilic AOA *Candidatus Nitrososphaera gargensis* (De Jonge et al., 2015) and thermophilic *Candidatus Nitrosocaldus yellowstonii* (de la Torre et al., 2008). A whole-genome based phylogenetic approach revealed that nonthermophilic crenarchaea, including the AOA, likely belong to a separate phylum, called Thaumarchaeota (Brochier-Armanet et al., 2008a; Brochier-Armanet et al., 2008b; Spang et al., 2010)

AOA are found in moderate temperature aquatic and terrestrial environments (Francis et al., 2005; Hallam et al., 2006; Treusch et al., 2005). The abundance of AOA oxidizers is affected by temperature (de la Torre et al., 2008; Reigstad et al., 2008; Tourna et al., 2008; Tourna et al.,

2011) ammonia availability (Hatzenpichler, 2012; Hatzenpichler et al., 2008; Kowalchuk and

Stephen, 2001), pH (He et al., 2007; Nicol et al., 2008; Reigstad et al., 2008), and salinity

(Bernhard et al., 2010; Caffrey et al., 2007; Santoro and Casciotti, 2011; Santoro et al., 2008).

2.1.2. AOB

The AOB phylogenetically consist of two monophyletic groups of bacteria in

Proteobacteria phylum based on an evolutionary study from 16S rRNA operon and *amoA* gene

sequences (Purkhold et al., 2003). Most AOB belong to the family *Nitrosomonadaceae* in the

class *Betaproteobacteria,* whereas marine AOB are in the genus *Nitrosococcus* and the class

Gammaproteobacteria. The AOB in the family *Nitrosomonadaceae* are further divided into three

genera of *Nitrosomonas, Nitrosospira,* and *Nitrosovibrio* (Purkhold et al., 2003). The ammonia

oxidation by AOB is performed with two consecutive reactions; ammonia (NH_3) to

hydroxylamine (NH_2OH) conversion by utilizing oxygen through membrane-bound ammonia

monooxygenase (AMO), and hydroxylamine to nitrite conversion by the octahaem cytochrome *c*

hydroxylamine oxidoreductase (HAO) occurring in the periplasm of the AOB (Andersson et al.,

1984; Arp et al., 2002; Stein and Arp, 1998; Hollocher, 1981). Nitrite, produced from the

oxidation of ammonia by AOB, is found to be detrimental to the AOB as nitrite reduces activity

of AMO (Stein and Arp, 1998). Because of the AOB's slow growth rate, high sensitivity to

environmental factors, and inability to outcompete heterotrophs, ammonia oxidation is a rate-

limiting step of nitrogen removal in wastewater treatment systems.

2.2. Role of AOM in Nitrification

Nitrification, the key and often rate-limiting step in N removal, entails the two-step

microbial oxidation of ammonia to nitrate via nitrite. Nitrification is sequential oxidation of

ammonia to nitrite via hydroxylamine by AOB and AOA (intermediate path is unknown), and

nitrite to nitrate by nitrite oxidizing bacteria (NOB). Both AOA and AOB are chemolithoautotrophs, who use oxygen as electron acceptor. They use ammonia or nitrite as electron donor. As their sole carbon source, they use carbon dioxide. An area of active research is the relative importance of archaeal and bacterial ammonia oxidizers to nitrification. Many researchers are also working to gain a better understanding of factors that cause niche separation between AOA and AOB to identify conditions that favor one over the other (Martens-Habbena et al., 2009, Caffrey et al., 2007).

2.2.1. Nitrification by AOB

Based on genome data of AOB, Nitrification reaction is started by a copper-containing AMO protein, which is encoded by multiple operons (called *amo*-operons) based on the AOB genome data. *Nitrosomonas eutropha* has two similar copies of *amo*-operon including genes encoding three subunits *(amoCAB),* and a single additional copy of *amoC* (Stein et al., 2007). On the other hand, *Nitrosospira multiformis* has three copies of *amo*-operon, and a fourth copy of *amoC* (Norton et al., 2002). A phylogenetic analysis was conducted with two conserved genes in beta- and gamma- AOBs which were directly following *amoCAB* operon, *orf4 (amoE)* and *orf5 (amoD)* (El Sheikh et al., 2008). Interestingly, the transcription levels of these two conserved genes *(orf4* and *orf5*) were significantly induced when *Nitrosomonas europaea* recovered from ammonia starvation. The similar incidence occurred in the case of *amoC* (El Sheikh et al., 2008). This concludes that *amoC, amoD,* and *amoE* are transcriptionally linked.

Temperature plays a critical role in nitrification by AOB. Nitrifying bacteria have been found to be extremely sensitive to low temperatures; nitrification rates have been reported to increase 12 - 50% with a 4°C increase and decrease 8 - 30% with a 1°C drop when compared to nitrification rates at 21.3°C (Barritt, 1933; Srna and Baggaley, 1975). The minimum generation

time of nitrifiers at 30°C (optimal temperature) is approximately 15 hours, whereas the generation time at 5°C is approximately 200 hours (Wijffels et al., 1995). The optimum temperature for nitrification is within a range of 28 - 36°C, thus classifying AOB as mesophilic bacteria (Sharma and Ahlert, 1977). Recent studies have observed bacterial population shifts in AOB with changes in temperature (Hallin et al., 2005; Layton et al., 2005; Siripong and Rittmann, 2007). *Nitrosospira*, another terrestrial AOB genera, has been found to co-exist with *Nitrosomonas* in small amounts but *Nitrosospira* is much more temperature sensitive (Park et al., 2006); therefore, *Nitrosomonas* becomes the dominant AOB genera at low temperatures.

Nitrification rate decreases significantly as pH decreases below 6.8. Optimal nitrification rates occur at pH 7.0 - 8.0. Specifically, optimum pH levels for *Nitrosomonas* and *Nitrobacter* are 7.9 - 8.2 and 7.2 - 7.6, respectively (Alleman, 1984). A study found that higher ammonia seemed to favor AOB in soils (Di et al., 2010); the nitrification rate also correlated with AOB abundance. *Nitrosomonas* are the dominant AOB in wastewaters (Ducey et al., 2010).

2.2.2. Nitrification by AOA

AOA are present in large numbers in the ocean and soils, suggesting a potential role for archaea, in addition to bacteria, in the global nitrogen cycle. Evidence for the existence of AOA has only appeared within the past 10-15 years. Könneke et al. (2005) successfully cultured an aerobic AOA that converted ammonia to nitrite while fixing inorganic-carbon. The generation time was at least 21 h for this AOA. A previous study had analyzed the genome of another marine AOA, which revealed that the ammonia monooxygenase gene present in this AOA were homologous to AOB for oxidizing ammonia (Hallam et al. (2006). With the genetic and metabolic evidence from the above studies, the existence of AOA has been confirmed.

Leininger et al. (2006) were among the first to investigate the relative numbers of AOA and AOB in soil environments. The study showed that AOA were predominant over AOB. The ratio of AOA to AOB were detected more in non-fertilized soils at a higher depth. However, the relative nitrification activity of AOA and AOB were not shown by the study. The study found that concentration of ammonia is main factor for niche separation between AOA and AOB (Schleper, 2010). As stated earlier, high affinity for ammonia by a strain of AOA proposes a competitive advantage of such AOA strain in low ammonia niches (Martens-Habbena et al., 2009).

Reed et al. (2010) observed that both AOA and AOB were stimulated by the addition of nutrients (sequential addition of organic carbon and then nitrogen) to groundwater. AOA were more abundant than AOB before nutrient addition occurred, and remained so throughout the experiment, but AOB showed a stronger response to the addition of nitrogen. In their research on the formation of nitrifying biofilms in fresh water flow channels, Herrmann et al. (2011) observed positive Spearman correlations (0.92 and 0.96, respectively) for both AOA and AOB *amoA* gene copy numbers with the ammonia concentration in water sources. The ratio of AOB/AOA gene copies was also positively correlated with ammonia ($R^2 = 0.87$). That is, AOB seem to have been favored at higher ammonia levels. Interestingly, AOB became dominant in all biofilms, even when AOA were higher in the source water. Sauder et al. (2012) conducted a study on ammonia-oxidizing microbial communities in a rotating biological contactor wastewater treatment system and found that AOA contributed a higher percentage of *amoA* gene copies when ammonium levels were low while AOB had greater relative abundances when ammonium levels were high. Other water quality parameters were not correlated significantly with the relative abundances of AOA and AOB in their study.

de Vet et al. (2009) conducted one of the first studies in a WTP environment that considered AOA and found that both bacteria and archaea contributed to ammonia oxidation in a full-scale sand filter treating groundwater. The magnitudes of their respective contributions to nitrification in the filter were not determined. Kasuga et al. (2010) investigated AOA in pilot-scale biological activated carbon (BAC) filters. AOA gene copies were higher (1-2 orders of magnitude) than those of AOB. The AOA abundance over AOB suggested that AOA may be the dominant ammonia oxidizers. In a study on full-scale granular activated carbon (GAC) filters, Kasuga et al. (2010) concluded that AOA were responsible for 75-93% of the ammonia removal. This number was estimated based on the fractions of AOA and AOB in the GAC filter. Moreover, the estimation relied on an assumption that only AOA and AOB could remove ammonia. van der Wielen et al. (2009) looked at AOB and AOA in three drinking water treatment plants using groundwater as a source, and distribution systems. The study found some distribution system locations where AOA were more numerous, somewhere AOB were more numerous, and in some cases the numbers of each type of ammonia oxidizer were similar. The total AOM numbers correlated well with ammonia removal in the treatment trains.

Other factors that have been suggested to explain AOA:AOB ratio or relative activities include pH (Nicol et al., 2008; Prosser and Nicol, 2008), susceptibility to chlorination or other treatment steps (Kasuga et al., 2010), and the concentration of organic carbon or metals (van der Wielen et al., 2009). A topic of interest is the way in which AOA differ from AOB. Martens-Habbena et al. (2009) found a very low half-saturation coefficient for ammonia for a strain of AOA. This high affinity for ammonia suggests it could successfully compete with heterotrophs for nitrogen and that it could thrive in low-substrate environments. It is not clear whether other species of AOA would share this capability.

2.3. Attached-Growth Biological Wastewater Treatment Systems

Attached growth processes can be used for biochemical oxygen demand (BOD) removal only, combined BOD removal and nitrification, or tertiary nitrification. TFs and MBBRs are capable of producing an effluent with total ammonia-nitrogen concentration lower than 1 mg/L at various flow rates and under conventional treatment temperatures (Rusten et al., 2006; Stenquist et al., 1978). MBBRs represent newer and more efficient technologies of biofilm systems compared to TFs and offer the advantage of less space requirement.

2.3.1. TF

The TFs include a solid substratum to support the growth of an active biofilm. The filter encompasses a bed of rocks or gravel or polyvinyl chloride (PVC) media. Wastewater is generally pumped to the rotating arm and is sprayed on the surface of the media bed. The dissolved oxygen (DO) needed for treatment is provided by a natural draft or forced air. The bed rock located in the upper portion of the filter contains thicker biofilm with heterotrophic bacteria whereas the bed rock located in the lower portion of the filter contains a thinner biofilm with autotrophic bacteria in a single stage filter. The wastewater flow approaches plug flow conditions in this system which can be operated at various hydraulic and feed loads.

Both BOD removal and nitrification can be achieved using a single stage or two stage TF with rock or plastic packing material at low organic loads (Stenquist et al., 1978). To initiate nitrification, BOD should be less than 30 mg/L while for complete nitrification BOD should be less than 15 mg/L (Bruce et al., 1975).

Operating costs are low to moderate, and the level of skill required to operate TFs is moderate. Although TFs require less energy compared to the conventional activated sludge process, there are a few disadvantages to consider. TFs are more suitable for low flow rates.

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Furthermore, as growth of biofilm may not be uniformly distributed on media, and the wastewater does not flow uniformly over the media, biofilm overgrowth may occur on some portions of the packed media which can cause clogging. High loading rates in the TF system, particularly under carbon removal conditions, can create excessive growth of biofilm/biomass, which in turn can clogs the pores of the media. Excessive growth of biofilm can directly affect the actual retention.

2.3.2. MBBR

MBBR uses biocarriers to maintain the growth of biofilm. Biocarriers are made by several suppliers and therefore they vary in shapes and sizes. The specific surface area provided for the growth of biofilm varies between 500 and 1200 m^2/m^3 based on the biocarrier type and corresponding manufacturer. The biocarriers are held in continual suspension and movement in the treatment basin. This allows biocarriers for optimal contact of the biofilm with DO and substrate in the bulk liquid. Biofilm grows inside the biocarrier to protect the biofilm, from external forces to a certain extent. The biofilm thickness depends on the hydrodynamic forces in the system. In general, the percentage fill (volume) of the media is 25 – 70% of the total reactor volume (Rusten et al., 2006). Thus, to increase treatment capacity of a MBBR, augmentation by adding more biocarriers to the system is carried out. Furthermore, carriers have been made to last very long time (15 years) with minimum impairment (Rusten et al., 2006).

Similar to the TFs processes, MBBRs can be used for both BOD removal and nitrification. MBBRs do not occupy a large area, they do not require sludge recycling, or backwashing and solids produced through the detachment of biofilm can be settled using a final clarifier. The cost associated with operation and maintenance of MBBR technologies are generally less in comparison to conventional suspended growth (activated sludge) processes, and

other attached growth processes; however this is depending on aeration rates, costs can also be high for activated sludge process.

2.4. Drinking Water Distribution Systems

Biofilms (or attached growth) formed on the inner surfaces of distribution pipes are generally believed to dominate microbial growth in distribution systems and therefore are considered to be responsible for the deterioration of drinking water quality. A significant fraction of bacteria growing in the distribution systems is associated with biofilms (Camper et al., 2003) and for nitrifiers, attachment to solid surfaces enhances their growth and provides more resistance to toxic substances (Wolfe et al., 1988).

Large metabolic differences between attached and free-living bacteria can occur (Boe-Hansena et al., 2002). In low nutrient conditions, bulk phase growth accounted for a significant part of the water treatment systems (Boe-Hansena et al., 2002). Moreover, bacteria occurred I the bulk water have been shown to dominate in portions of a DS that have low chlorine disinfection residual (Srinivasan et al., 2008), therefore presenting a necessity to study both bulk phase and biofilm for both chlorinated and chloraminated DS.

Nitrifiers can form biofilm inside DS pipelines. The type of disinfection practice (chloramination versus chlorination) affects in the development of nitrifiers in the DS. Ammonia used in the chloramination process can promote the growth of nitrifying bacteria in the DS (Gomez-Alvarez et al., 2014; Lee et al., 2011a; Wahman et al., 2016), which can cause increased nitrate levels in finished water. The chloramine residual is more stable and longer lasting than free chlorine or chlorine dioxide, thereby can protect against bacterial regrowth. However, excess ammonia in the network may support biofilm formation in pipeline. Chlorination may be effective in reducing the growth of nitrifiers, but it is undesirable due to potential formation of

harmful disinfection by-products (DBPs). Therefore, when using chloramine as a secondary

disinfectant, it may be necessary to periodically switch to free chlorine for biofilm control in the

DS.

Apart from different types of disinfection practices, the effects of pH (Allison and

Prosser, 1993), chloramine concentration (Wolfe et al., 1988) and temperature (Pintar and

Slawson, 2003), on nitrifying organisms in the DS were observed. The optimum pH for AOB

depends on ammonia concentration but normally is 7.5-8.0 (Allison and Prosser, 1993). By

increasing chloramine concentration, the growth of AOB can be ceased in the DS. Increased

concentration of chlorine reduces ammonia level before the water enters into the DS; thus, in

turn, limits ammonia-dependent growth of AOB (Wolfe et al., 1988). Higher temperatures are

more favorable for nitrification. Wilczak et al. (1996) reported that most nitrification occurred

when temperatures were above 15°C, but it was also observed below 10°C. In a bench-scale

study, Pintar and Slawson (2003) confirmed that AOB could become established in low-

temperature reactors (6-12°C).

2.4.1. Chlorination

Earlier studies on nitrifying microorganisms in DS were limited to AOB (Gomez-Alvarez

et al., 2014; Regan et al., 2002; Wahman and Pressman, 2015). Factors associated with the

presence or abundance of nitrifying bacteria have been identified. Lipponen et al. (2004) studied

the development of biofilms containing nitrifiers on PVC pipes that received water from full-

scale DS. Nitrifiers were more prevalent further from the WTP. There was a positive correlation

between nitrifiers and heterotrophs. Both heterotrophic and nitrifying microorganisms were

positively correlated with turbidity and retention time and negatively correlated with pH and

total chlorine. Lipponen et al. (2004) surveyed AOB and NOB in water and sediment samples

from DS in Finland. They found positive correlations between heterotrophic plate count and AOB in both water and sediments. Piping material and the use of GAC filtration were not found to significantly affect the number of nitrifiers, and DO was not a limiting factor in any of the systems they studied. A study performed by Kasuga et al. (2010) raised the possibility that archaea may be more susceptible than bacteria to chlorination.

2.4.2. Chloramination

Nitrification occurs in a wide range of environments; chloraminated DS are one of these environments due to the presence of ammonia, which is added to the water to react with free chlorine and form a chloramine disinfectant residual (mono-chloramine is the most common form). In contrast to other disinfectant residual options used in the DS, chloramination adds a substrate for microorganisms, in addition to a disinfectant (Zhang and Edwards, 2009). Many utilities in North America have adopted chloramines because they form lower amounts of DBPs, are better at penetrating and disinfecting biofilms, and in many situations, are more persistent than free chlorine in the DS (Zhang et al., 2015a).

Chloramine is widely used to minimize the formation of DBPs such as trihalomethanes. These DBPs have been linked with carcinogenesis in animals. About thirty percent of water treatment facilities in the USA currently practice chloramines. The chloramine practice is projected to rise due to the application of Stage 2 DBP Rule (Seidel et al., 2005). Even though chloramine is more stable and efficient in controlling DBPs, residual ammonia triggers nitrification in the DS and generates a chain of consequences as shown in Figure 1, which cause biological instability in the water by fostering bacteria and in turn increasing chloramine demand. The major chloramine-nitrogen reactions in the DS are listed in Table 1.

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In DS, AOB oxidize ammonia to nitrite, which can be a substrate for NOB to produce

nitrate, which is directly related to the deterioration of the water quality. Proliferation of these

bacteria increases the chloramine demand of the system. Deterioration of chloramine can create

increased ammonia residuals, which promote the growth of AOB and nitrification. Eventually,

this increased ammonia residual (that is the initial step in nitrification) leads to the loss of

residual chloramine concentration, changes in pH and alkalinity, DO depletion, production of

nitrite and/or nitrate, and the increase of the nitrifying organisms in the system (Cunliffe, 1991;

Wolfe et al., 1990).
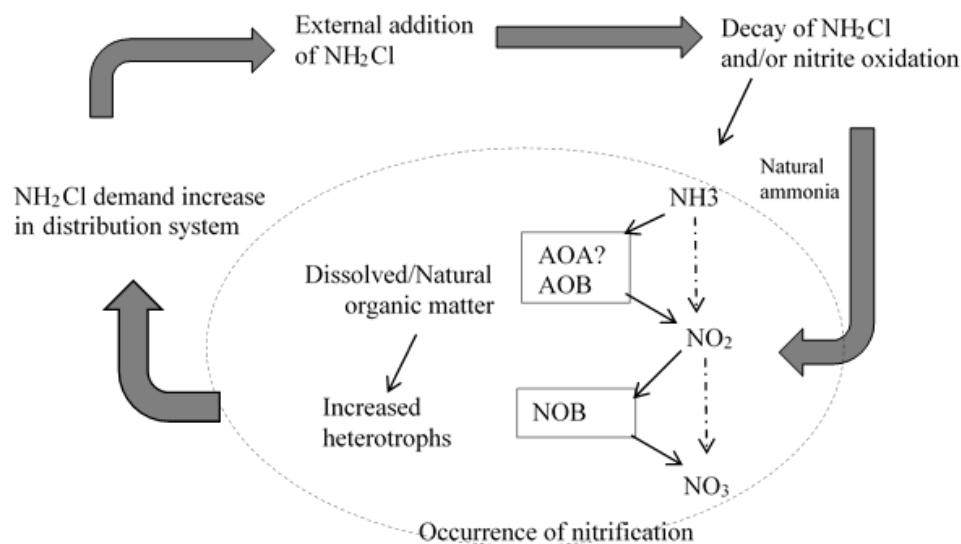
Figure 1. The chain of consequences after chloramine (monochloramine) decay in the DS
(modified from Park, 2011)

One of the known remedies for nitrification is going back to alternative disinfection

strategies such as short-term free chlorine and reducing ammonia concentration, increasing

chloramine residuals, and reducing water age or flushing DS. Cunliffe (1991) investigated the

abundance of nitrifying bacteria in chloraminated distribution systems in Australia. Using

stepwise multiple logistic regression and Spearman correlations, it was found that total chlorine

and NO_2^- plus NO_3^- were statistically significant indicators for the presence of nitrifying bacteria;

temperature and standard plate counts were not statistically significant indicators of nitrifiers.

Regan et al. (2002) investigated specific species of AOB that are present in pilot-scale WTP and

DS. The most abundant AOB species was related to *Nitrosomonas oligotropha*. They suggested

that this type of AOB might be selected in the DS because of a strong affinity for ammonia.

AOA have been detected in WTPs and DSs with or without disinfections process (Kasuga et al.,

2010; van der Wielen et al., 2009) as discussed earlier in Section 1.1.

Table 1. Overview of major chlorine-nitrogen (chloramine) reactions in DS (Park, 2011)

Reaction description	Stoichiometry
Development of chloramine	$NH_3 + HOC1 = NH_2C1 + H_2O$
Auto-breakdown of chloramine	$3NH_2C1 = N_2 + NH_3 + 3C1- + 3H+$
Nitrite oxidation by chloramine	$NH_2Cl + NO_2- + H_2O = NH_3 + NO_3- + HCl$
Organic matter oxidation by chloramine	$0.1C_5H_7O_2N + NH_2C1 + 0.9H_2O = 0.4CO_2 + 0.1HCO_3- + 1.1NH_4+ + Cl-$
Ammonia release by chloramine reaction with corrosion residues at pipe wall	$0.5NH_2C1 + H+ + Fe_2+ = Fe_3+ + 0.5NH_4+ + 0.5Cl-$

2.5. Previous Molecular Detection of AOA and AOB

Molecular techniques, especially qPCR, are increasingly being adopted to study nitrifiers.

Ammonia oxidation-related microorganisms are low in number and are hardly detectable using

16S rRNA (Junier et al., 2010). Therefore, alternative functional markers such as *amoA*, which is

involved in ammonia oxidation, have been used. To amplify the *amoA* gene from AOA and

AOB, an improved approach is used to release ammonia-oxidizing microorganisms that are

firmly attached to biofilms. PCR primers targeting the *amoA* genes that are specific to AOB and AOA have been developed (de la Torre et al., 2008; Rotthauwe et al., 1997). However, PCR and qPCR detections are dependent on the purity of deoxyribonucleic acid (DNA) that is extracted from environmental samples. Commonly used DNA extraction methods are sometimes inadequate since the archaeal cell wall and membrane structures are distinct from bacteria. Moreover, biofilms are made up of rich humic acid and complex protein substances that hinder desired gene expression during conventional PCR. Commercially available bead beating type DNA extraction kits are now often used for different water and wastewater samples because of their reliability (Agogué et al., 2009; Jiang et al., 2005). Although most of the protocols associated with these kits incorporate some form of mechanical disruption to insure more uniform DNA extraction than traditional methods that rely entirely upon enzymatic digestion and/or chemical disruption (Hybbinette et al., 1999), their suitability for the concerted analysis of AOA and AOB populations has not been fully evaluated.

2.5.1. Wastewater Systems

AOA have been found in both natural and engineered systems along with AOB (Beman et al., 2008; Caffrey et al., 2007; Francis et al., 2005; Hansel et al., 2008; Hatzenpichler, 2012; Hatzenpichler et al., 2008; Kasuga et al., 2010; Santoro and Casciotti, 2011; Santoro et al., 2008; Tourna et al., 2008; Urakawa et al., 2008); however, their precise role in nitritation in different environments remains unclear. For biological wastewater treatment systems, AOA have been detected in both suspended growth (Limpiyakorn et al., 2013; Park et al., 2006b; Wells et al., 2009) and attached growth systems (Sauder et al., 2012). In the only study to date of AOA in a RBC, Sauder et al. (2012) showed that the relative abundance of AOA increases as ammonia concentration decreases along the flow path of the RBC.

The most common molecular tool for the detection of AOA and AOB is the ammonia monooxygenase subunit A (*amoA*) as its sequence is highly conserved. Several studies detected AOA specific *amoA* in industrial and municipal wastewater (Lehtovirta-Morley et al., 2011; Mussmann et al., 2011; Park et al., 2006; Tourna et al., 2011). Different studies on activated sludge found that AOA outnumbered AOB (Leininger et al., 2006; Limpiyakorn et al., 2013; Zhang et al., 2009a) while in some studies (Jin et al., 2010; Mosier and Francis, 2008; Wells et al., 2009) AOA were present in less numbers than AOB.

Quantitative and qualitative AOA data have not been reported for other widely-used fixed-film processes, such as TF and MBBR systems. Bacterial ammonia oxidizers such as *Nitrosomonas* and *Nitrospira* have been reported in fixed film processes (Mobarry et al., 1997; Shore et al., 2012; Wagner et al., 1996).

In terms of ammonia oxidation, mixed opinions prevail regarding the contribution of AOA (Nicol et al., 2008). Studies suggested that AOB could play a central role in ammonia oxidation despite the predominance of AOA (Wells et al., 2009; Wu et al., 2013a). Wells et al. (2009) suggested that AOA were minor contributors to ammonia oxidation in highly aerated activated sludge systems. Other studies have demonstrated that AOA are indeed involved in nitrification in soil environment (Herrmann et al., 2011; Offre et al., 2009).

2.5.2. Water Treatment Plants and Distribution Systems

Nitrosomonas europaea is the most studied AOB pure culture in WTP and DS. Its ammonia oxidation pathway under chloramine exposure has been studied (Oldenburg et al., 2002). Several studies have been conducted with nitrifying biofilm in the presence of chloramine (Maestre et al., 2013, 2016; Wahman et al., 2016). de Vet et al. (2011) conducted one of the first studies in a WTP environment that considered AOA and claimed that both AOB and AOA

contributed to ammonia oxidation in a full-scale sand filter treating groundwater. Kasuga et al. (2010) investigated AOA in pilot-scale BAC filters. AOA gene copies were one to two orders of magnitude higher than those of AOB. The AOA predominance suggested that they may be the dominant ammonia oxidizers in the filter. In another study (Kasuga et al., 2010) on full- scale GAC filters, the authors concluded that AOA were responsible for 75–93% of the ammonia removal. The conclusion was simply based on the fractions of AOA and AOB detected.

van der Wielen et al. (2009) looked at AOB and AOA in three WTPs using groundwater as a source, and DS. The study found the number of AOA could be more, less or similar to the number of AOB in different locations of the DS. The total ammonia oxidizer numbers correlated well with ammonia removal in the treatment trains. Wahman and Pressman (2015) studied a laboratory scale annular reactor to simulate the DS. AOA were below detection level and only AOB were detected in their reactor. They suggested that the operational conditions during initial reactor setup might not be favorable for AOA growth.

2.6. Metal Inhibition, AOA and AOB Enrichment, AOA and AOB Culture Preparation

Inhibition of nitrification in WWTPs by heavy metals is a common concern. A loss in viability and changes in the community structure in activated sludge, loss of floc structure (Neufeld, 1976), and/or decreases in treatment efficiency (Beyenal et al., 1997) may occur at high concentrations of heavy metals. Due to their effect on nitrifying population, copper have been found as a constant threat to the nitrification efficiency of WWTPs (Hu et al., 2002, 2004; Tchobanoglous et al., 2003). Although AOA along with AOB participate in ammonia oxidation in various environments, it is unclear if AOA are subject to similar inhibitory effects on nitrification in the presence of copper in the WWTP. The effect copper on nitrification rate is related to the type and concentration of copper present (Mertoglu et al., 2008). Microbial

nitrification processes are typically not affected by low concentrations of copper; however, as concentrations increase copper can inhibit activity (Hu et al., 2002, 2004). The concentration at which copper becomes toxic depends on bacterial physiology.

The inhibition by copper on nitrification in wastewater has been widely studied on AOB. Similar studies on AOA have not been reported because pure cultures of AOA are not widely available. Moreover, for the mixed cultures containing AOB and AOA, selective inhibitors that allow separation between AOB and AOA activities are needed. The inhibition should be instantaneous and complete for the target population and should not affect other populations. It is not known whether AOB inhibitor, such as allylthiourea (ATU) has an effect on AOA. On the contrary, 2-phenyl-4,4,5,5, -tetramethylimidazoline-1-oxyl-3-oxide (PTIO) can inhibit AOA population; however, it is not known if PTIO affects AOB. Moreover, it takes months or years to develop a pure culture of AOA in the laboratory because of their extremely slow growth. Studies have been using AOA enrichment cultures to evaluate the physiology and nitrification activity of AOA (Park et al., 2006b; Santoro and Casciotti, 2011; Zhang et al., 2009b). Enrichment of AOA have been studied using AOA specific growth conditions, especially from soil samples, open ocean (Kim et al., 2008; Park et al., 2006; Santoro and Casciotti, 2011), and fresh water environment (French et al., 2012). However, AOA enrichment by inhibiting AOB population in the mixed consortia of nitrifying biofilm in WWTP has not been reported.

Heavy metal toxicity assays used to screen for the presence and effects of toxicants in wastewater include respirometry (Ginestet et al., 1998; Kelly et al., 2004), measurement of growth inhibition based on cell viability (Alsop et al., 1980), and chemical analyses and microscopic analyses (Curds and Cockburn, 1970; Madoni et al., 1996; Madoni et al., 1999). Respirometric inhibition methods are the most commonly used toxicity assessment method in

WWTPs (Kong et al., 1996; Spanjers et al., 1996). The OUR of activated sludge decreases when the wastewater contains toxicants. This method examines the metabolic rate of the entire sludge community. The respirometric approach was performed to evaluate stoichiometry of the nitrification (Liu and Wang, 2012). Respirometry also has been used to analyze partial nitrification by nitrifying enrichment media developed from activated sludge (Kapoor et al., 2016; Kapoor et al., 2015; Li et al., 2016). Inhibitory effects on AOB in the presence of metal contaminants have been quantified by sOUR along with specific ammonia uptake rate (sAUR) (Chandran and Love, 2008).

2.7. Copper Inhibition on AOA and AOB

Copper concentrations between 1.27 and 12.7 mg/L are important for optimal AMO enzyme function (Ensign et al., 1993). High copper level can inhibit nitrification in both *Nitrosomonas* and *Nitrobacter* (Braam and Klapwijk, 1981; Hu et al., 2002, 2004; Lee et al., 1997). At higher copper concentrations (greater than 30 mg/L), AOB, such as *Nitrosomonas*, show signs of growth delay greater than that of nitrite oxidizers, such as *Nitrobacter* (Lee et al., 1997). A study showed that a copper concentration of only 0.5 mg/L causes significant reduction (50%) in nitrification rates for *Nitrosomonas* (Sato et al., 1988).

Studies have shown that adsorbed copper concentration has no direct correlation with nitrification inhibition even though the biomass has a higher sorption affinity for copper than other heavy metals (Zn, Cd, and Ni) (Hu et. al., 2002, 2003). On the other hand, some studies have shown that inhibition to the growth of AOB, such as *Nitrosomonas europaea* was correlated to Cu(II)-amine species, regardless of the total copper activity present in the medium (Lee et al., 1997; Sato et al., 1988). Copper has also been found to increase the ammonia oxidizing activity of cell-free extracts of AOB (Ensign et al., 1993). Due to the fact that AMO

enzyme contains a copper active site, metal binding compounds such as ATU can bind to the site

to inhibit nitrification (Hooper and Terry, 1973).

3. SEASONAL VARIATION AND EX-SITU NITRIFICATION ACTIVITY OF AMMONIA OXIDIZING ARCHAEA IN BIOFILM BASED WASTEWATER TREATMENT PROCESSES

3.1. Introduction

As the first step of nitrification, the oxidation of ammonia to nitrite (nitritation) is performed by AOP which are AOB and AOA. It was thought that only AOB are involved in nitritation, but AOA are also known to play a significant role (Konneke et al., 2005; Park et al., 2006). AOA have been found with AOB in natural and engineered systems (Beman et al., 2008; Caffrey et al., 2007; Francis et al., 2005; Gao et al., 2013; Hansel et al., 2008; Hatzenpichler et al., 2008; Kasuga et al., 2010; Könneke et al., 2005; Leininger et al., 2006; Park et al., 2006; Hatzenpichler, 2008; Santoro et al., 2008; Tourna et al., 2008; Urakawa et al., 2008; Wuchter et al., 2006; Yin et al., 2016), but their precise role in nitritation varies among different environments. The abundance and community structure of AOA and AOB can be affected by temperature (Avrahami and Conrad, 2003; de la Torre et al., 2008; Hatzenpichler et al., 2008; Herrmann et al., 2011; Reigstad et al., 2008; Tourna et al., 2008), ammonia availability (Hatzenpichler et al., 2008; Kowalchuk and Stephen, 2001), pH (Nicol et al., 2008; Reigstad et al., 2008), and salinity (Bernhard et al., 2010; Caffrey et al., 2007; Santoro et al., 2008).

The abundance of AOA and AOB is generally determined by quantifying the *amoA* gene in extracts of total DNA. Using this approach, several studies have reported finding AOA in industrial and municipal WWTPs (Lehtovirta-Morley et al., 2011; Mussmann et al., 2011; Park et al., 2006; Shu et al., 2015; Tourna et al., 2011; Wang et al., 2017). Some studies of activated sludge have found that AOA outnumber AOB (Leininger et al., 2006; Limpiyakorn et al., 2011),

while others have found that AOB predominate (Jin et al., 2010; Mosier and Francis, 2008; Wells et al., 2009; Zhang et al., 2011).

For biological wastewater treatment systems, AOA have been detected in suspended growth (activated sludge) processes in several studies (Limpiyakorn et al., 2013; Limpiyakorn et al., 2011; Park et al., 2006; Wells et al., 2009; Zhang et al., 2009). However, research on the presence of AOA in major attached growth (fixed-film) processes is extremely rare and limited to one process. In the only study to date of AOA in a fixed-film municipal wastewater treatment process, Sauder et al. (2012) showed that the relative abundance of AOA increases as ammonia concentration decreases along the flow path of a RBC system. However, the relative abundance of AOA in other widely-used fixed-film processes, such as TF and MBBR systems, is not known. A TF is a common wastewater treatment process, and although it is less effective than activated sludge, it can reliably provide organic carbon removal and nitrification. The MBBR process is well established and widely used for full-scale nitrification, and it offers a number of advantages over nitrifying activated sludge process, including high effluent quality, no bulking problems, and lower cost.

The aim of research work described in this chapter was to determine the relative abundance and nitrification activity of AOA and AOB in TF and MBBR systems. Biofilm samples were collected during a 17-month period from full-scale TF and MBBR systems at separate WWTPs. The abundance of AOA and AOB in total DNA extracted from biofilms was determined using a real-time qPCR approach targeting archaeal and bacterial *amoA* genes. Temperature data was obtained from both WWTPs and examined for relationships with AOA and AOB abundance. By inhibiting AOA and AOB separately in the biofilm samples, nitrification activity of AOA and AOB dominated cultures was determined by measuring

ammonia reduction, and nitrite and nitrate production. This research work is the first to investigate AOA in TF and MBBR and their nitrification activity and contributes to extremely limited information on AOA in full scale fixed film wastewater treatment process. The monitoring period of 17 months is the longest compared to similar previous studies and provides a complete and reliable picture of seasonal variation of AOA abundance. In addition, it comes up with a novel inhibition approach to determine the nitrification activity of AOA in mixed cultures.

3.2. Materials and Methods

3.2.1. Abundance of AOA and AOB in Full-Scale WWTPs

3.2.1.1. Biofilm Sample Collection

Biofilm samples were collected from the City of Fargo WWTP (Fargo, ND, USA) and the City of Moorhead WWTP (Moorhead, MN, USA), which are in the same metropolitan area and primarily treat municipal wastewater. The City of Fargo has a capacity to treat an average flow of 57,000 m^3 day^{-1}, with a peak pumping capacity of 111,000 m^3 day^{-1}. The plant employs a two-stage TF system consisting of a biochemical oxygen demand TF (BTF) and a nitrifying TF (NTF) in series. Biofilm samples were collected from PVC media stacked in the BTF and NTF. The samples were collected for 17 months, biweekly for the first 6 months and monthly thereafter. For each type of TF, 3-3.5 g of biofilm (wet weight) was collected from 30-60 cm below the top of the media stack in duplicate from three random locations (a total of 18-21 g). Biofilm samples (~5 mm thickness) were scraped from the media using a modified spatula and placed in a 50 mL collection tube containing 20 mL of phosphate buffered saline (PBS). Biofilm samples were kept on ice before transporting to the laboratory (less than 1 h) where they were prepared immediately for DNA extraction.

The City of Moorhead WWTP treats 17,000 m^3 of wastewater day^{-1}, on average. The plant operates a 3,000 m^3 MBBR for nitrification after a high purity oxygen activated sludge process. The MBBR is filled with 30% (v/v) small plastic media (2.1 cm diameter) that move throughout the reactor by aeration-induced mixing. A discharge screen retains the plastic media in the reactor. Approximately 20 pieces of MBBR media, corresponding to 10-12 g of biofilm (wet weight) were collected from two locations in the MBBR, immediately after the inlet and before the outlet of the basin. Biofilm (a total of 20-24 g) was removed from the MBBR media using a brush and placed in a 50 mL collection tube containing 20 mL PBS. The sampling period, frequency, and transportation were as described above for the City of Fargo WWTP.

Water quality data, including ammonia concentration, pH, dissolved oxygen, and chemical oxygen demand, were collected from the NTF influent at the Fargo WWTP and from the nitrification basin at the Moorhead WWTP (APPENDIX, A1).

3.2.1.2. DNA Extraction

Biofilm samples from TFs and MBBR were vortexed to disperse the biofilm, and centrifuged at 5,000×g and 4°C for 5 min. The supernatant was discarded and 1.5-2.0 g of pellet were placed in separate tubes and resuspended in 900 µL of TE buffer (100 mM Tris-Cl, pH 7.6, 10 mM EDTA) and 50 µL of lysozyme (10 mg mL^{-1}). The resuspended biofilm samples were extensively vortexed for 10 min followed by incubation for 2 h at 37°C and 10 min at 90°C. One hundred microliters of proteinase K (10 mg mL^{-1}) were added and the samples were incubated for another 1.5 h at 55°C. One hundred microliters of guanidinium thiocyanate (Sigma, Saint Louis, MO) and 100 µL diatomaceous earth (Sigma) were added, the mixture was centrifuged at 5,000×g for 45 s, and the supernatant was removed. DNA was further purified using a biofilm DNA isolation kit in accordance with the manufacturer's instructions (PowerBiofilm™ DNA

Isolation Kit, MO BIO, Carlsbad, CA), with slight modifications as follows. Bead beating

duration was increased from 15 to 45 s at 3,200 rpm. Two hundred microliters of BF3 solution

was added to the sample, vortexed, and centrifuged at 13,000×g. This step was performed twice

instead of once to achieve clear supernatant (after centrifuging). DNA extracted from individual

aliquots were merged into one at the end. DNA was quantified using a NanoDrop®

spectrophotometer (ND-1000, Thermo Fisher Scientific, USA) and stored at -20°C or

immediately used for PCR or qPCR application.

3.2.1.3. Quantitative PCR Assays

Archaeal and bacterial *amoA* genes were amplified from serial 10 fold dilutions of DNA

extracts using a real-time PCR assay. The primers Arch-amoAF (5'-STAATGGTCTGGCTTAG

ACG-3') and Arch-amoAR (5' -GCGGCCATCCATCTGTATGT-3') were used to amplify the

archaeal *amoA* gene (Francis et al., 2005). The bacterial *amoA* gene was amplified using the

primers *amoA* 1F (5'- GGGGTTTCTACTGGTGGT-3') and *amoA* 2R (5' -CCCCTCKGSAAA

GCCTTCTTC-3') (Rotthauwe et al., 1997). These primers were used in previous studies where

AOA and AOB were detected in a wide range of environments such as soil, wastewater, and

marine (Limpiyakorn et al., 2011; Santoro et al., 2008; Wells et al., 2009; Wuchter et al., 2006).

Identical conditions were used to amplify archaeal and bacterial *amoA* genes. Reactions included

12.5 µL of Biorad SYBR Green QPCR Master Mix (Biorad, USA), 1 µL (0.4 µM) of each

primer, and 1 µL of template DNA in a total volume of 25 µL. The PCR cycling conditions were

an initial denaturation of 95°C for 10 min followed by 40 cycles of 95°C for 60 s, 56°C for 60 s,

and 72°C for 30 s. Data capture for each cycle was at 78°C for 15 s. To confirm the single target

fragment of the PCR amplified products, dissociation curves were analyzed and plotted at the

end of every qPCR reaction. All reactions were performed in triplicate and run in an iCycler iQ qPCR detection system (BioRad).

3.2.1.4. Quantification of AOA and AOB

AOA and AOB abundance was determined from the copy number of the archaeal and bacterial *amoA* genes, respectively. Standard curves were created using 3×10^1 to 3×10^7 copies of pGEM-T Easy Vector (Promega, USA) containing PCR amplified fragments of the archaeal or bacterial *amoA* gene as inserts.

3.2.1.5. Sequence Analysis of the Archaeal amoA Gene

The PCR protocol was modified from Limpiyakorn et al. (2011). The archaeal *amoA* gene (~635 bp) was amplified using the same primers used for qPCR (Francis et al., 2005). PCR was carried out in 50 μL reactions containing 20-30 ng of extracted DNA, 1× GoTaq Flexi buffer (Promega), 2 mM $MgCl_2$ (Promega), 100 μM of each dNTP (Invitrogen, USA), 0.2 μM of each forward and reverse primer, and 1.25 units of GoTaq Flexi polymerase (5 U μL^{-1}, Promega). Amplification conditions included an initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 45 s, 53°C (AOA) or 55°C (AOB) for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min. Amplified products were visualized following electrophoresis on 1.5% agarose gels.

PCR amplicons were purified using a PCR quick-spin™ purification kit (Promega, Madison, WI), and cloned using a pGEM T-vector cloning kit (Promega, Madison, WI). Plasmids were purified from 5-10 clones per combined extracted DNA sample, using a NucleoSpin® Plasmid cleanup kit, and the inserts were sequenced in both directions using a BigDye Terminator v3.1 cycle sequencing kit with M13F and M13R primers in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA).

Sequences were assembled using SeqMan (DNAStar, Madison, WI), aligned with previously published sequences using the MAFFT version 7 online server with automatic selection of alignment strategy (http://mafft.cbrc.jp/alignment/server) (Katoh and Standley, 2013). Alignments were manually edited, including trimming at both ends, and phylogenetic analyses were performed using MEGA 6.0 (Tamura et al., 2013). The Neighbor-Joining (NJ) method incorporating distance estimates was used to infer phylogenetic trees based on the Kimura 2-parameter distance model and pairwise deletions. Bootstrap values were determined from 1000 pseudoreplicates (Kimura, 1980; Saitou and Nei, 1987). Phylogenetic trees were edited for style using Adobe Illustrator CS5. Sequences from this research are available in GenBank under the accession numbers KX139131–KX139135.

3.2.2. Laboratory Nitrification Activity Experiments

3.2.2.1. Sample Collection and Experimental Setup

About 55 NTF media pieces (square/rectangular shape, 1.0-1.5 cm length and width) and 55 pieces of MBBR media, were collected from the same two WWTPs described above for nitrification activity experiments. All chemicals used in the experiments were purchased from Sigma (Sigma-Aldrich, St. Louis, MO) unless otherwise mentioned. Two batch reactors of 500 mL were filled separately with 250 mL of NTF effluent water and MBBR basin water (collected from the same two WWTPs described above) and amended with 3 mg ammonia-N L^{-1} through granular ammonium sulfate (purity $\geq$ 99.0%) addition. Then each reactor was added with 25 NTF or MBBR media pieces accordingly. After 3 days, ammonia-N decreased 0.5-1.0 mg L^{-1} and then ammonium sulfate was added to increase ammonia-N by 0.5 mg L^{-1} so the concentration on day 4 went to 3.0 mg/L or slightly lower. The AOB inhibitor ATU or the AOA inhibitor PTIO was added on day 4 to the reactors at a final concentration of 20, 50, or 100 µM

to select for AOA- and AOB-dominated populations in the media samples. Stock solutions of 1.4 mM of PTIO and 1.0 mM ATU were prepared and stored at 4°C. The solutions were added directly to the reactors to achieve the targeted concentrations. Several studies have shown that PTIO (Jung et al., 2014; Yan et al., 2012) and ATU (Lehtovirta-Morley et al., 2013) specifically inhibit AOA and AOB, respectively. The reactors were incubated for 7 more days (a total of 10 days) in the dark on an orbital shaker (60 rpm) at room temperature (20-22°C). A reactor without ATU or PTIO were included as a control. pH was monitored (Thermo Orion 250A meter + electrode) and was in a range of 7.2 to 7.5 throughout the experiments. The nitrification activity experiments were conducted in duplicate.

3.2.2.2. Reactor Sample Preparation and Analytical Methods

Approximately 2.0 g of biofilm, was scraped from each type of media (MBBR and TF) daily from day 4 to day 10. Biofilm samples from the duplicated experiments were combined into one and immediately prepared for DNA extraction, or they were stored at -80°C before DNA extraction at a later date. DNA extraction and qPCR to determine the archaeal and bacterial *amoA* gene copy number were performed as described earlier.

Ten mL water samples were collected daily from each reactor, filtered through a 0.22 µm pore-size polycarbonate filter, and analyzed for ammonia-N, nitrite-N, and nitrate-N. The salicylate method (Hach method # 10023) was used to measure ammonia-N (low range; 0.02 - 2.50 mg/Las NH_3-N), with 'N Tube Amver test kits. The diazotization method (Hach method # 10019) was used to measure nitrite-N (low range; 0.003 - 0.500 mg/Las NO_2-N), with 'N Tube NitriVer3 test kits. A second derivative UV spectrophotometric (SDUS) method (APHA et al., 2005) was used to measure nitrate-N (0.0 and 3.0 mg/Las NO_3-N). A Hach DR 5000 spectrophotometer was used for all N analyses.

3.2.3. Statistical Analysis

The sampling period was divided into cold months (October to February) and warm months (March to September). A paired t-test was performed to determine whether differences in the log base ten values of *amoA* gene copy numbers of archaeal and bacterial ammonia oxidizers under warm and cold conditions in MBBR and TFs were significant based on a p-value criterion < 0.05. A calculation of Pearson's correlation coefficients was conducted to assess correlations between the abundance of *amoA* genes of archaeal and bacterial ammonia oxidizers and ambient wastewater temperature. The calculation was carried out based on a t-test and p-value. The Satterthwaite method was used to verify that there was no uneven distribution. SAS statistical software version 9.3 was used for all statistical calculations and analyses. Nitrification activity and log-transformed *amoA* gene copy numbers were compared by one-way analysis of variance (ANOVA) followed by Student–Newman–Keulstest to check for quantitative variance between ATU and PTIO treatments. One-way ANOVA was also performed to determine whether the differences in nitrification activity by the AOA and AOB dominated cultures (obtained from PTIO and ATU inhibitions) were significant ($\alpha < 0.05$).

3.3. Results

3.3.1. AOA and AOB Abundance

AOB (9.2 ± 0.7 to 128.0 ± 4.0 bacterial *amoA* copies μL^{-1}) were more abundant than AOA (2.2 ± 0.3 to 7.8 ± 0.9 archaeal *amoA* copies μL^{-1}) in the BTF (Figure 2). AOA were frequently undetectable during cold months. Although AOB were detected consistently during cold and warm months, they were significantly more abundant during warm months ($p = 0.001$) (Figure 3). AOA ($4.5 \pm 0.1 \times 10^5$ to $1.9 \pm 0.3 \times 10^6$ archaeal *amoA* copies μL^{-1}) were more abundant than AOB ($4.5 \times 10^3 \pm 0.1 \times 10^3$ to $1.1 \times 10^4 \pm 0.1 \times 10^4$ bacterial *amoA* copies μL^{-1}) in the

NTF (Figure 2). Similar to observations in the BTF, AOA and AOB were most abundant in the NTF during warm months (Figure 3).

AOA ($2.2 \times 10^6 \pm 0.1 \times 10^6$ to $1.0 \times 10^7 \pm 0.1 \times 10^7$ archaeal *amoA* gene copies μL^{-1}) were more abundant than AOB ($3.4 \times 10^4 \pm 0.3 \times 10^4$ to $1.0 \times 10^5 \pm 0.3 \times 10^5$ bacterial *amoA* gene copies μL^{-1}) in the MBBR (Figure 2). AOB were significantly more abundant in warm months than cold months ($p = 0.03$) (Figure 3). AOA were similarly more abundant in warm months than cold months (Figure 3), but the difference was not significant ($p = 0.534$). AOA and AOB were most abundant in June and least abundant in September (Figure 2). The numbers of archaeal and bacterial *amoA* gene copies were highest in June and lowest in September (Figure 3)

3.3.2. Correlation Between Ambient Wastewater Temperature and *amoA* Gene Copy Numbers

Table 2 shows Pearson correlation coefficient (r) between temperature and the *amoA* gene copy number in archaea (AOA abundance), bacteria (AOB abundance), and prokaryotes (AOP abundance; AOA and AOB combined). In the NTF and MBBR, AOA abundance was significantly positively correlated with ambient wastewater temperature during warm months ($p = 0.001$), but not during cold months. AOA, AOB, and AOP were significantly correlated with temperature in the TFs, with AOA and AOB significantly more abundant in warm months than cold months ($p = 0.021$). Also, AOA was significantly more abundant in the NTF in warm months than cold months (Figure 3). In contrast, the increase AOB abundance during warm months was not significant ($p = 0.0864$). AOA, AOB, and AOP were not significantly correlated with temperature in the MBBR.
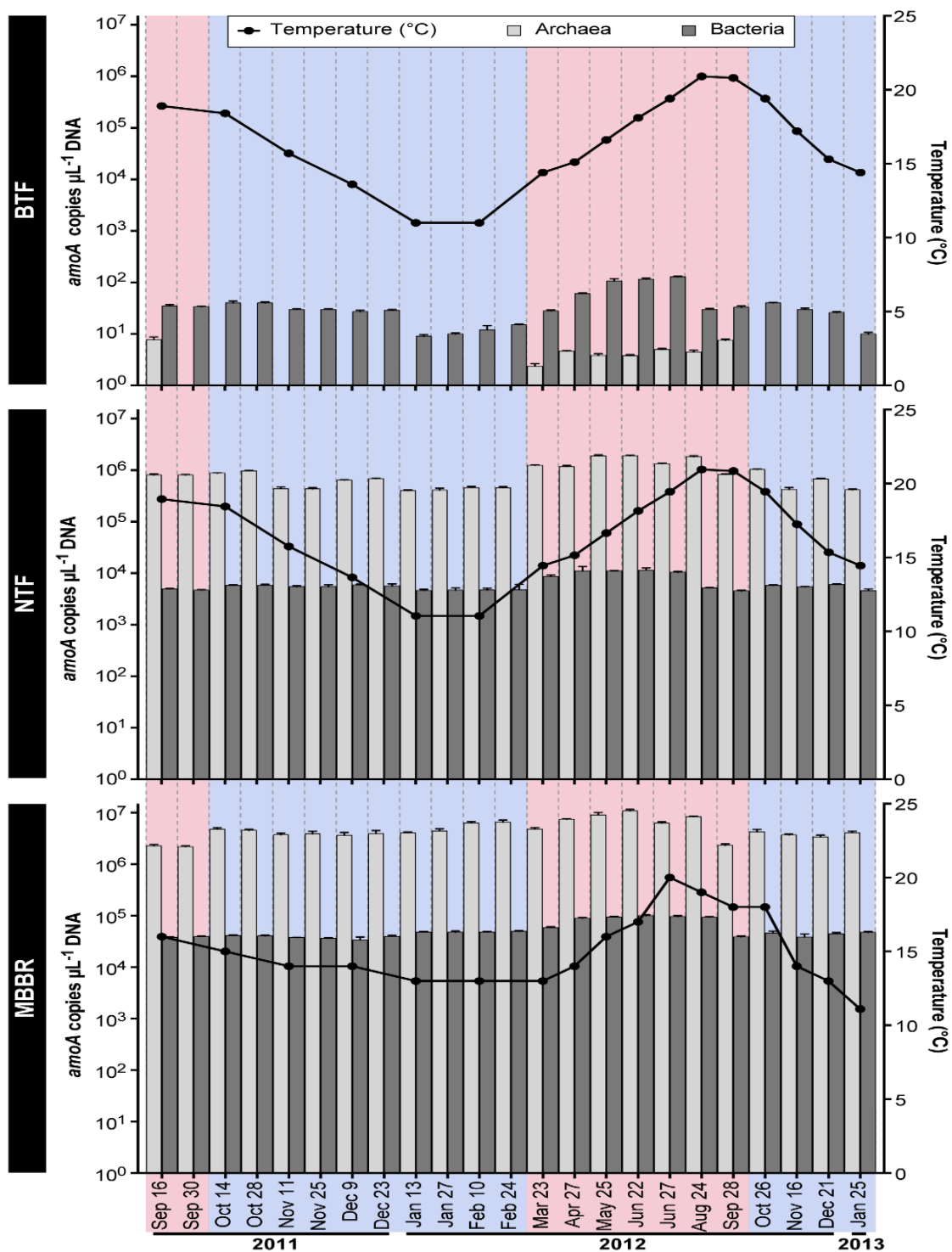
Figure 2. Archaeal and bacterial *amoA* copies in DNA of biofilm samples from a BTF, a NTF, and a moving bed biofilm reactor (MBBR). Data highlighted with a red background and blue background are from biofilm samples collected during warm months (March to September) and cold months (October to February), respectively. The data are based on the triplicate of qPCR amplicon.
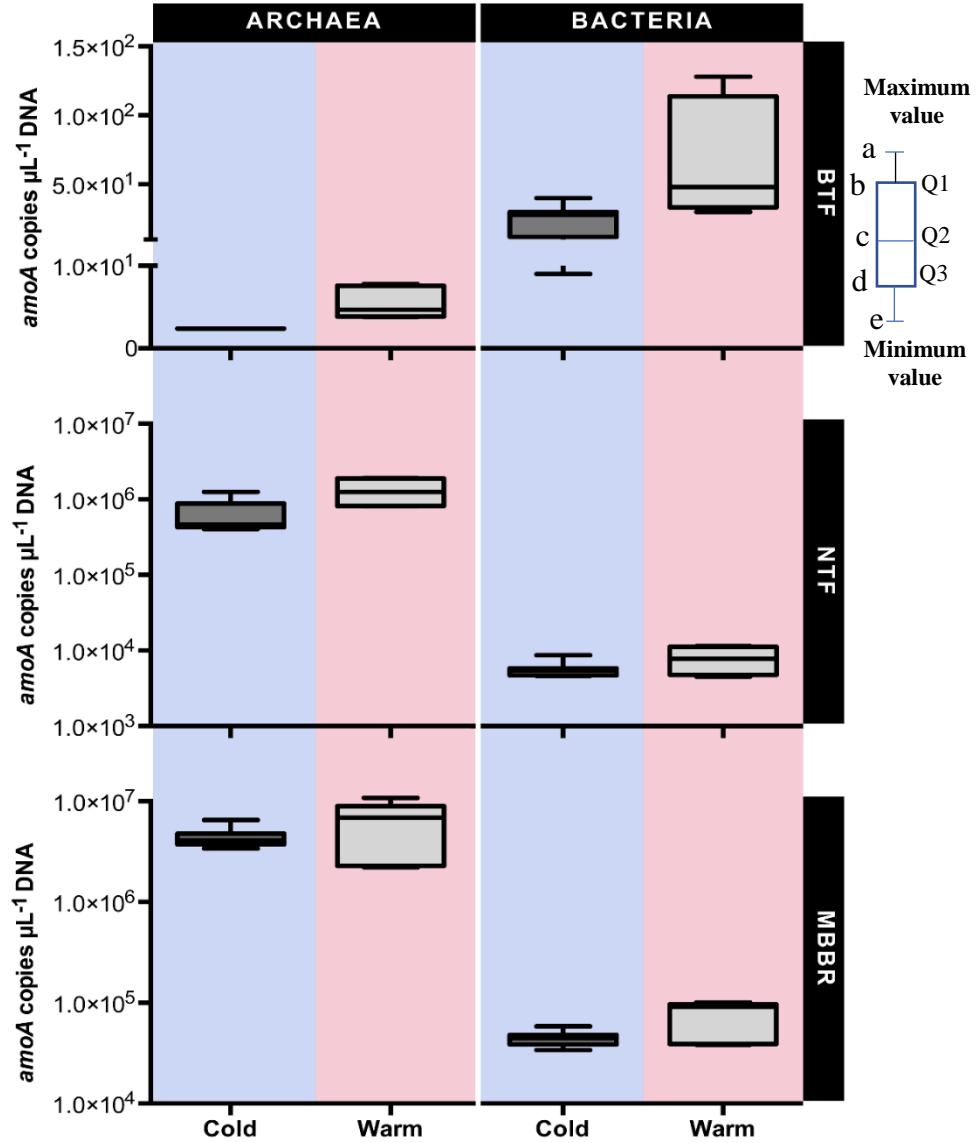
Figure 3. Box and whisker plot of archaeal and bacterial *amoA* copies in DNA of biofilm samples collected during warm months (March to September; red background) and cold months (October to February; blue background) from a BTF, a NTF, and a moving bed biofilm reactor (MBBR). The archaeal *amoA* gene was not detected in the BTF during cold months. The box and whisker plot is based on the following formula: a = Q1, b = Median-Q1; c = Q3-Median; d = Q1-Minimum value; e = Maximum value-Q3; where Q1 and Q3 represents first and third quartile.

Table 2. *Pearson*'s correlation coefficients describing the relationships temperature and the abundance of AOA, AOB, and prokaryotes (AOP; AOA+AOB).

	MBBR	NTF	BTF
AOA versus temperature (°C)	0.63*	0.50*	0.63*
AOB versus temperature (°C)	0.27	0.11	0.41
AOP versus temperature (°C)	0.63*	0.50*	0.44

Values with an asterisk are significant based on a *p*-value < 0.05.

3.3.3. Phylogenetic Analysis

A phylogenetic tree was constructed to show the evolutionary relationships of *amoA* gene sequences from archaea in this and other studies (Figure 4). Sequences from the present research clustered in the marine clade as *Nitrisoarchaeam limnia* and were most similar to AOA-S1-41 (Accession no. KM116989), AOA-834-205-27 (Accession no. KC293237), MM-10 (Accession no. DQ278561), and SF_NB1_1 (Accession no. DQ148633), which were previously isolated from soil, marine environments, and WWTPs (Francis et al. 2005; Hong et al. 2014; Park et al. 2006; Zhang et al. 2015).

3.3.4. Nitrification Activities of AOA- and AOB- Dominated Populations from the NTF and MBBR

The inhibitor PTIO reduced AOA abundance in a dose-dependent manner, with respective ratios of archaeal *amoA*: bacterial *amoA* copies in 0, 20, 50, and 100 µM ATU of 2.0, 0.7, 0.4, and 0.1 in the NTF (Figure 5a) and 2.0, 0.6, 0.3, and 0.2 in the MBBR (Figure 5b). Conversely, the inhibitor ATU reduced AOB abundance in a dose-dependent manner, with respective ratios of archaeal *amoA*: bacterial *amoA* copies in 0, 20, 50, and 100 µM ATU of 1.9, 2.5, 6.2 and 6.2 in the NTF (APPENDIX Figure A1. a) and 1.9, 2.5, 4.8, and 6.4 in the MBBR (APPENDIX Figure A1. b).
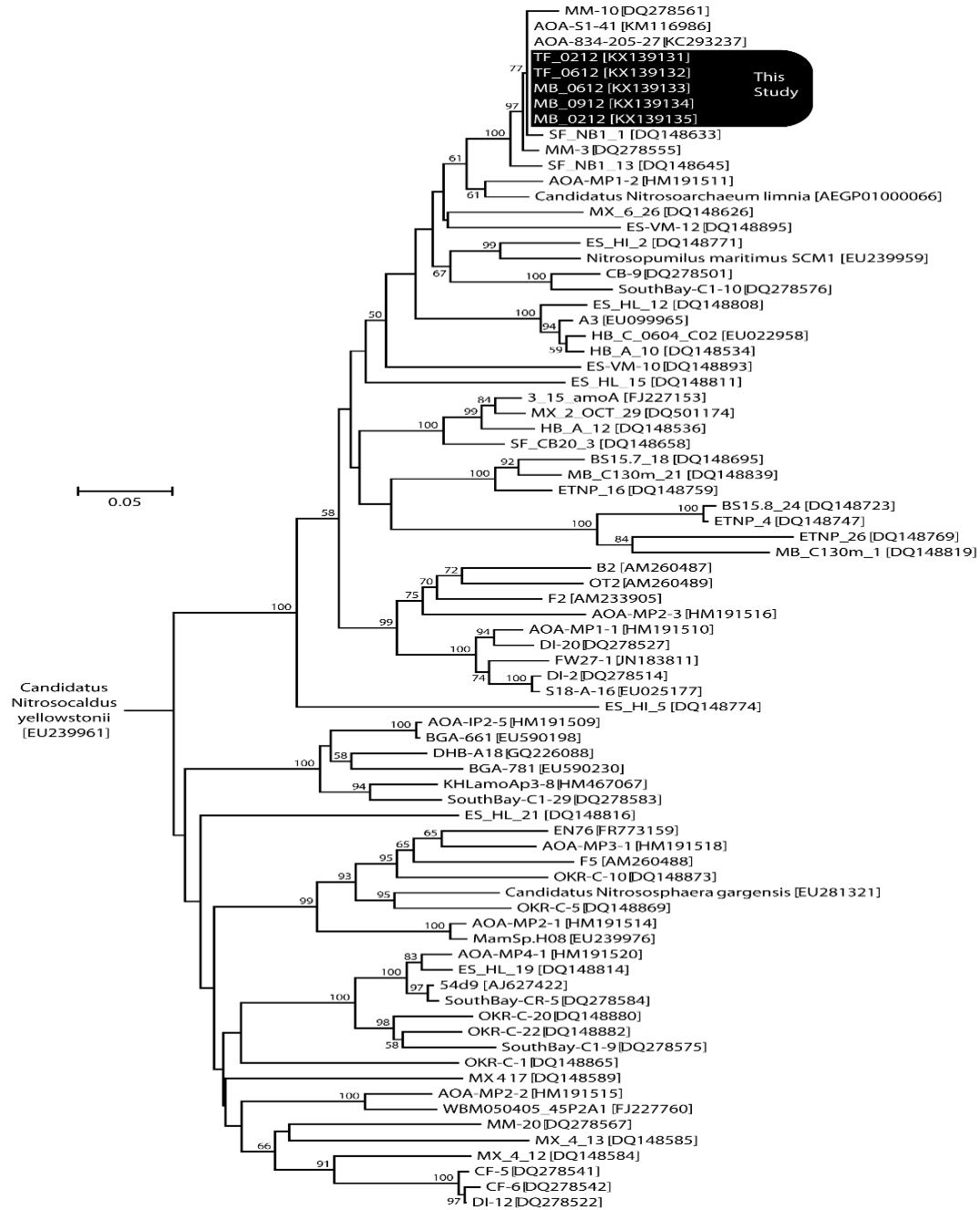
Figure 4. Neighbor-joining tree of representative partial archaeal *amoA* gene sequences from this research and GenBank. Sequences from this research are highlighted in white with a black background. Sequence names with TF and MB prefixes are from nitrification trickling filter and moving bed biofilm reactor, respectively.
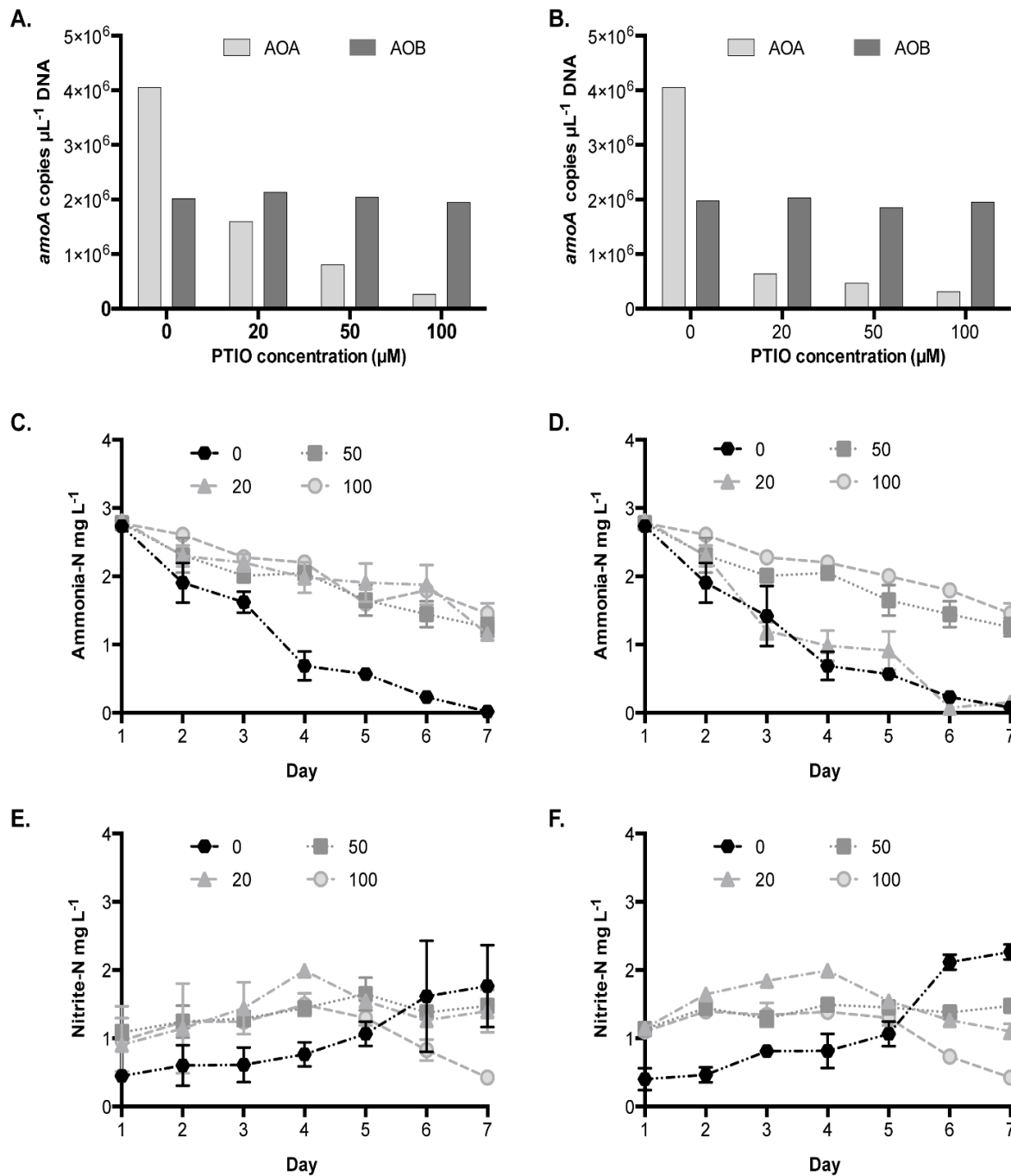
Figure 5. AOA and AOB *amoA* copy number/µL at different concentrations of the AOA inhibitor PTIO in batch experiments with samples of a) the NTF and b) the MBBR; ammonia oxidation in batch experiments with samples of c) the NTF and d) the MBBR; nitrite-N production in batch experiments with samples of e) the NTF and f) the MBBR; Day 1 is the first day of sample collection after nitrite generation in the batch experiment. Data shown are means ± SD (n = 3).

Nitrification activity – ammonia reduction and nitrite production – decreased with increasing inhibition of either AOA or AOB populations (Figure 5c-f and APPENDIX Figure A1. c-f), and nitrification activities were not significantly different between AOA- and AOB-dominated populations ($p \geq 0.05$; $p = 0.12$). These data indicate that both AOA and AOB populations in the NTF and MBBR contributed to nitrification. Nitrate production was limited in all treatments, within a range of 0.0-0.5 mg/L (data not shown).

3.4. Discussion

This research found low levels of AOA and AOB in the BTF compared to the NTF and MBBR processes, which is not surprising given that BTF including that at the City of Fargo WWTP is designed primarily for organic carbon removal, not nitrification. The greater levels of organic carbon in the BTF likely supported the growth of heterotrophs, which compete for oxygen with nitrifiers, resulting in lower levels of AOA and AOB relative to the NTF, where organic carbon is limited. This is consistent with the observation by Wells et al. (2009) that AOA abundance was lower at a location in the treatment system with higher levels of organic matter.

AOA were more abundant than AOB in the NTF and MBBR, where ammonia concentrations were lower than in the BTF. This agrees with previous work showing that the relative abundance of AOA is greater at lower ammonia concentrations. Sauder et al. (2012) found AOA to be more abundant than AOB in a RBC, which is a fixed-film wastewater treatment system, similar to the NTF and MBBR in the present research. They reported that AOA abundance was negatively correlated with ammonia concentration. AOB was detected inconsistently in the studied RBC system. In contrast, AOB were consistently found in the NTF in the present research. Limpiyakorn et al. (2011) similarly found AOA to be substantially more abundant than AOB in municipal WWTPs with lower levels of ammonia in their influent (5.6–

11.0 mg N L^{-1}) and effluent (0.2–3.0 mg N L^{-1}). These ammonia concentrations are comparable

to the levels in the NTF and MBBR in the present research. The lower abundance of AOA

relative to that of AOB at higher ammonia concentrations as found in the present research and

previous work (Limpiyakorn et al., 2011; Sauder et al., 2012; Wells et al., 2009), suggests that

AOA have a minor role when ammonia levels are high.

AOA abundance was positively correlated with warmer temperatures (22-30°C), and

AOA and AOB were more abundant during warm months. This is consistent with the report by

Sauder et al. (2012) that AOA were more abundant in a RBC WWTP during warmer (July –

September) than colder (November – January) periods. Outside of WWTPs, Zeng et al. (2014)

found that AOA were more abundant in freshwater sediment at higher (35°C) than lower (15°C)

temperatures. Other studies have found that AOA are more abundant in soil at higher

temperatures (Offre et al., 2009; Tourna et al., 2008; Tourna et al., 2011; Wu et al., 2013). Wu et

al. (2013) found that the greater abundance of AOA in soil at 37°C than 4-25°C was related to

autotrophic growth of ammonia oxidizing archaea at that temperature.

Archaeal *amoA* gene sequences from WWTPs in the present research were similar to

sequences found in various environments, including a full-scale activated sludge process

(Clusters B and D in phylogeny reported by Park et al. (2006)), seawater column and sediments

(Marine lineage group 1.1a in the phylogeny reported by Francis et al. (2005)), water in

Chesapeake Bay (Hong et al. 2014), and soil (Group 1.1b in the phylogeny reported by Zhang et

al. (2015)). *amoA* gene sequences from the NTF and MBBR in this research are closely related to

the marine clade *Nitrisoarchaeam limnia*, which is found in low salinity environments (Mosier et

al., 2012). In addition, the sequences observed in this research were similar to those reported in a

previous study on a fixed film RBC system (Sauder et al., 2012) which are closely related to the

environmental sequences derived from ammonia rich environments such as agricultural soil, WWTP activated sludge, and landfills. This suggests a possibility of AOA found in the present research to be adaptive to environments with varying nutrient levels.

In laboratory batch experiments with biofilm samples from the TF and MBBR, nitrification activity decreased with increasing inhibition of either AOA or AOB, confirming that both prokaryotic populations play a role in autotrophic ammonia oxidation. Although the contribution of AOB to autotrophic ammonia oxidation is well supported, the role of AOA has been less clear (Mussman et al., 2012). The findings in this research are consistent with the finding of Mosier et al. (2012) that the closely related *Nitrisoarchaeam limnia* participates in autotrophic ammonia oxidation. This research provides a novel and simple method to probe the involvement in nitrification by AOA in mixed nitrifying cultures.

3.5. Summary

The abundance and seasonal variation of AOA and AOB in NTF and MBBR biofilm were studied. AOA were dominant in both NTF and MBBR. AOA were detected at two magnitudes higher than AOB in warmer months. A strong correlation between ambient temperature and AOA and AOP (AOA and AOB together) was observed. The oxidation of ammonia to nitrite decreased when AOA populations from the NTF and MBBR were inhibited, demonstrating that AOA contributed to nitrification. This research has shown that AOA outnumber AOB and contribute to ammonia oxidation in nitrifying biofilm processes under ex-situ conditions for two full-scale wastewater treatment plants. Thus, AOA cannot be ignored for controlling biofilm based nitrification process in WWTPs.

4. COPPER INHIBITION OF WASTEWATER NITRIFICATION BY AMMONIA-OXIDIZING ARCHAEA IN ATTACHED AND SUSPENDED GROWTH CULTURES

4.1. Introduction

Nitrification in WWTPs can be inhibited by heavy metals from industrial waste. Copper affects nitrification by inhibiting the nitritation, the first step of nitrification in WWTPs (Hu et al., 2004; Juliastuti et al., 2003). Copper also causes oxidative stress to nitrifying bacteria (Chandran and Love, 2008; Nies, 1999; Wuchter et al., 2006), leading to cell damage or death and a reduction in nitrification efficiency (Chandran and Love, 2008; Hu et al., 2004; Juliastuti et al., 2003).

Studies using pure cultures of AOB (Chandran and Love, 2008; Park and Ely, 2008) and mixed nitrifying cultures (Cecen et al., 2010a; Cecen et al., 2010b; Juliastuti et al., 2003; Mertoglu et al., 2008; Semerci and Cecen, 2007) have shown that copper inhibits nitrifying prokaryotes. A copper concentration of 0.5 mg/L reduced nitrification by the AOB genus *Nitrosomonas* (Sato et al., 1988). Ammonia oxidation by *Nitrosomonas europea* decreased by 90% following exposure to 8 μM copper chloride (Park and Ely, 2008). In a separate study, 0.09 mg/L copper caused a 50% inhibition of a mixed culture of nitrifiers (Juliastuti et al., 2003).

Studies have shown that AOA also participate in the nitrification process (Martens-Habbena et al., 2009a; Martens-Habbena et al., 2009b; Sauder et al., 2012), and may be better adapted to nitrification under conditions with low ammonia levels (Martens-Habbena et al., 2009b). An extremely low half saturation constant (K_m = 133 nM of total ammonium) shows that *Nitrosopimulus maritima,* and perhaps other AOA, have a high affinity for NH_4^+-N (Martens-Habbena et al., 2009b). The archaeal *amoA* gene, which encodes ammonia monooxygenase subunit A, an enzyme necessary for nitrification, has been detected in natural systems and

WWTPs (Caffrey et al., 2007; Francis et al., 2005; Hatzenpichler et al., 2008; Könneke et al., 2005; Leininger et al., 2006; Park et al., 2006; Wuchter et al., 2006). Moreover, by analyzing AOA specific core membrane lipids, it has been shown that AOA participate in ammonia oxidation in a RBC (Sauder et al., 2012).

The number of *amoA* gene copies has been used as a surrogate for AOP abundance, and domain-specific *amoA* primers allow the relative proportions of AOA and AOB to be determined. Archaeal and bacterial *amoA* gene copies in soil decreased with increasing copper concentration, indicating that copper is toxic for both AOA and AOB (Li et al., 2014), but copper appears less toxic for AOA than AOB in long term exposure experiments (Mertens et al., 2010).

The aim of research work described in this chapter was to determine the inhibitory effect of copper on nitrifying cultures with different proportions of AOA. Cultures were prepared from samples obtained from full-scale WWTPs, and the proportions of AOA were adjusted using specific inhibitors of AOA and AOB. sOUR, sAOR, and archaeal *amoA* gene copy number were measured during respirometric experiments under both suspended and attached growth conditions.

4.2. Materials and Methods

4.2.1. Preparation of Nitrifying Cultures with Different Proportions of AOA

The NTF media in the Fargo WWTP, Fargo, ND, USA were cut onsite into multiple pieces (~4 cm in width and length) and transported to the Environmental Engineering Laboratory, North Dakota State University in NTF effluent. After that, the pieces were further cut to produce 36-40 pieces of square/rectangular shape, 1.0-1.5 cm length and width. Approximately 80 MBBR media were collected from the Moorhead WWTP, MN, USA. These NTF and MBBR media pieces provided attached growth nitrifying cultures. Suspended growth

nitrifying cultures were prepared from biofilm samples because the WWTPs did not have

suspended growth nitrification systems. Approximately 50 g of biofilms were scraped from NTF

media onsite and from MBBR media that were brought back to the laboratory. The biofilms were

scraped by using a sterile scalpel and homogenized by vortexing (to disperse biofilm) in filtered

(0.22 µm pore-size polycarbonate membrane filter) NTF effluent and MBBR basin effluent.

PTIO (purity ≥ 99% TLC) and ATU (purity ≥98.0% HPLC), specific inhibitors of AOA

and AOB, respectively, were purchased from Sigma and solutions of 1.4 mM of PTIO and 1.0

mM ATU were prepared and stored at 4°C. The solutions were added directly to the prepared

cultures to achieve various inhibitor concentrations (20, 50, and 100 µM) to manipulate the AOA

proportion in attached and suspended growth nitrifying cultures. The proportion of AOA in

nitrifying cultures was determined using qPCR to quantify archaeal and bacterial *amoA* gene

copies. Cultures were washed with a phosphate buffer solution before copper inhibition

experiments.

4.2.1.1. Assessing the Effect of Copper on Nitrifying Cultures with Different Proportions of AOA by Measuring sOUR

Oxygen uptake rate (OUR) was measured using a closed respirometric unit

(BODTrack^{TM}, Hach, Loveland, CO), which comprised a 300 mL amber-glass vessel with sealed

cap. Gas produced by microbial metabolism in the vessel was absorbed by crystals of lithium

hydroxide in the sealed cap, causing partial pressure to decline in the vessel. The pressure change

was measured by a mercury manometer, which was attached to the sealed cap by vinyl tubing

and connected to a computer for data storage. The change in partial pressure was converted to a

change in dissolved oxygen (DO) concentration in the sample.

NTF or MBBR basin effluent (approximately 2 L) was aerated (1-2 mins), filtered through a 0.22 µm pore-size sterilized polycarbonate membrane filter, and later supplemented with 5 mg/L NH_4^+-N, by adding granular ammonium sulfate (Sigma-Aldrich, purity $\geq$ 99.0%). One hundred fifty mL of this prepared effluent was poured into each respirometric vessel (reactor) and augmented with copper (0, 2, 5, 10, 15 mg/L) by adding granular copper chloride (Sigma-Aldrich, purity $\geq$ 99.0%). The measured concentrations of copper in the reactors for 2, 5, 10, 15 mg/L were 2.1, 4.9, 10.1 and 14.9 mg/L. A 0.05 N sodium hydroxide solution was used to adjust the pH of the solution (7.2-7.4) in the reactor.

Suspended and attached cultures were added to the vessel. Five NTF media pieces (square/rectangular shape, 1.0-1.5 cm length and width) or 5 pieces of MBBR media cut in half were added to the reactors. A magnetic stirrer was used to stir the suspended growth culture; an orbital shaker was used for the attached growth culture to avoid detachment of cells from the plastic media. The test was conducted at room temperature (20-22°C) for 10 hours. OUR, calculated from the slope of DO depletion over time, was divided by VSS to obtain the total sOUR [mg O_2/(g VSS)/h]. All experiments were carried out in duplicate. Percent inhibition of nitrification activity was expressed as the difference between the measured sOUR with and without copper exposure ($sOUR_{sample}$ and $sOUR_{control}$) divided by $sOUR_{control}$ as shown in Equation 1. All sOUR experiments were performed in duplicate.

$$\text{Nitrification inhibition (\%)} = \frac{sOUR_{control} - sOUR_{sample}}{sOUR_{control}} \times 100\% \qquad (1)$$

4.2.1.2. Assessing the Effect of Copper on Nitrifying Cultures with Different Proportions of AOA by sAOR

Ammonia oxidation rates (AOR) of suspended and attached cultures were obtained using an approach similar to that used for OUR. The pH and the concentrations of ammonia-N, nitrite-

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N, nitrate-N, and copper were measured hourly for the first 10 hours and daily for 7 days. AOR was calculated using the slope of combined nitrite and nitrate concentration versus time and divided by VSS to determine the total sAOR [mg N/(g VSS)/ h]. The nitrification inhibition was determined using Equation 2.

$$\text{Nitrification inhibition (\%)} = \frac{\text{sAORcontrol} - \text{sAORsample}}{\text{sAORcontrol}} \times 100\% \qquad (2)$$

sAORcontrol is sAOR without copper addition and sAORsample is sAOR with copper addition. All sAOR experiments were performed in duplicate.

4.2.1.3. Assessing the Effect of Copper on AOA Abundance

Abundance of AOA was measured during the sAOR experiment. Approximately 0.1 g of suspended and attached growth samples (scrapped from attached growth) was collected on days 0, 3, 5 and 7 from the sAOR experiment and was subject to DNA extraction and qPCR as described below. Copper effect on AOA abundance was determined based on reduction of *amoA* copy number by using the following equation.

$$amoA \text{ reduction (\%)} = \frac{amoA \text{ copy on day } 0 - amoA \text{ copy on day } 7}{amoA \text{ copy on day } 0} \times 100\% \qquad (3)$$

4.2.1.4. Analytical Methods

Water samples collected from the sOUR and sAOR experiments were filtered through a 0.22 µm pore size polycarbonate membrane. They were preserved by adding 0.1 mL of 4 M sulfiric acid and stored at 4°C before chemical analyses. The salicylate method with the 'N Tube Amver test kit was used to measure ammonia-N (low range; 0.02 - 2.50 mg/L as NH_3-N), (Hach method # 10023). The diazotization method with the Test 'N Tube NitriVer3 test kits was used to measure nitrite-N (low range; 0.003 - 0.500 mg/L as NO_2-N), (Hach method # 10019). A second derivative UV/visible spectroscopy method was used to measure nitrate-N (0.0 and 3.0

mg/L as NO_3-N) (APHA, 2012). A DR 5000 spectrophotometer was used to measure nitrogen species. DO (Thermo Orion 850A meter, Thermo Orion DO 083005D probe), pH (Thermo Orion 250A + pH Electrode), and VSS were determined according to Standard Methods [23]. Soluble copper concentration was measured using a Perkin Elmer SCIEX inductively coupled plasma mass spectrometer (ICP-MS, Elan DRC-e). Quality control for ICP-MS included certified reference materials (EnviroMat ES-H-2 and NIST 1640a), internal standard, continuous control verification, and spiking samples for matrix interference detection.

4.2.2. DNA Extraction

DNA was extracted from cultures on days 0, 3, 5 and 7 of the sAOR experiment. The extraction method was modified from a previous study (Roy et al., 2014). In brief, culture cells were centrifuged at 5,000g and 4°C for 5 min. Attached growth cultures were vortexed before centrifugation. The supernatant was discarded, and 1.5-2.0 g of pellet was placed in separate tubes and resuspended in 900 µL of TE buffer (100 mM Tris-Cl, pH 7.6, 10 mM EDTA) and 50 µL of lysozyme (10 mg/mL). Tubes were extensively vortexed for 10 min followed by incubation for 2 h at 37°C and 10 min at 90°C. One hundred microliters of proteinase K (10 mg/mL) was added, and samples were incubated for 1.5 h at 55°C. One hundred microliters of guanidinium thiocyanate (Sigma, Saint Louis, MO) and 100 µL diatomaceous earth (Sigma) were added, the mixture was centrifuged at 5,000g for 45 s, and the supernatant was removed. DNA was further purified using a biofilm DNA isolation kit in accordance with the manufacturer's instructions (PowerBiofilm™ DNA Isolation Kit, MO BIO, Carlsbad, CA), with the exception that the initial bead beating duration was increased 15 to 45 s at 3,200 rpm. Two hundred microliters of BF3 solution was added twice to achieve clear supernatant. DNA extracted from separate aliquots of a sample was combined into a single DNA sample after

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purification, quantified using a NanoDrop® spectrophotometer (ND-1000, Thermo Fisher

Scientific, USA), and stored at -20°C or used immediately in a real-time qPCR assay.

4.2.3. qPCR Assays

Archaeal and bacterial *amoA* genes were amplified from serial 10-fold dilutions of DNA

extracts using a qPCR approach. The primers Arch-amoAF (5'-STAATGGTCTGGCTTAG

ACG-3') and Arch-amoAR (5' -GCGGCCATCCATCTGTATGT-3') were used to amplify the

archaeal *amoA* gene (Francis et al., 2005). The bacterial *amoA* gene was amplified using the

primers amoA 1F (5'- GGGGTTTCTACTGGTGGT-3') and amoA 2R (5' -CCCCTCKGSAAA

GCCTTCTTC-3') (Rotthauwe et al., 1997). Identical conditions were used to amplify archaeal

and bacterial *amoA* genes. Reactions included 12.5 µL of Biorad SYBR Green QPCR Master

Mix (Biorad, USA), 1 µL (0.4 µM) of each primer, and 1 µL of template DNA in a total volume

of 25 µL. The PCR cycling conditions were an initial denaturation at 95°C for 10 min followed

by 40 cycles of 95°C for 60 s, 56°C for 60 s, and 72°C for 30 s. Data capture for each cycle was

at 78°C for 15 s. To confirm the single target fragment of the PCR amplified products,

dissociation curves were analyzed and plotted at the end of every qPCR reaction. All reactions

were performed in triplicate by using an iCycler iQ qPCR detection system (BioRad).

Archaeal and bacterial *amoA* gene copy numbers, were used as surrogates for AOA and

AOB abundance, respectively. Standard curves were created using 3×10^1 to 3×10^7 copies of

pGEM-T Easy Vector (Promega, USA) containing PCR-amplified fragments of the AOA or

AOB specific *amoA* gene as inserts.

4.2.4. Statistical Analyses

sOUR and sAOR results were expressed as average percent inhibition. Regression analysis was performed between sOUR/sAOR and AOA proportions at the highest copper concentration. All statistical analyses were performed using Microsoft Excel (2013).

4.3. Results and Discussion

4.3.1. Use of PTIO and ATU to Produce Cultures with Different Proportions of AOA

ATU and PTIO inhibit AOB and AOA, respectively (Taylor et al., 2013; Yan et al., 2015), in a concentration dependent manner (Roy et al., 2014), and were used here to achieve cultures with different proportions of AOA. At a concentration of 100 µM, ATU can begin to inhibit AOA (Lehtovirta-Morley et al., 2013), so this was the highest concentration that was used in this research. Prior to treatment with ATU or PTIO, the proportion of AOA in suspended and attached growth cultures was 66-75% (Table 3). Treatment with PTIO at 20, 50, and 100 µM resulted in cultures with AOA proportions of 37-41%, 28-33%, and 7-10%, respectively. At the same concentrations, application of ATU resulted in AOA proportions of 71-74%, 83-86%, and 86-87%. For subsequent copper inhibition experiments, ATU concentrations of 20 µM and 100 µM were used to achieve higher AOA proportions and PTIO concentrations of 20 µM, 50 µM, and 100 µM were used to achieve lower AOA proportions.

Table 3. Archaeal *amoA* gene copies (AOA) as a proportion of total *amoA* gene copies (archaeal + bacterial) in attached and suspended cell cultures prepared from NTF and MBBR samples after 0, 20, 50 and 100 µM of ATU and PTIO treatments.

		AOA (%)				AOA (%)			
		ATU (µM)				PTIO (µM)			
		0	20	50	100	0	20	50	100
NTF	Attached	66	71	86	87	75	41	29	9
	Suspended	66	74	86	87	74	37	28	9
MBBR	Attached	66	71	83	86	67	41	29	7
	Suspended	67	72	84	87	67	37	33	10

4.3.2. Effect of Copper on sOUR and sAOR

Copper caused concentration-dependent reductions in sOUR and sAOR in attached and suspended cultures with different proportions of AOA (Figures 6-9), which is similar to the findings of previous studies on AOB (Hu et al., 2002, 2004; Kapoor et al., 2015). Reductions were greater in suspended than attached cultures, particularly at copper concentrations of 2 and 5 mg/L. Similarly, in a study on the AOB species *Nitrosomonas europaea*, copper had a greater effect on suspended than attached growth (biofilm) systems (Stehr et al., 1995). It has been shown previously that inhibition of nitrifiers by copper and other heavy metals can be reduced by the presence of extracellular polymeric substances in biofilm cultures (Vu et al., 2009).

The effect of copper of sOUR and sAOR increased with decreasing proportion of AOA in the cultures (Figures 6-9). For example, at 15 mg/L of copper, sOUR reductions of 60, 80, ~80, 90, 100 and 100% were observed for attached growth cultures from the NTF with AOA proportions of 87, 71, 66, 41, 29 and 9%, respectively (Figure 6a). Similar trends were observed

for suspended growth cultures from the NTF and the attached and suspended growth cultures from the MBBR (Figure 6b, 7a, and 7b.). These data are evidence that AOA are more resistant than AOB to copper inhibition. AOA and AOB oxidize ammonia to nitrite by different mechanisms, and it has been proposed that slow internalization kinetics of AOA account for the reduced susceptibility to copper (Lehtovirta-Morley et al., 2013).

In cultures with higher proportions of AOA ($> 70\%$), similar reductions in sOUR and sAOR were observed following treatments with 10 and 15 mg/L of copper (Figures 6-9). However, at <40% AOA, the 15 mg/L copper treatment resulted in greater sOUR and sAOR reductions than the 10 mg/L treatment. The trend that sOUR and sAOR were more inhibited for cultures with lower proportions of AOA was most evident at 15 mg/L of copper. Applying a linear regression between the proportion of AOA and percent sOUR/sAOR inhibition for 15 mg/L of copper resulted in R^2 values of 0.84, 0.79, 0.64, 0.56, 0.67, 0.47, 0.75, and 0.45 for the data in Figures 6a, 6b, 7a, 7b, 8a, 8b, 9a, and 9b, respectively. Several studies have used sOUR and sAOR to assess the inhibitory effects of heavy metals on AOB in biological wastewater treatment, and a wide range of inhibitory concentrations have been reported (Hu et al., 2002,2004; Li et al., 2016). This research is the first to examine copper inhibition of AOA in attached and suspended growth cultures, and the data are similar to previous findings for AOB, where attached cultures were more resistant to copper than suspended cultures (Ong et al., 2005).
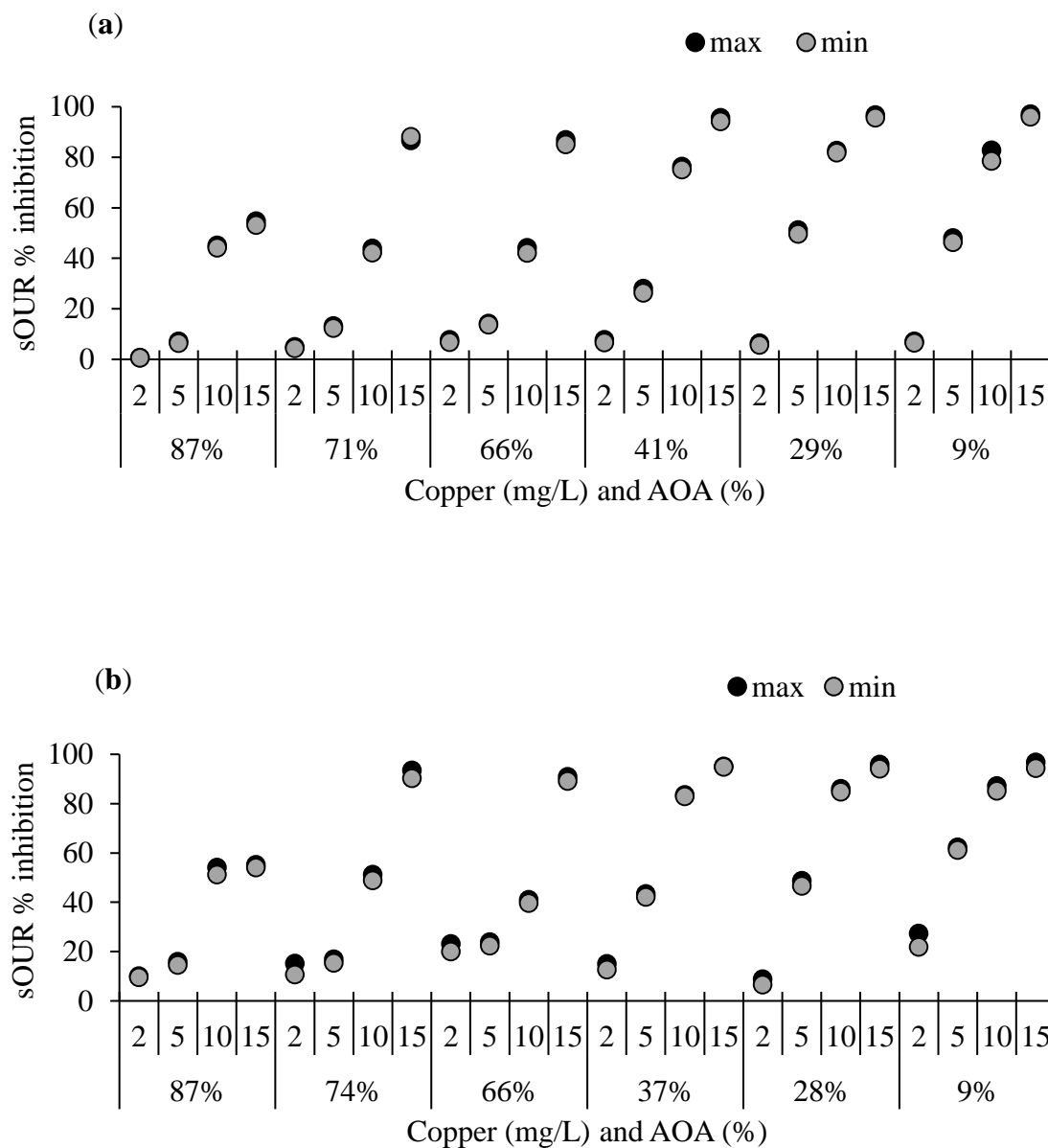
Figure 6. sOUR inhibitions in two independent experiments (maximum and minimum) as a function of total copper concentrations (2, 5, 10, 15 mg/L) for different fractions of AOA in (a) attached and (b) suspended growth conditions prepared from NTF culture.
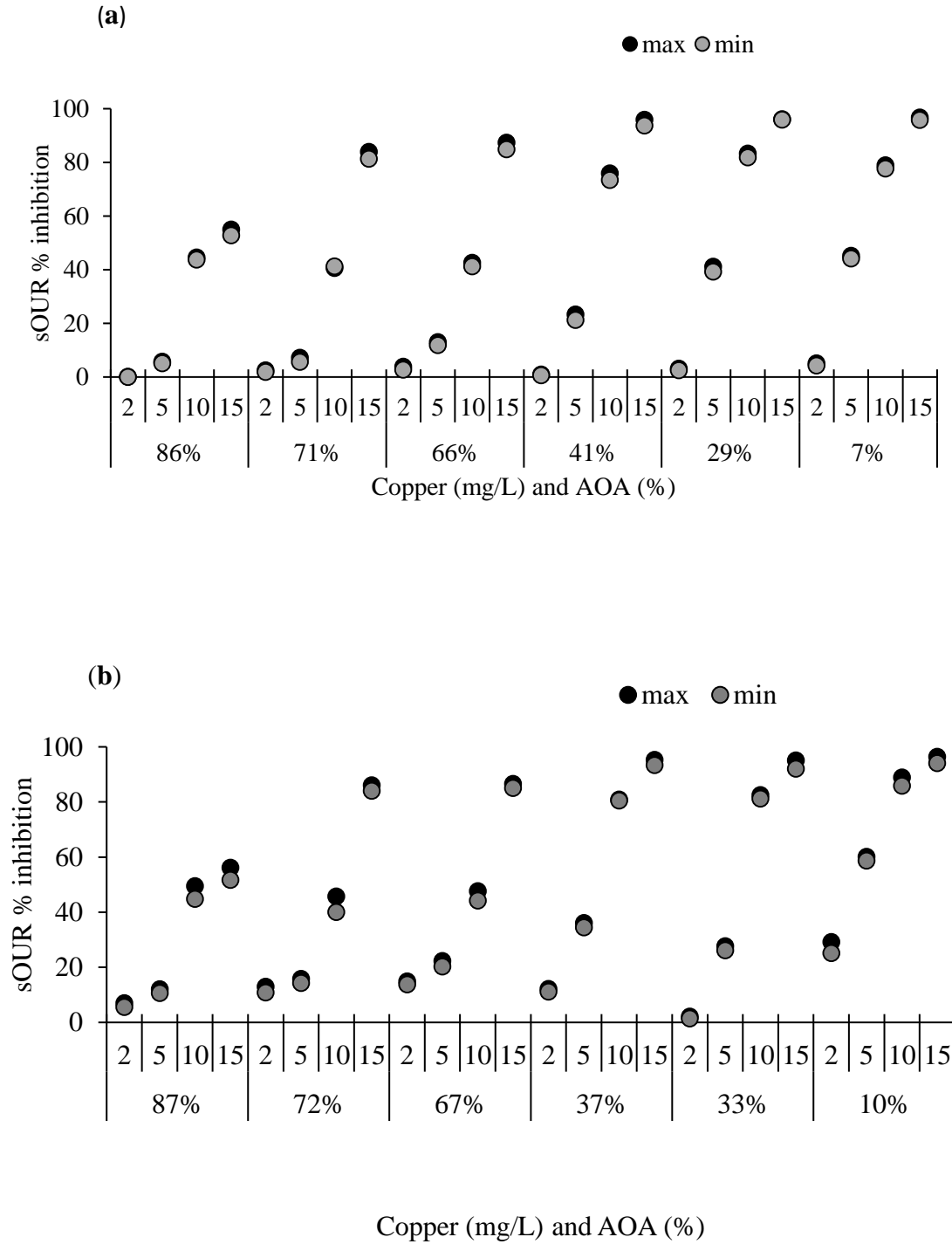
Figure 7. sOUR inhibitions in two independent experiments (maximum and minimum) as a function of total copper concentrations (2, 5, 10, 15 mg/L) for different fractions of AOA in (a) attached and (b) suspended growth conditions prepared from MBBR culture.
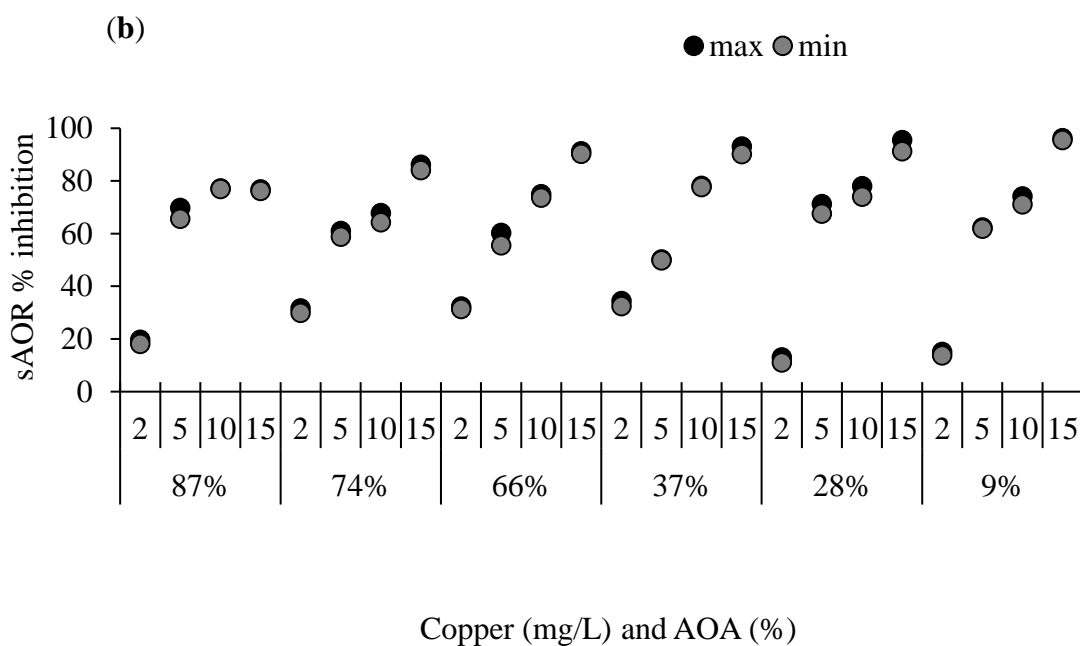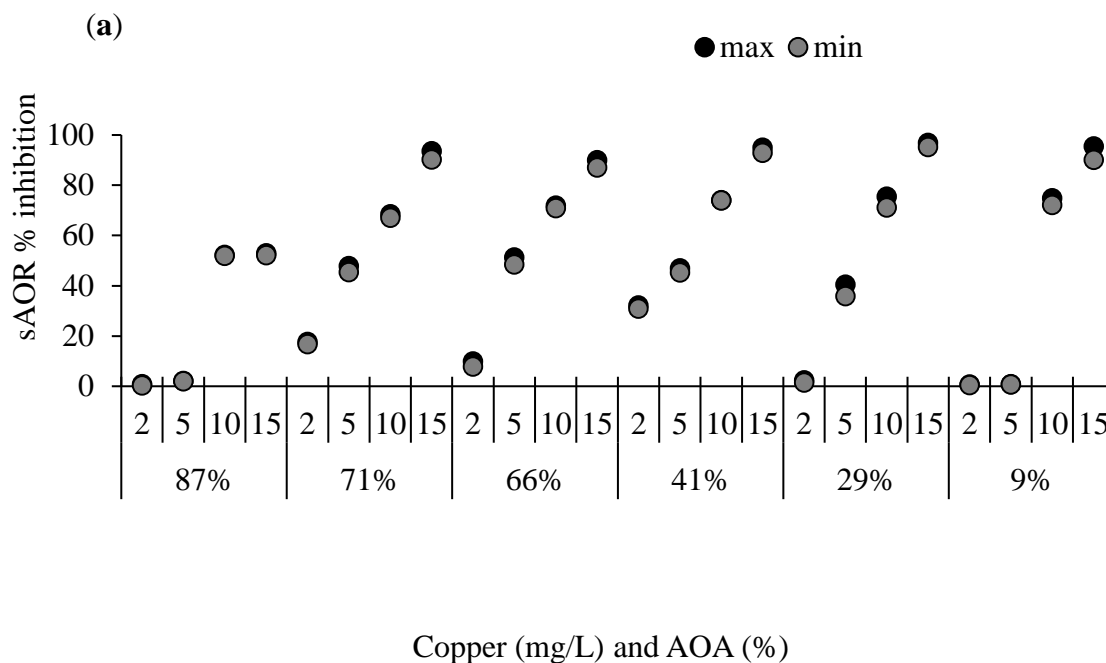
Figure 8. sAOR inhibitions in two independent experiments (maximum and minimum) as a function of total copper concentrations (2, 5, 10, 15 mg/L) for different fractions of AOA in (a) attached and (b) suspended growth conditions prepared from NTF culture.
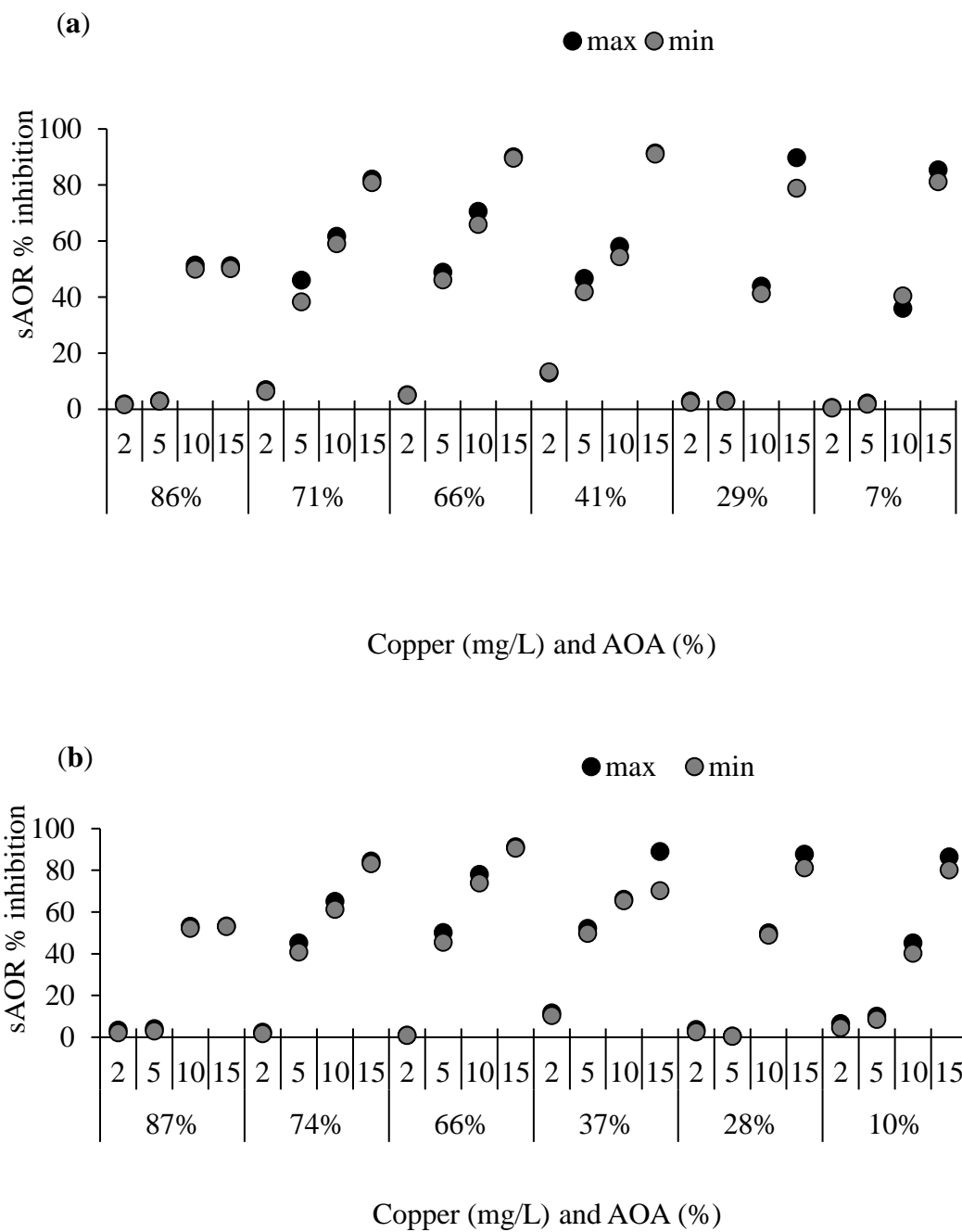
Figure 9. sAOR inhibitions in two independent experiments (maximum and minimum) as a function of total copper concentrations (2, 5, 10, 15 mg/L) for different fractions of AOA in (a) attached and (b) suspended growth conditions prepared from MBBR culture.

4.3.3. Effect of Copper on AOA Abundance

The effect of copper on cultures with different proportions of AOA was determined by quantifying archaeal and bacterial *amoA* gene copies. Attached cultures were less affected by copper than suspended growth cultures particularly at higher proportions of AOA for the cultures from both NTF (Figure 10) and MBBR (Figure 11). AOA abundance in all the cultures decreased at higher copper concentrations, regardless of the growth conditions; however, the abundance reductions in low fractions of AOA were greater than highly dominated AOA cultures concurring with sOUR and sAOR results.

The abundance of AOA in suspended and attached cultures decreased with increasing copper concentration regardless of the initial AOA proportion. This concentration-dependent inhibition is similar to the findings from a previous study on the effect of copper (II) on a suspended growth culture of AOB (Kapoor et al., 2015; Ochoa-Herrera et al., 2011). At 2 mg/L copper and higher proportions of AOA ($> 60\%$), *amoA* abundance after day 3 remained steady, indicating that AOA was relatively tolerant to this lower concentration of copper. At the same copper concentration and lower proportions of AOA ($< 50\%$), *amoA* abundance decreased over time. Reductions in *amoA* copy number, when combined with reductions in sOUR and sAOR, suggest that AOA is likely to be involved in nitrification. Moreover, the changes in *amoA* abundance due to copper inhibition for different fractions of AOA reconfirm the existence of various nitrification mechanisms between AOA and AOB. A previous study reported similar results that the difference in heavy metal tolerance between AOA and AOB in soil showed a different pathway of nitrification by AOA and a possible niche differentiation between AOA and AOB exists (Lehtovirta-Morley et al., 2013).

For both the cultures from NTF and MBBR, reduction in *amoA* of attached growth was less than suspended growth. Cultures with lower fractions of AOA were completely inhibited at 10 and 15 mg/L of copper. For lower fractions of AOA, the inhibitory effect was not dependent on AOA fractions.
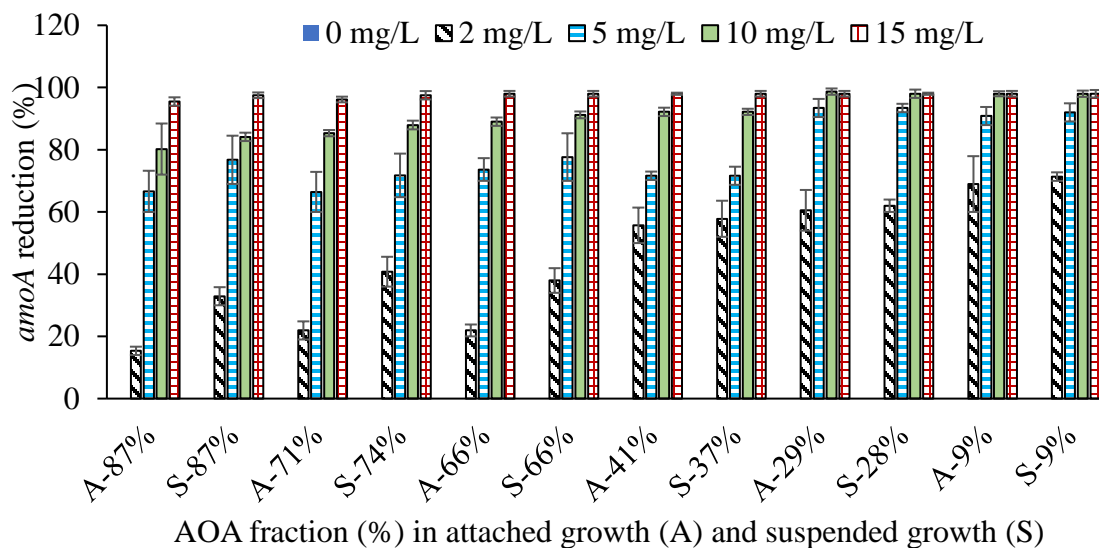
Figure 10. Archaeal *amoA* reduction (%) in prepared attached and suspended growth nitrifying cultures from NTF exposed to different copper concentrations (0, 2, 5, 10, and 15 mg/L). Error bars represent minimum and maximum values from duplicated experiments.
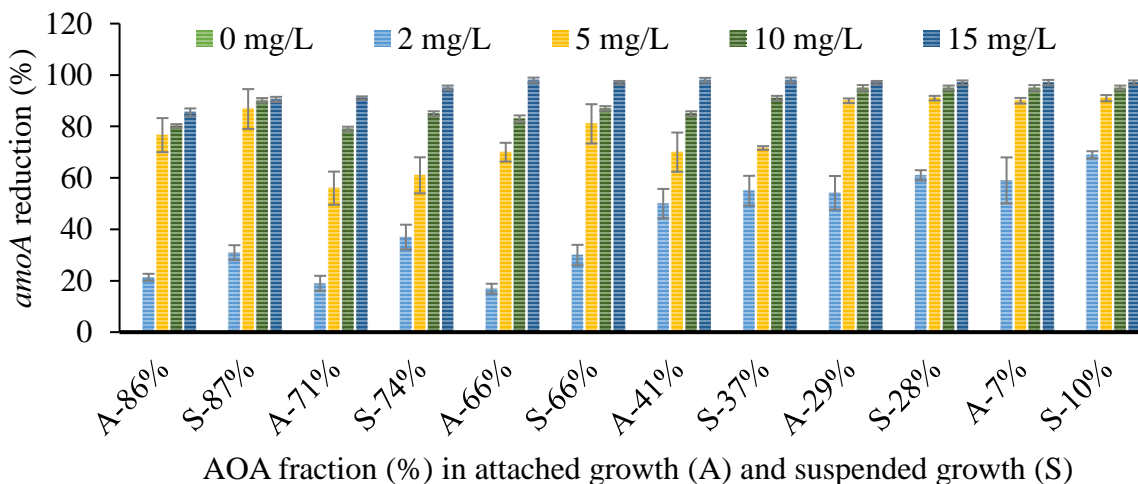
Figure 11. Archaeal *amoA* reduction (%) in prepared attached and suspended growth nitrifying cultures from MBBR exposed to different copper concentrations (0, 2, 5, 10, and 15 mg/L). Error bars represent minimum and maximum values from duplicated experiments.

4.4. Summary

This research shows the involvement of AOA in wastewater nitrification and the susceptibility of AOA and their nitrification activity to inhibition by copper. However, the higher fractions of AOA cultures were found more to be tolerant to copper inhibition than the lower fractions of AOA cultures. Municipal WWTPs usually receive wastewater with less than 2 mg/L of copper but concentrations up to 15 mg/L, which is shown to inhibit nitrification in this research, are not uncommon. At high levels of copper, which is possible at WWTPS through industrial shock loads, the nitrifying culture containing higher fractions of AOA would be beneficial given their greater tolerance compared to the AOB dominated culture. The unique approach to develop different fractions of AOA cultures in this research opens up an enormous research scope to unveil the nitrification efficiency and kinetics of AOA in the presence of environmental contaminants.

5. ABUNDANCE AND ACTIVITY OF AMMONIA OXIDIZING ARCHAEA AND BACTERIA IN BULK WATER AND BIOFILM IN WATER SUPPLY SYSTEMS PRACTICING CHLORINATION AND CHLORAMINATION: FULL AND LABORATORY SCALE INVESTIGATIONS

5.1. Introduction

Chlorine and chloramines are the two widely used disinfectants by WTPs. Chloramines are formed when ammonia is added along with chlorine. Chloramines are generally preferred over chlorine by WTPs because of lower potential for DBPs formations, longer persistence in DSs, and enhanced biofilm penetration (Lechevallier et al., 1981). However, due to the presence of ammonia, chloramination can promote the growth of nitrifiers, and hence nitrification. Nitrification is a common problem in WTPs, which can lead to operational or regulatory compliance challenges, including decline in disinfectant residual, increased biofilm production, and corrosion. WTPs practicing chloramination experience more nitrification than those employing chlorination. For instance, two-thirds of WTPs in the United States that applied chloramination experienced some degree of nitrification (Wilczak, et al., 1996).

Ammonia oxidation or nitritation is the first and rate-limiting step of nitrification, in which ammonia oxidation to nitrite is performed by autotrophic AOM including AOA and AOB. Nitritation is a major concern for WTPs as the process deteriorates the water quality (Lipponen et al., 2002; Skadsen, 1993) because the produced nitrite is a precursor for nitrate production. Nitritation can occur in the DSs and as well as treatment units. In the treatment train with ammonia present in source water, nitritation tends to occur mainly in a filtration unit. AOMs that perform nitritation in both of these locations (filters and DSs) are mainly in a biofilm form. A significant fraction of bacteria growing in the DSs is associated with biofilms (Camper et al.,

2003), and for nitrifiers, attachment to solid surfaces enhances their growth and provides more resistance to toxic substances (Wolfe et al., 1988).

The *amoA* gene of AOA and AOB, has been detected in WTPs (Bai et al., 2013; Berry et al., 2006; Pinto et al., 2012; van der Wielen et al., 2009). AOA were detected in three WTPs and DSs in the Netherlands using groundwater as a raw water source (van der Wielen et al., 2009). They were found in source water, and throughout the treatment train all the way to finished water that was not disinfected. The ammonia concentration removed in each step of the treatment showed a correlation with the copy number of *amoA* (AOA and AOB combined). Ammonia, which is nitrified during biological filtration in the WTPs, might play a vital role in harboring AOA in the filter. A few studies reported AOA on the surface or in the biofilm of anthracite/sand media of biologically active filtration systems (Bai et al., 2013; Berry et al., 2006; Pinto et al., 2012; van der Wielen et al., 2009).

In chloraminated DSs, AOB increased from the proximal to the distal parts due to an increase in ammonia (Lipponen et al., 2004; Lipponen et al., 2002). On the contrary, in a DS without disinfectant residual, there was no substantial difference in the number of AOB in the proximal and distal zones, whereas AOA were found in greater numbers in the distal portion compared to the proximal portion (van der Wielen et al., 2009).

In comparison to chloramine (primarily monochloramine), chlorine is a less effective disinfectant. Biological instability was observed in a chlorinated drinking water system where concomitant loss of disinfection residual and bacterial growth occurred (Nescerecka et al., 2014). A chlorine to ammonia ratio is a major factor for WTPs to maintain a stable DS by minimizing nitrification. Higher disinfectant residual appears to limit nitrification. However, once nitrification occurs, the disinfectant residual is quickly degraded. Temperature also influences

the occurrence of nitrification in DSs. A study showed that nitrification occurs mostly in summer months (Lee et al., 2011). A combination of disinfectant type and temperature had a cumulative effect on nitrification (Pintar and Slawson, 2003). A study found that AOA and AOB might differ in their resistance to chlorine (Kasuga et al., 2010). Indeed, the relative abundance and respective roles of AOA and AOB in nitrification have been topics of recent research (Hatzenpichler, 2012; Nicol et al., 2008; Prosser and Nicol; 2008, Schleper, 2010).

Detection of nitrifiers including AOM was mostly investigated by collecting bulk water because biofilm sampling from DS pipeline is more challenging. Studying both bulk water and biofilm is important because large metabolic differences between attached (biofilm) and free bacteria can occur (Boe-Hansena et al., 2002). A study reported the detection of AOA in bulk water in a distal zone of a DS that involved natural ammonia in the source water (van der Wielen et al., 2009). Free bacteria including nitrifiers (mostly AOB) in bulk water have been shown to dominate in portions of a distribution system (Regan et al., 2002). However, these studies were conducted without disinfectants and limited to bulk water.

This research focuses on the abundance and activity of AOA and AOB in chloraminated (primarily monochloramine), and chlorinated water supply systems based on both bulk and biofilm phases. The occurrence of AOA along with AOB in DS is of interest, as these two microbial groups may have different growth and survival characteristics against disinfectants. It has been shown that distribution systems have AOA and AOB, but it is unclear whether their presence has any relationships with the type and level of disinfection particularly monochloramine versus chlorine. There has been an extremely limited number of studies on AOA in drinking water systems as AOB has been the main focus for most nitrification studies. As reported in a previous study (van der Wielen et al., 2009) and described above, AOA

potentially contribute to nitrification in WTPs and DSs. Therefore, it is important to understand how disinfection practices (chloramination and chlorination) affect the abundance of AOA.

In this research, the abundance and activity of AOA and AOB in bulk water and biofilm samples from full-scale WTPs, and distal and proximal locations of their DSs were investigated. Temperature, chlorine, chloramine, total ammonia, nitrite, and nitrate in bulk water were measured to find a relationship with the abundance of AOA and AOB. A laboratory-based experiment was also conducted to simulate DS conditions using a Center for Disease Control (CDC) reactor. The experiment involved incubating biofilm at different concentrations of chorine and chloramine and two temperatures.

5.2. Materials and Methods

5.2.1. Sample Collections from Water Supply Systems and Preparations

5.2.1.1. Bulk Water Samples

Five liters of grab water samples were collected from three WTPs - Plants A, B, and C monthly from March 2014 to February 2015, April 2015 to March 2015, and June 2014 to May 2015, respectively. Plant A is in Minnesota, USA while Plants B and C are in North Dakota, USA. Diagrams of the treatment steps and sample collection points for all three plants are shown in APPENDIX (Figures A2, A3 and A4). Water samples were mainly from source water (surface water or groundwater), blended water (surface plus groundwater) wherever applicable, and filter effluent (prior to chlorination or chloramination). For DS, water samples were collected from clear well, tower, and proximal and distal zones. Samples were collected from two distal zones (DS2 and DS3) for Plants A and C and only one distal zone (DS2) for Plant B. Tap water samples within the WTPs represented proximal zone samples while distal zone samples were collected 3-10 km away. For Plant A, initially March 2014 to August 2014, the samples were

collected from one distal zone. Later another sampling location based on nitrification occurrence was added from September 2014 to February 2015.

5.2.1.2. Biofilm Samples

Along with bulk water samples, approximately 1 kg of sand/anthracite samples was obtained from the filters of the three WTPs studied. The sand/anthracite was collected using a polypropylene bucket with a lid and brought back to a laboratory and stored at 4ºC until biofilm extraction was performed. In addition, for some of the water sampling events, 5 L of backwash water samples were collected. Biofilm samples in a distal zone of the DS of Plant A were also obtained during five water main break events in a 4-month span. A procedure to obtain a pipe section for biofilm sample collection is described in Section 5.2.1.3. Water leaking from the broken pipe was also collected and analyzed for ammonia-N, nitrate-N, nitrite-N, and chloramine.

5.2.1.3. Sample Preparations

Bulk and backwash water samples were filtered through sterile 0.22 μm pore-size polycarbonate membrane filters (EMD MIlipore, GTTP04700). Specifically, 1 L of water samples and 2 L of backwash water samples were filtered. The filter membranes with retentate was transferred to sterile microcentrifuge tubes and stored at -80°C or immediately used for DNA extraction. Twenty grams of each sand/anthracite sample were added to 150 mL phosphate buffer containing 0.1 M sodium citrate, and vigorously vortexed for 30 min to detach biofilm. One milliliter of the liquid phase was subject to DNA extraction.

5.2.1.4. Procedure for Obtaining Pipe Section for Biofilm Sample Collection

Bulk water samples were filtered through sterile 0.22 μm pore-size polycarbonate membrane filters (EMD MIlipore, GTTP04700). Specifically, 1 L of water samples and 2 L of

backwash water samples were filtered. The filter membranes with retentate was transferred to sterile microcentrifuge tubes and stored at -80°C or immediately used for DNA extraction. Twenty grams of each sand/anthracite sample were added to 150 mL phosphate buffer containing 0.1 M sodium citrate, and vigorously vortexed for 30 min to detach biofilm. One milliliter of the liquid phase was subject to DNA extraction.

After the DS pipe section (from the water main break event) reached the laboratory at North Dakota State University, one cap was removed, and the dechlorinated water (retained in the pipe, described in section 5.2.1.3) was discarded. The interior of the pipe section was rinsed gently with membrane (0.22 μm pore-size polycarbonate) sterilized tap water to remove unattached or settled solids. Biofilm samples were collected from three separate, evenly-spaced sections of the pipe interior. For each section, biofilm material was obtained by scraping the entire interior circumference of the pipe with a sterile spatula. The collected biofilm was transferred to a sterile 10 mL vial, and 3 mL ultrapure water was added. The suspension was homogenized by vortexing and large particles such as corrosion byproducts were allowed to settle. From this suspension, 2 mL was transferred to a 2 mL microcentrifuge tube and centrifuged at 10,000×g for 10 minutes. The supernatant was then discarded, and the pellet was immediately used for DNA extraction or stored at -20°C.

5.2.2. Laboratory Incubation Experiments

A CDC reactor with 290 mL of water-volume and PVC coupons (1.5 × 1.7 cm) was used for incubation experiments. Each experiment started with biofilm establishment for 25-30 days. During this period, an ultra-low peristaltic pump (0.05 to 0.1 mL/min) was used to feed the reactor with water sample collected from sand/anthracite media filter effluent of Plant A in which AOA and AOB were previously detected. This sample served as a sole source of

indigenous microorganisms (inoculum) to the reactors. To simulate premises plumbing (where maximum nitrification is typical observed in the distribution system pipeline, Rahman et al. (2011)), periods of flushing and stagnation were included in the startup of the reactors and incubation experiments described below. The reactor was flushed by the ultra-low peristaltic pumps and a paddle in the reactor (0.2 m/s) for five minutes followed by eight hours of stagnation periods. The reactor was kept in the dark to simulate conditions similar to the DSs. The startup was considered complete based on stable nitrification as measured by conversion of ammonia to nitrate.

A 96-h incubation experiment was conducted under the same operational conditions as the startup period using different concentrations of chlorine and monochloramine and at two different temperatures (10-12°C and 25°C). Three chlorine residual concentrations in the reactor, 1.5, 2.0 and 2.5 mg/L were tested. For chloramine residual, high (1.5-1.6 mg/L), medium (0.3-0.4 mg/L), and low (0.05-0.1 mg/L) concentrations were examined. The chlorine and chloramine solutions were added to the membrane sterilized filter effluent water followed by additions of 0.01 N sodium hydroxide to achieve pH 7.4-7.6 and phosphate buffer. To control the temperature at 10-12°C and 25°C, the influent was placed inside a temperature controlled refrigerator (4ºC), and at room temperature respectively. Biofilm samples were collected from two to three coupons every 24 h interval for the entire experiment. Biofilm was scrapped off the coupon by a bottle brush in water placed in a micro centrifuge tube containing 1 mL of deionized water which was later centrifuged at 13,000× g to collect the pellet. The sample was stored at -80°C or used immediately for DNA extraction. Control experiments using 0.22 µm pore-size polycarbonate membrane filter sterilized effluent with added disinfectants were included.

5.2.3. Preparation of Chemicals for Laboratory Incubation Experiment

All the solutions were prepared using organic free water (Hach # 2641549). All the

chemicals were purchased from Sigma-Aldrich[TM] unless otherwise mentioned. Solutions with

different chlorine concentrations (1.5, 2.0 and 2.5 mg/L) were prepared by diluting a stock

sodium hypochlorite solution (20 mg/L, HACH, # 1426810). Monochloramine solutions (1.5-

1.6, 0.3-0.4, and 0.05-0.1 mg/L) were prepared by adding a stock ammonia-N solution (100

mg/L, HACH, # 2406549) to water while stirring, and then adding an aliquot of the stock

chlorine solution based on a 3:1 (w/w) chlorine:ammonia-N dosing ratio to reach 1.5-1.6, 0.3-

0.4, and 0.05-0.1 mg/L. The monochloramine solutions were mixed at 200-300 rpm for 15 min

before use. The monochloramine formation was verified using the method described in Section

5.2.4. Total ammonia-N, chlorine (free and total), nitrite-N and nitrate-N in the influent and

effluent were measured daily according the methods described in Section 5.2.4.

5.2.4. Analytical Methods

Total and free ammonia-N, nitrite-N, nitrate-N, free and total chlorine, monochloramine

and pH were the studied water quality parameters. For the field samples, chlorine (combined free

and total) was measured immediately on-site with a pocket colorimeter (Hach, Pocket

Colorimeter™ II, 5870000) using the N,N-diethyl-p-phenylenediamine (DPD) colorimetric

method. For laboratory analysis, chlorine (combined total and free chlorine) was analyzed by

Hach method # 8167 and #8021 (Hach, 2008), which is based on Standard Methods (APHA.

2005). Monochloramine concentration was determined using the indophenol method according

to Hach method # 10200 (Hach, 2008). Free ammonia was analyzed by the DPD method (HACH

#10020). Dissolved organic carbon (DOC), conductivity, low range ammonia-N, nitrite-and

nitrate-N, and pH measurements followed Standard Methods (APHA. 2005). The three nitrogen

analyses were performed calorimetrically with a DR/6000 spectrophotometer (HACH Company, Loveland, CO). DOC, pH and conductivity were measured according to Standard Methods (APHA et al., 2005). DOC analysis was conducted using a UV/persulfate oxidation total organic carbon analyzer (Phoenix 8000, Tekmar Dohrmann, OH, USA). The pH and conductivity were measured using a pH and conductivity meter (Orion Star™ A211, Thermo Scientific, MA, USA).

5.2.5. DNA Extraction, PCR, Quantitative PCR, Cloning and Sequencing

DNA was extracted according to the following procedure. Approximately 1.25 g of the watery biofilm sample was centrifuged at $5,000\times$ g for 7 min at 4°C. The supernatant was discarded, and the pellet was mixed with 900 µL of TE buffer (100 mM Tris-Cl, pH 7.6, 10 mM EDTA) and 50 µL of lysozyme (10 mg/mL). The mixture was vortexed for 2 min, followed by incubation periods of 1 h at 37°C, and then 3 minutes at 100°C. One hundred microliters of proteinase K (10 mg/ml) were added, and the mixture was incubated for 1 h at 55°C. DNA was further purified using a biofilm DNA isolation kit in accordance with the instruction by the manufacturer (PowerBiofilm™ DNA Isolation Kit, MO BIO, Carlsbad, CA) with a slight modification in which an initial bead beating duration was increased from 30 to 45 s at 3,200 rpm. Then, 200 µL of a BF3 solution (included in the kit) was added twice to obtain clear supernatant. DNA was quantified using a NanoDrop® spectrophotometer (ND-1000, Thermo Fisher Scientific, USA). An internal control was used to check for inhibitions in the extraction procedure by mixing a known bacterial DNA with the extracted DNA at different ratios. Successful PCR amplification of the known DNA suggests that the extraction was not inhibited.

PCR, qPCR, cloning and sequencing followed a protocol by Roy et al. (2014). In brief, archaeal amoA genes (~635bp) were amplified using primers Arch-amoAF (5′-

STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′)

(Francis et al., 2005). Bacterial amoA genes (~595bp) were amplified by amoA-1F (5′-

GGGGTTTCTACTGGTGGT-3′) and amoA-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′)

(Rotthauwe et al., 1997). Amplified products were visualized following electrophoresis on 1.5%

agarose gels. Triplicate PCR reaction products were pooled, purified using a PCR quick-spin™

purification kit (Promega, Madison, WI), and cloned using a pGEM T-vector cloning kit

(Promega, Madison, WI). Plasmids were purified with NucleoSpin® Plasmid clean up kits and

inserts were sequenced in both directions using a BigDye Terminator v3.1 cycle sequencing kit

with M13F and M13R primers in an ABI Prism 3130 genetic analyzer (Applied Biosystems,

Carlsbad, CA).

     The evolutionary history was inferred by using the Maximum Likelihood method based

on the Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-

11144.4102) is shown. The percentage of trees in which the associated taxa clustered together is

shown next to the branches (values lower than 50% are not shown). Initial tree(s) for the

heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to

a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL)

approach, and then selecting the topology with superior log likelihood value. A discrete Gamma

distribution was used to model evolutionary rate differences among sites (5 categories (+G,

parameter = 0.6968)). The rate variation model allowed for some sites to be evolutionarily

invariable ([+I], 32.6610% sites). The tree is drawn to scale, with branch lengths measured in the

number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et

al., 2013).

5.2.6. Statistical Analyses

The sampling period was divided into cold months (October to April) and warm months (May to September). A paired t-test was performed to determine whether differences in the $\log_{10}$ values of *amoA* gene copy numbers (for AOA and AOB) in WTPs and DSs between the warm and cold months were significant ($p < 0.05$). The Pearson's correlation coefficients were calculated to assess correlations between the abundance of *amoA* genes of AOA and AOB and water temperature. The correlation coefficient (r) was also determined between the concentration of ammonia and the AOA, AOB and AOM (combined AOA and AOB) abundance in DS water samples. The Satterthwaite method was used to verify that there was no uneven distribution in the AOA, AOB and AOM abundance. The SAS statistical software version 9.3 was used for all statistical calculations and analyses.

5.3. Results and Discussion

5.3.1. WTP and DS Practicing Chloramination (Plant A)

For source water for Plant A, AOA were more abundant than AOB in surface water for the majority of the samples (Figure 12 and APPENDIX, Figure A5). The opposite trend was observed in groundwater and blended water. Physical and chemical characteristics of these source water samples are listed in APPENDIX (Table A2). The difference of mean abundance between cold and warm months was not significant for AOA for both surface water ($p = 0.331$) and groundwater ($p = 0.218$) but for AOB for both surface water ($p = 0.001$) and groundwater ($p = 0.002$) suggesting that AOA population remained stable regardless of the season. Cell wall of AOA is more resistant to temperature perturbation than that of AOB (Martens-Habbena et al., 2009; Urakawa et al., 2008). Previous studies also reported that the abundance of AOB in WTPs was relatively sensitive to seasonal changes (Scott et al., 2015; van der Wielen et al., 2009). The

effect of seasonal changes on the AOA and AOB abundance in blended water followed the statistical trends for surface water and groundwater.

The biofilm of sand-anthracite media showed slightly more AOA than AOB (APPENDIX, Figure A6). The mean abundance difference between cold and warm months was significant for both AOA ($p = 0.001$) and AOB ($p = 0.002$) suggesting that temperature has an effect on AOA under the biofilm condition. Previous studies reported that temperature had a positive correlation with AOA and AOB combined (AOM) in biofilm-based wastewater systems, and AOA and AOB individually increased with the wastewater temperature (Roy et al., 2014; Sauder et al., 2012). In the filter effluent, AOA and AOB were detected substantially more during warm period, and a significant difference between cold and warm months exists for AOB ($p = 0.002$) but not for AOA ($p = 0.398$) suggesting that warm temperature did not affect AOA growth in bulk water.

The difference in ammonia-N concentration in surface water (1.37 ± 0.11 mg/L) and groundwater (2.91 ± 0.16 mg/L) possibly influenced the abundance of AOA and AOB in Plant A. More AOB abundance than AOA was observed in groundwater agreeing with previous studies that showed more AOB than AOA at similar concentrations of ammonia-N in groundwater (Kasuga et al., 2010; van der Wielen et al., 2009). Ammonia concentration was a determinant for AOA and AOB abundance in other environments including marine, soil, wastewater treatment plants, where AOA dominance over AOB was associated with low concentrations of ammonia (Martens-Habbena et al., 2009; Sauder et al., 2012; Verhamme et al., 2011). Martens-Habbena et al. (2009) found a half-saturation coefficient for ammonia for an AOA strain that was lower than any reported for AOB, suggesting that some AOA strains may be better adapted for growth at low ammonia concentrations.

In the DS of Plant A, AOA and AOB were not detected in chlorine chamber, tower and proximal zone of the distribution system where disinfection residual was high and hence the data are not shown in Figure 12. They were detected only in distal zones (DS2 and DS3). The differences between cold and warm months were not significant for AOA ($p = 0.289$) but for AOB ($p = 0.001$) in distal zone (combined DS2 and DS3) again suggesting that AOA is more adaptable with seasonal variation. The DS2 and DS3 zones were further from the chloramination point where chloramine dissipation was more likely resulting in ammonia-N release and promoted the growth of AOA and AOB. Kasuga et al. (2010) showed a similar result where proximal zone had no or less combined AOA and AOB than distal zone. Regrowth of AOA and AOB in distal zones (DS2 and DS3) and simultaneous nitrification episodes suggest that AOA were able to oxidize ammonia in the chloraminated system.

A significant correlation between ammonia-N concentration and AOA abundance and ammonia concentration was found for the distal zones of the DS of Plant A (APPENDIX, Table A3). This finding coincides with a significant correlation between AOA and AOB abundance and ammonia concentration associated with chloramine degradation observed by Scott et al. (2015). Studies have shown that AOB can grow in a chloramine concentration range of 0.05-3.0 mg/L (Wahman et al., 2016; Wahman and Pressman, 2015). Also, the growth of AOB was not inhibited after prolonged contact period at that range of chloramine concentration (Kasuga et al., 2010) suggesting that AOB is adapted to chloramine at that level. This research shows that both AOA and AOB were detected in distal DS zones suggesting that both AOA and AOB were adapted to chloramine (0.05 to 1.5 mg/L). Studies have found that AOB have long inactivation time with chloramine; maintaining a disinfectant residual does not always prevent their growth

(Lee et al., 2011; Maestre et al., 2013; Oldenburg et al., 2002; Wahman et al., 2016). As demonstrated by the results of this research, these findings are also applicable to AOA.

5.3.2. WTP and DSs and DSs Practicing Chlorination (Plants B and C)

In both Plants B and C, AOA and AOB were detected throughout the sampling period for groundwater, sand-anthracite biofilm (only Plant B), backwash water, and filter effluent (Figure 13, Figure 14, and APPENDIX, Figure A6). In groundwater for Plant B, AOB was slightly more abundant than AOA only in warm months (Figure 13). In Plant C, AOA was always more abundant than AOB (Figure 14). For both plants, the differences between cold and warm months were not significant for AOA ($p = 0.247$) but for AOB ($p = 0.003$) similar to Plant A. The biofilm of sand-anthracite media had more AOA than AOB in both Plants B and C (APPENDIX, Figure A6). The difference between cold and warm months was significant for both AOA ($p = 0.001$ and 0.002) and AOB ($p = 0.002$ and 0.001) for both Plants B and C. AOA and AOB in the bulk water phase contributed to the microbial population in the filter as the filter effluent for both Plants B and C had less AOA and AOB compared to groundwater.

Figure 12. Quantity of *amoA* of AOA and AOB in water samples collected from Plant A and DS from March 2014 to February 2015; DS2 = Distal zone (4.8 km away from WTP); DS3 = Distal zone (9.6 km away from WTP); No sampling during March-August for DS3.

AOA abundance in Plant C was two orders of magnitude lower compared to Plant B (Figure 13) which is attributed to higher ammonia-N concentration in groundwater for Plant C than Plant B (Figure 14). For both Plants B and C, ammonia-N was oxidized in the sand-anthracite filter resulting in low ammonia-N in filter effluent (0.20-0.78 mg/L and 0.36-0.19 mg/L respectively). For Plant B, the numbers of AOA and AOB in the backwash water have

strong relationships with ammonia-N concentration ($p = 0.01$ and $r^2 = 0.80$ for AOA; $p = 0.01$ and $r^2 = 0.78$ for AOB). That is also the case for Plant C ($p = 0.001$ and $r^2 = 0.86$ for AOA; $p = 0.002$ and $r^2 = 0.88$ for AOB).

For DS, proximal zones had lower AOA and AOB abundance than distal zones for Plants B (Figure 13) and C (Figure 14). AOA and AOB were not detected in the clear wells and towers for Plants B and C and hence the data are not shown. As the concentration of chlorine residual decreased in distal zones, increases in both AOA and AOB in both plants were observed suggesting that there are relationships between chlorine decay and AOA and AOB growth. The difference between cold and warm months was not significant for AOA ($p = 0.182$ for DS 2 and $p = 0.190$ for DS 3) but for AOB ($p = 0.002$ for DS 2 and $p = 0.001$ for DS 3) in distal zones of Plant C indicating that AOA was stable with seasonal variation but AOB varied which is in agreement with a previous study in which AOB decreased at lower temperatures in a chlorinated system (Pintar and Slawson, 2003; Pintar et al., 2005). Results on the relationships between temperature and AOA and AOB abundance for Plant B followed the same trends as Plant C.
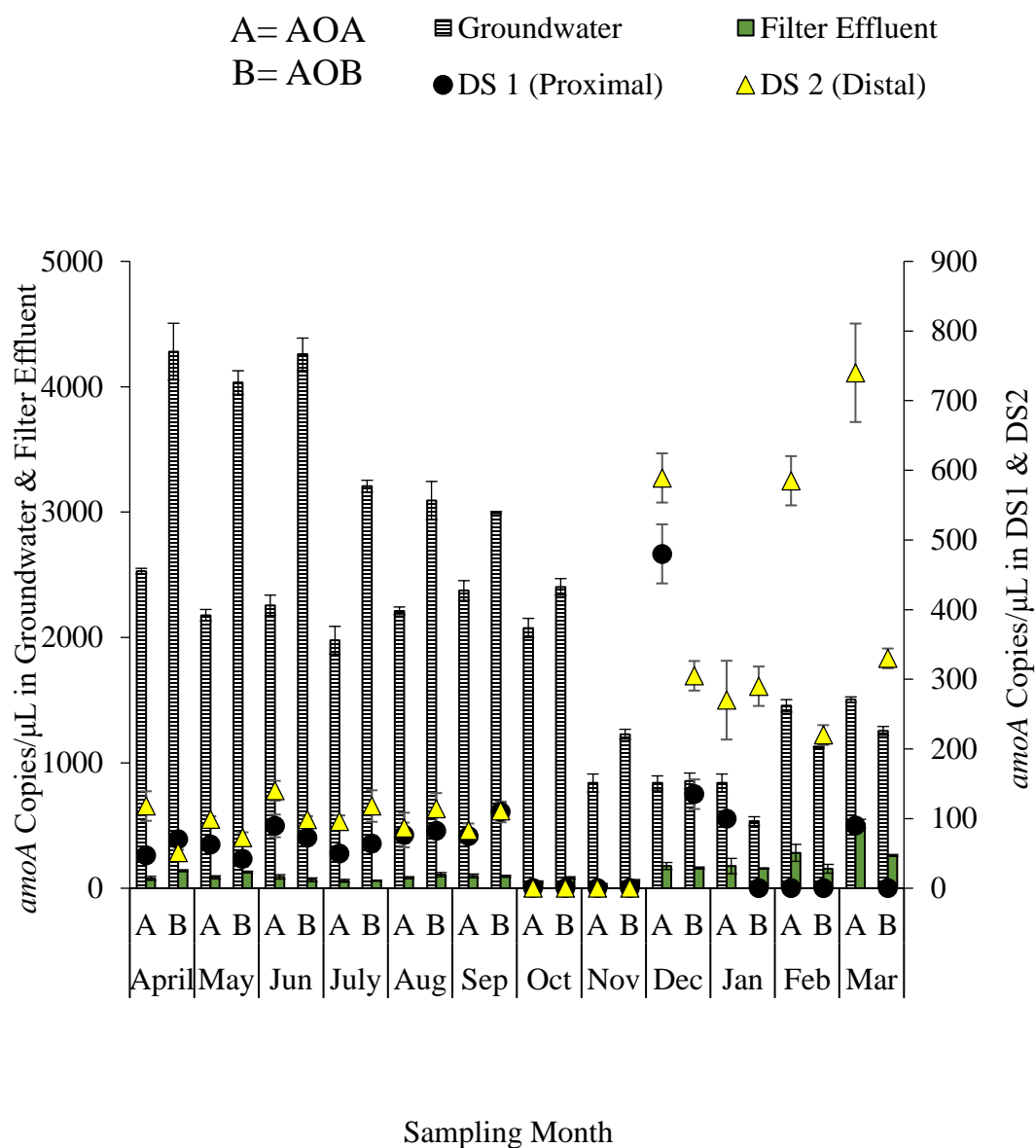
Figure 13. Quantity of *amoA* of AOA and AOB in water samples collected from Plant B and DS from April 2014 to March 2015; DS1 = Proximal zone; DS2 = Distal zone (6.2 km away from WTP), No sampling in October and November due to plant maintenance.
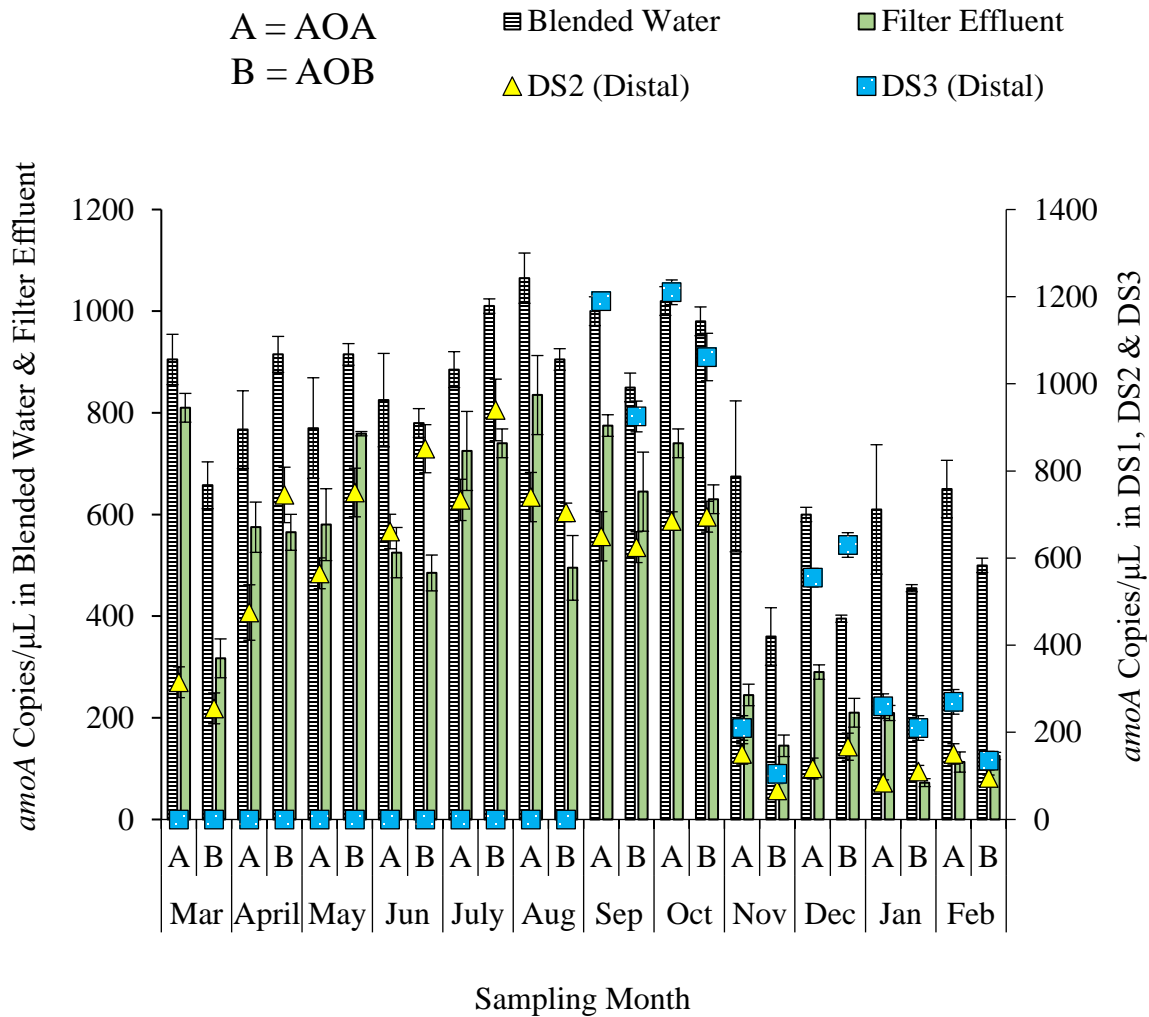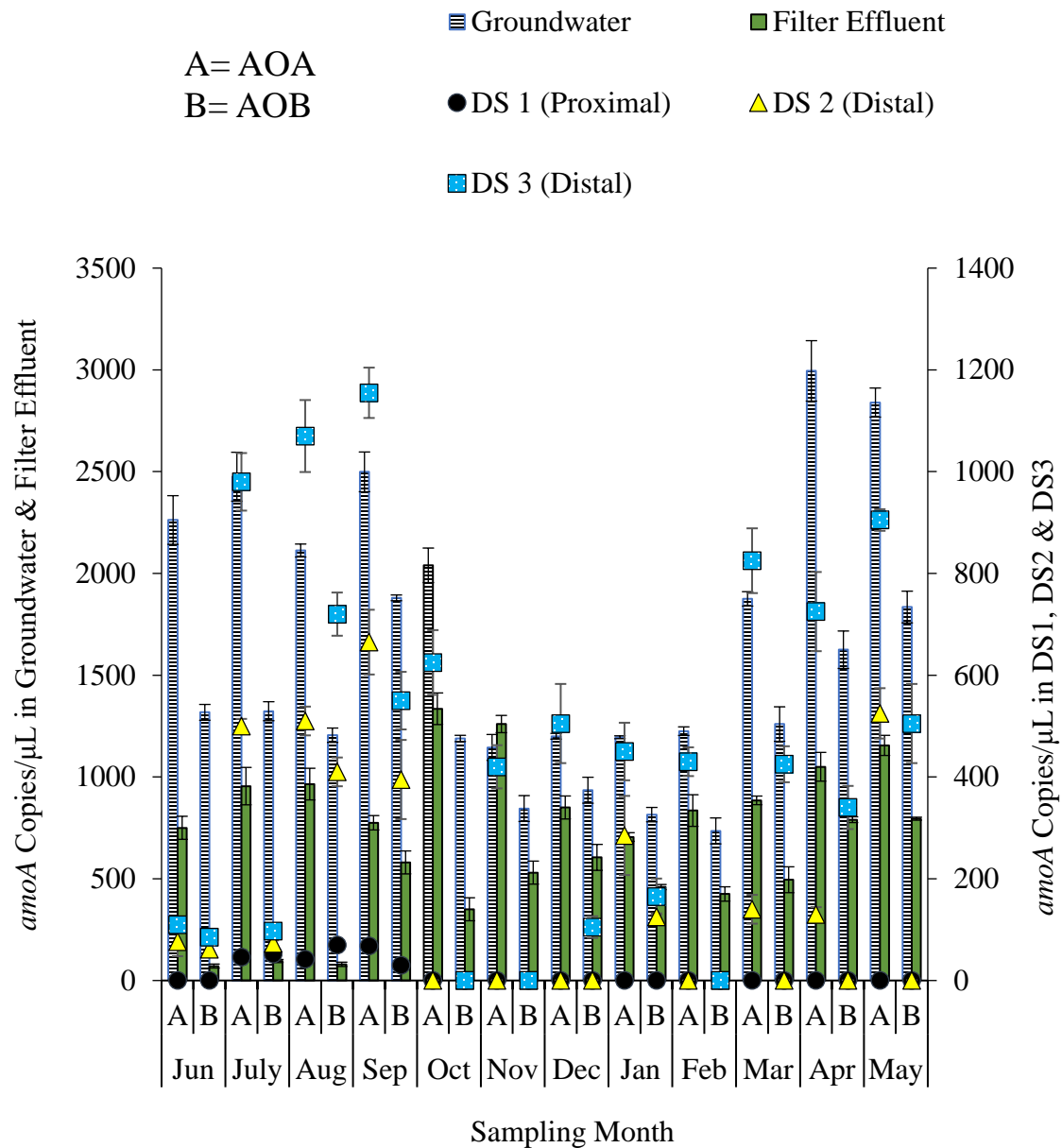
Figure 14. Quantity of *amoA* of AOA and AOB in water samples collected from Plant C and DS from June 2014 to May 2015; DS1 = Proximal zone; DS2 = Distal zone 1 (approximately 7.8 km away from WTP); DS3 = Distal zone 2 approximately 8.8 km away from WTP).

Significant correlations were found between ammonia concentration and AOA quantity for both Plants B and C (APPENDIX, Table A3). Regrowth of AOA and AOB in distal zones for Plants B and C and simultaneous increase in combined nitrite-nitrate concentration in the water in comparison to proximal zone indicates that AOA was able to oxidize ammonia in the chlorinated system in distal zones. The source of ammonia was from the groundwater for both plants. A correlation between AOA abundance and increase in combined nitrite-nitrate concentration was found for Plant B ($p = 0.01$; $r^2 = 0.91$). AOA and AOB abundance in a chlorinated distribution system based on their resistance was studied by Kasuga et al. (2010), and the result showed that AOB is less tolerant to chlorination than AOA. AOA showed higher chlorine resistance than AOB for both plants in this research.

5.3.3. AOA and AOB Abundance in Pipelines from Water Main Break Events

For biofilm in the distal zone pipelines of Plant A, AOA was found in higher numbers than AOB for most of the sampling events (APPENDIX, Figure A7). Physical and chemical characteristics of DS water samples collected along with the pipe sections are presented in APPENDIX (Table A4). Biofilm samples from the warm months had significantly higher AOA and AOB than the cold months, and there was a significant positive correlation between AOA quantity and water temperature ($r^2 = 0.81$; $p < 0.001$).

There was a significant positive correlation between the quantity of AOA and ammonia concentration ($r^2 = 0.79$; $p < 0.001$) which is indicative of the involvement of AOA in ammonia-N oxidation. Previous studies (Kasuga et al., 2010; van der Wielen et al., 2009) found similar correlations between AOA quantity and ammonia concentration. There was also a significant correlation between AOA abundance and combined nitrite-nitrate concentration ($r^2 = 0.84$; $p < 0.001$) confirming the participation of AOA in the ammonia-N oxidation the DS biofilm. There

were no significant correlations between level of chloramine concentration and AOA ($r^2 = 0.009$, $p = 0.50$) as well as AOB ($r^2 = 0.012$, $p = 0.22$) suggesting that AOA and AOB were not affected by the level of chloramine residual, similar to the results found for the bulk water in distal zones of the DS of Plant A.

5.3.4. CDC Incubation

5.3.4.1. Chlorine Incubation

For the biofilm development and activity prior to the incubation, AOA and AOB abundance and production nitrite-nitrate N concentrations were monitored (APPENDIX, Figure A8). Growth of AOA and AOB in the biofilm and nitrification were observed. Incubation experiments showed that increased chlorine concentration decreased both AOA and AOB abundance in the biofilm (Figure 15). Neither AOA and AOB nor the reduction in disinfectant was found in the control experiment (0.22 µm pore-size polycarbonate membrane filter sterilized effluent with added disinfectants). Scott et al. (2015) examined AOA and AOB in of a full-scale DS system and reported lower levels of both AOA and AOB at a sampling point that had higher free chlorine than other sampling points with less or no free chlorine. The current study observed more inhibition of AOB than AOA particularly at low temperature suggesting that AOA is able to survive better than AOB in cold chlorinated system. In previous studies, AOB was not detected in the DS with chlorine residual in winter months (Pintar and Slawson, 2003; Scott et al., 2015).

Results from this research showed that under typical chlorine residual concentrations (1.5-2.0 mg/L), it would take longer time to inactivate AOA than AOB. However, both AOA and AOB were inactivated quickly at 2.5 mg/L, meaning that if nitrification occurred, higher residual of chlorine would be required. Temperature is considered as a major factor influencing

nitrification, and results from this research showed that colder temperature may promote

inhibitory effect by chlorination more on AOB and less on AOA. Available research on this issue

is mostly focused on AOB (Maestre et al., 2013; Oldenburg et al., 2002; Wahman et al., 2016).

This research fills in the gap to understand the temperature effect on AOA under different

concentrations of chlorine residual in DS. In a full-scale survey of DS in the United States

(Wilczak et al., 1996), nitrification occurred in the majority of samples during periods when the

water temperature exceeded 15°C. This supports the results from this research that the growth of

AOA and AOB and nitrification occurred more preferably at higher temperatures.
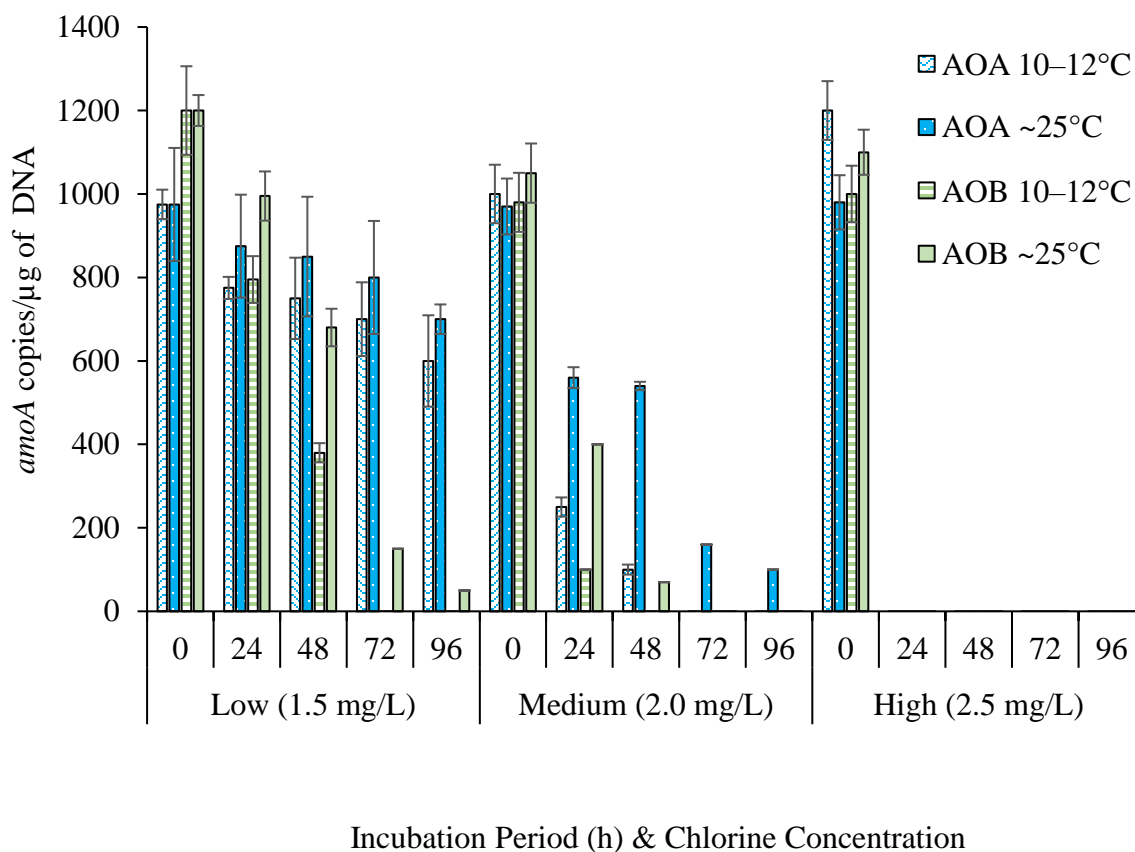
Figure 15. Abundance of AOA and AOB at different chlorine residuals (1.5; 2.0; and 2.5 mg/L) during incubation in a CDC reactor at 10–12°C and ~25°C.

5.3.4.2. Chloramine Incubation

Overall, the inhibitory effect of chloramine increased with its concentration (high > medium > low, Figure 16). The initial abundance of AOA was more than that of AOB in the biofilm. Both AOA and AOB experienced less inhibition at 25°C than 10-12°C, particularly at low residual chloramine concentration (0.05-0.1 mg/L). AOB was low or undetectable after 72 hours at 10-12°C for almost all residual chloramine concentrations agreeing with a previous bench scale drinking water distribution system study under comparable temperature and chloramine concentration (Pintar and Slawson, 2003). At the medium residual chloramine concentration (0.3-0.4 mg/L), AOA was detected after 48 hours only at 10-12°C while AOB was not detected in any incubated samples. A study by Wolfe et al. (1990) found that no AOB were detected in chloraminated reservoirs when the water temperature dropped below 18°C at a similar range of chloramine residual.

The above results showed that AOA and AOB differ in their inhibitory response to chlorine and chloramine. Kasuga et al. (2010) suggested that this observation could be due to metabolic differences between AOA and AOB. This study demonstrates that the maintaining chloramine residual for short time did not completely inhibit AOA and AOB. In general, AOA was less inhibited in the presence of low to medium concentrations of chloramine than AOB. This is because likely AOA was more tolerant to chloramine and/or was able to thrive on low levels of ammonia-N released from the decay of low to medium levels of chloramine better than AOB.
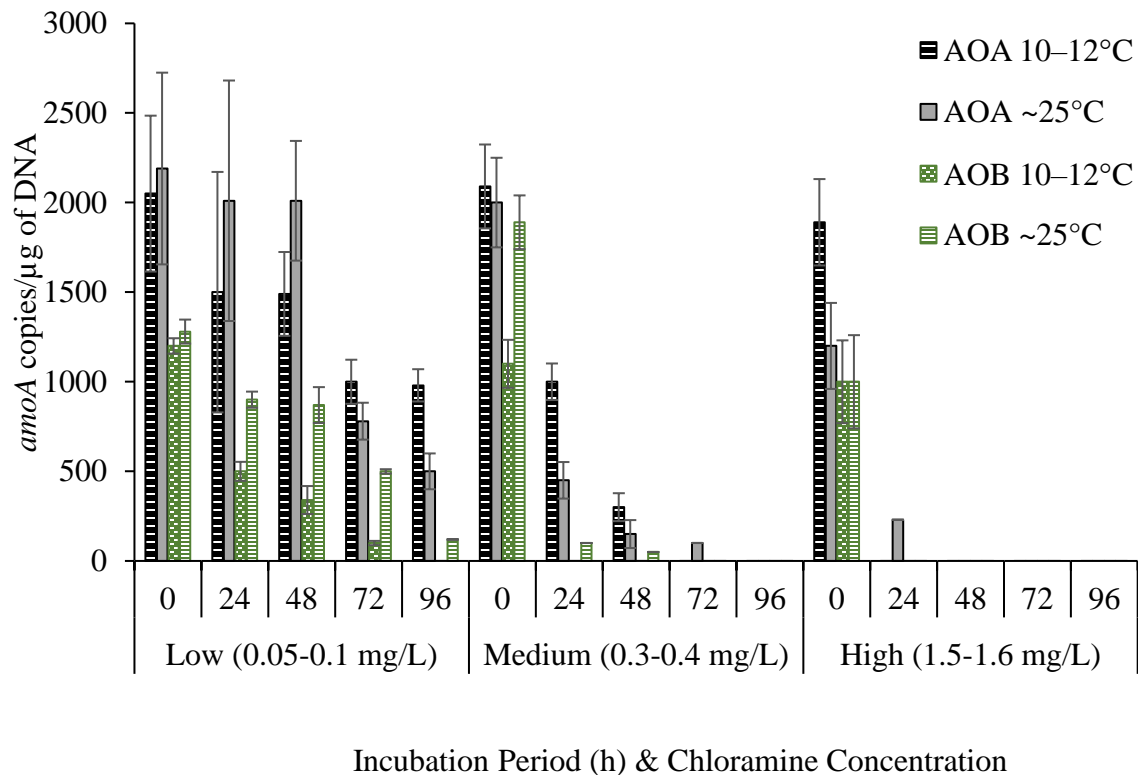
Figure 16. Abundance of AOA and AOB at different chloramine concentrations (low, medium, and high) during incubation in a CDC reactor at 10–12°C and ~25°C.

5.3.5. Phylogenetic Analysis

A phylogenetic tree was constructed to show the evolutionary relationships of *amoA* gene sequences from AOA in this and other studies (APPENDIX, Figure A9). The sequences from the present research clustered in the marine clade as *Nitrisopimulus maritima* and were most similar to SF_NB1_13 (Accession no. DQ148645), AOA-834-205-27 (Accession no. KC293237), MM-10 (Accession no. DQ278561), and SF_NB1_1 (Accession no. DQ148633), which were previously isolated from soil, marine environments, and wastewater treatment plants (Francis et al., 2005; Hong et al., 2014; Park et al., 2006; Zhang et al., 2009). This concurs with a previous study where sequences from marine (*N. maritima*) and soil environment (*Cenarchaeum*

symbiosum), were detected in the DS (van der Wielen et al., 2009). The sequences from this research were deposited in GenBank under the accession numbers MF598374-MF598400.

5.4. Summary

The abundance of AOA in the bulk water (raw and finished waters) was not affected by temperature variation for all three WTPs studied. However, AOA in the filter biofilm increased during warm months. More AOA were detected in the chloramination plant compared to the chlorination plants. Observed correlations between ammonia-N and AOA indicate likelihood of its involvement in nitrification in DSs practicing chloramination and chlorination. Based on the laboratory scale experiments in this study, at a low temperature (10-12°C), chloramine and chlorine provided similar inhibition trends; AOB was inhibited more than AOA. At a high temperature (~25°C), chloramine was less inhibitory to AOA and AOB than chlorine. AOA was inhibited less than AOB in the presence of low to medium concentrations of both chlorine and chloramine. High chlorine (2.5 mg/L) and chloramine (1.5-1.6 mg/L) could eliminate both AOA and AOB within short periods of time. However, increasing disinfectant residual in the DS may not be desirable by many utilities as it would increase DBP formations. Short-term switching between chloramination and chlorination may mitigate nitrification. This approach is recommended for future research. This research is the first to report how common disinfection practices affect AOA and AOB based on both full scale and laboratory efforts. Most importantly, it provides strategies for controlling AOA and AOB in water supply systems.

6. CONCLUSIONS AND FUTURE WORK RECOMMENDATIONS

6.1. Conclusions

In WWTPs studied, AOA were dominant in both NTF and MBBR. AOA were detected at two magnitudes higher than AOB in warmer months. Strong correlations between ambient temperature and AOA and AOP (AOA and AOB together) was observed. Laboratory-based nitrification activity using an AOA specific inhibitor, PTIO, showed that the oxidation of ammonia to nitrite decreased when the AOA populations from the NTF and MBBR were inhibited, demonstrating that AOA contributed to nitrification. This dissertation research has demonstrated that AOA outnumbered AOB and contributed to ammonia oxidation in nitrifying biofilm processes under ex-situ conditions for two full-scale WWTPs. Therefore, AOA is relevant when it comes to controlling nitrification process in biofilm based WWTPs.

Copper inhibition on nitrification based on sOUR, sAOR and *amoA* showed that the cultures with higher fractions of AOA are more tolerant to copper and therefore more beneficial to WWTPs. Municipal WWTPs usually receive wastewater with less than 2 mg/L of copper but concentrations up to 15 mg/L, which is shown in this dissertation research to inhibit nitrification, are not uncommon. Reduction in overall *amoA* of attached growth cultures was less than suspended growth cultures corresponds to a greater copper effect on AOA and AOB in suspended growth cultures than attached growth cultures.

AOA and AOB were detected through the treatment trains and distribution systems of three different WTPs, one with surface water plus groundwater as a raw water source and chloramination as a disinfection process, and other two with groundwater as source water and chlorination practice. The abundance of AOA and AOB differed between the bulk water and biofilm phases. AOA in biofilm increased in quantity during warm months and decreased in cold

months. However, AOA in the bulk water did not show any significant effect from temperature variation for both chloraminated and chlorinated DSs. Observed correlations between ammonia-N and AOA indicate likelihood of its involvement in nitrification. The DS practicing chloramination showed more AOA than AOB than the chlorinated DS. At a low temperature (10-12 ℃), chloramine and chlorine provided similar inhibition trends; AOB was inhibited more than AOA. At a high temperature (25 ℃), chloramine was less inhibitory to AOA and AOB than chlorine. AOA was inhibited less than AOB in the presence of low to medium concentrations of both chlorine and chloramine. Detection of more AOA than AOB in the presence of chloramine implies that low concentrations of ammonia resulting from chloramine degradation select for AOA rather than AOB.

6.2. Future Work Recommendations

This research provides a method to obtain cultures with different fractions of AOA from WWTP nitrifying cultures. The unique approach to develop different fractions of AOA cultures opens up an enormous research scope to unveil the nitrification efficiency and kinetics of AOA in the presence of environmental contaminants. Nitrifying cultures with higher fractions of AOA (>80%) can be used for bioaugmentation of WWTPs that frequently encounter heavy metal shock loads. This idea is worth a future study. AOA are adaptable to lower ammonia-N concentrations released by chloramine in DSs while chlorine is more inhibitive on both AOA and AOB but provides less residual effect. A possible scheme for effective control of AOA and AOB in chloraminated DS is an intermittent chlorine application. This scheme should be investigated particularly on dynamics of AOA and AOB (population and activity) in chloramination systems in response to different doses and frequencies of intermittent chlorine application.

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APPENDIX

Table A1. NH$_3$-N, pH, soluble chemical oxygen demand (SCOD), and DO of NTF influent of the Fargo WWTP and of nitrification basin wastewater of the Moorhead WWTP.

	NH3-N (mg N/L)	pH	SCOD (mg/L)	DO (mg/L)
Fargo WWTP (NTF influent)	0.3–7.2	5.1–7.7	N/A	2–5
Moorhead WWTP (nitrification basin wastewater)	11.7–2.7	5.7–7.8	758–920	5

Table A2. Water quality data for the source water samples collected from three different WTPs.

Parameter	Plant A	Plant B	Plant C
Water source	Blended Water	Groundwater	Groundwater
pH	7.51 ± 0.17	7.47 ± 0.18	7.45 ± 0.16
DOC (mg/L)	2.62 ± 0.83	3.57 ± 1.28	2.89 ± 0.34
Conductivity (μS/cm)	317 ± 32	701 ± 57	568 ± 45
Alkalinity (mg/L as CaCO3)	80.2	223	178
Ammonia-N (mg-N/L)	1.37 ± 0.11 (surface water) 2.91 ± 0.16 (groundwater)	2.09 ± 0.16	3.08 ± 0.05
Nitrate (mg-N/L)	0.46 ± 0.12	1.98 ± 0.35	1.39 ± 0.11
Nitrite (mg-N/L)	0.001 ± 0.002	0.004 ±0.001	0.009 ± 0.001
DS (Proximal Zone) monochloramine or chlorine	1.8 ± 0.02	2.0 ± 0.04	1.9 ± 0.11
DS (Distal zones combined) monochloramine or chlorine	1.6 ± 0.03	1.8 ± 0.02	1.3 ± 0.02

Values listed are means ± standard deviations. Alkalinity data was provided by the treatment plants

Table A3. Correlations between ammonia-N concentration and the log number of AOA, AOB and AOM in DS of Plants A, B, and C (considered significant at $p < 0.01$).

Plant A

Sampling location	DS1 (Proximal)			DS2 (Distal)			DS3 (Distal)		
	na	na	na	AOM	AOA	AOB	AOM	AOA	AOB
p value	na	na	na	<0.01	<0.01	0.02	<0.01	<0.01	<0.01
r^2 value	na	na	na	0.69	0.83	0.31	0.83	0.78	0.81

Plant B

Sampling location	DS1 (Proximal)			DS2 (Distal)					
	AOM	AOA	AOB	AOM	AOA	AOB	na	na	na
p value	0.03	0.04	0.03	<0.01	<0.01	0.03	na	na	na
r^2 value	0.13	0.38	0.23	0.83	0.78	0.23	na	na	na

Plant C

Sampling location	DS1 (Proximal)			DS2 (Distal)			DS3 (Distal)		
	AOM	AOA	AOB	AOM	AOA	AOB	AOM	AOA	AOB
p value	0.04	0.03	0.03	<0.01	<0.01	0.04	<0.01	<0.01	0.03
r^2 value	0.48	0.43	0.23	0.78	0.73	0.43	0.83	0.78	0.23

na- not applicable

Table A4. Quality data of water in distribution system pipelines associated with biofilm sampling during water main breaks for Plant A.

Parameter	Sampling date in 2015				
	Feb 18	Mar 13	Mar 25	Apr 9	May 15
Temp (°C)	12	13	12	15	18
pH	7.5	7.58	7.64	7.30	7.12
Nitrate-N (mg/L)	0.08	0.12	0.10	0.09	0.40
Nitrite-N (mg/L)*	N/A	N/A	N/A	N/A	N/A
Free ammonia-N (mg/L)	0.14	0.35	0.41	0.32	0.42
Monochloramine (mg/L)	1.7	1.6	1.8	1.6	1.9
Conductivity (μmhos/cm)	549	486	470	523	495

N/A- Below detection limit; *Detection limit = 0.02 mg/L.
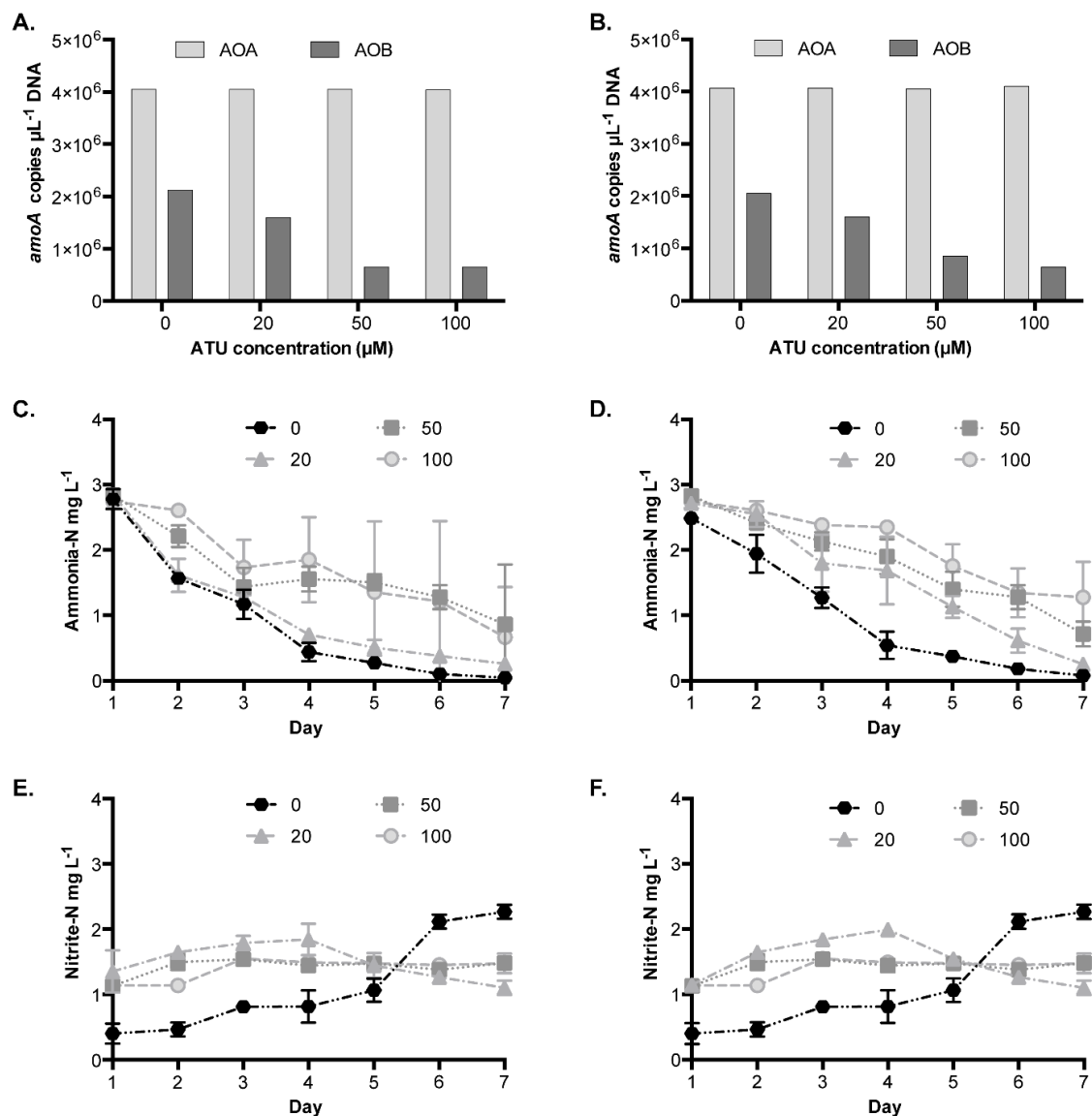
Figure A1. AOA and AOB *amoA* copy number/μL at different concentrations of the AOB inhibitor, ATU, in batch experiments with samples from a) NTF and b) MBBR; ammonia concentration in batch experiments with samples from c) NTF and d) MBBR; nitrite-N production in batch experiments with samples from e) NTF and f) MBBR; Day 1 is the first day of sample collection after nitrite generation in the batch experiment. Data are shown as means ± SD (n = 3).
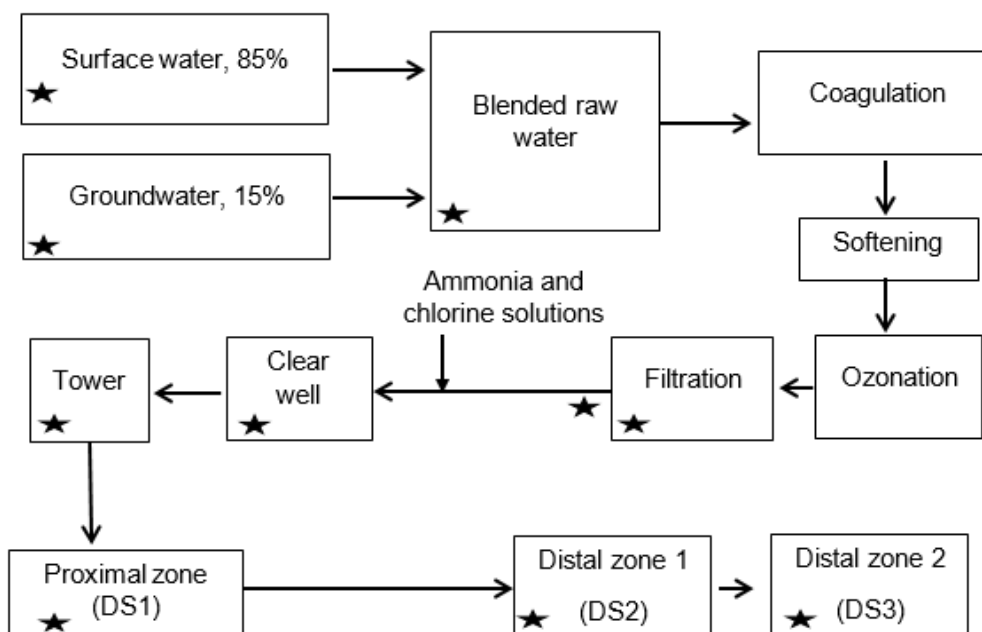
Figure A2. A simplified flow diagram and sampling locations (marked with stars) of Plant A.
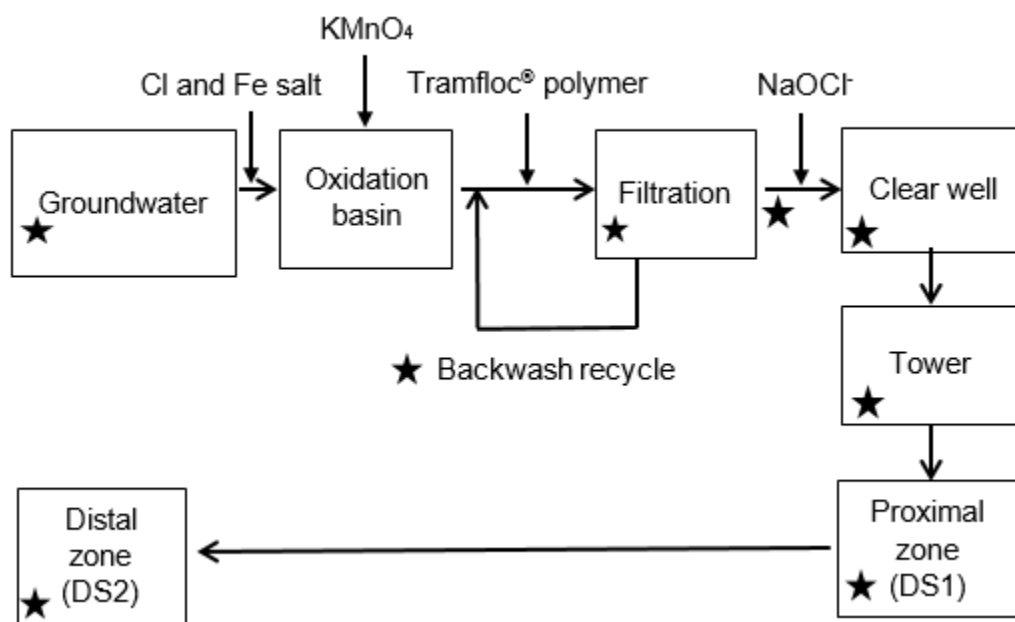
Figure A3. A simplified flow diagram and sampling locations (marked with stars) for Plant B.
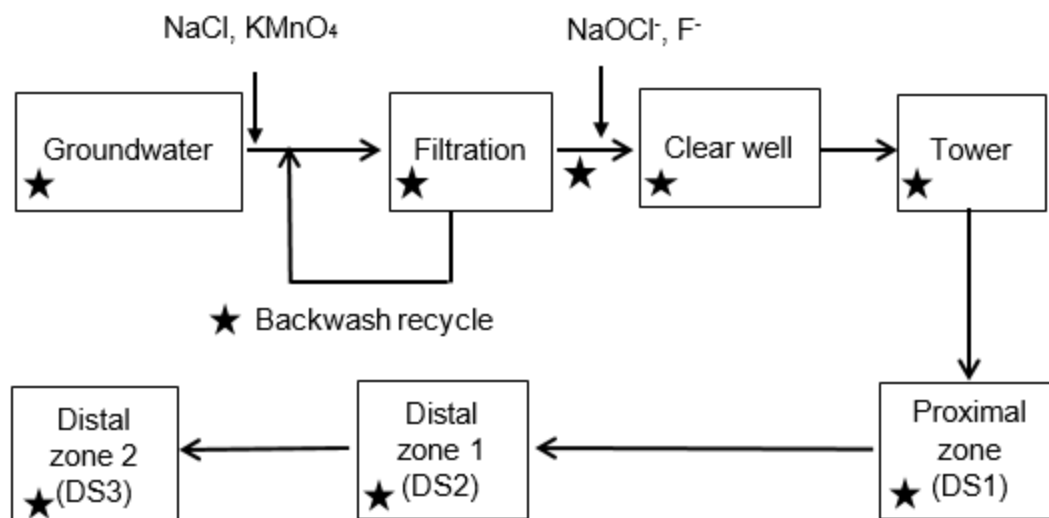
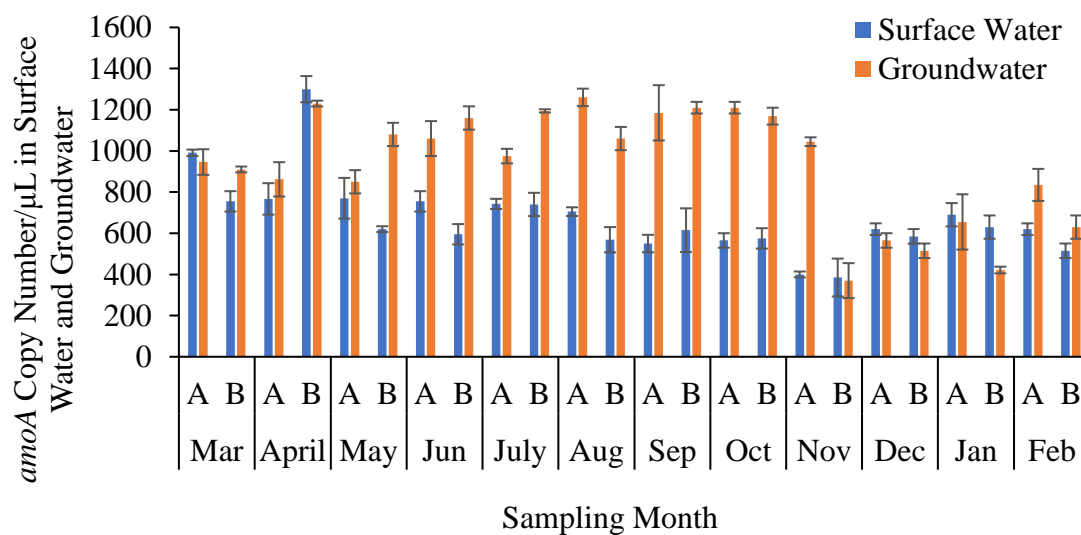Figure A4. A flow diagram and sampling locations (marked with stars) for Plant C.

Figure A5. Abundance of *amoA* of AOA (A) and AOB (B) in source water samples collected from Plant A from March 2014 to February 2015.
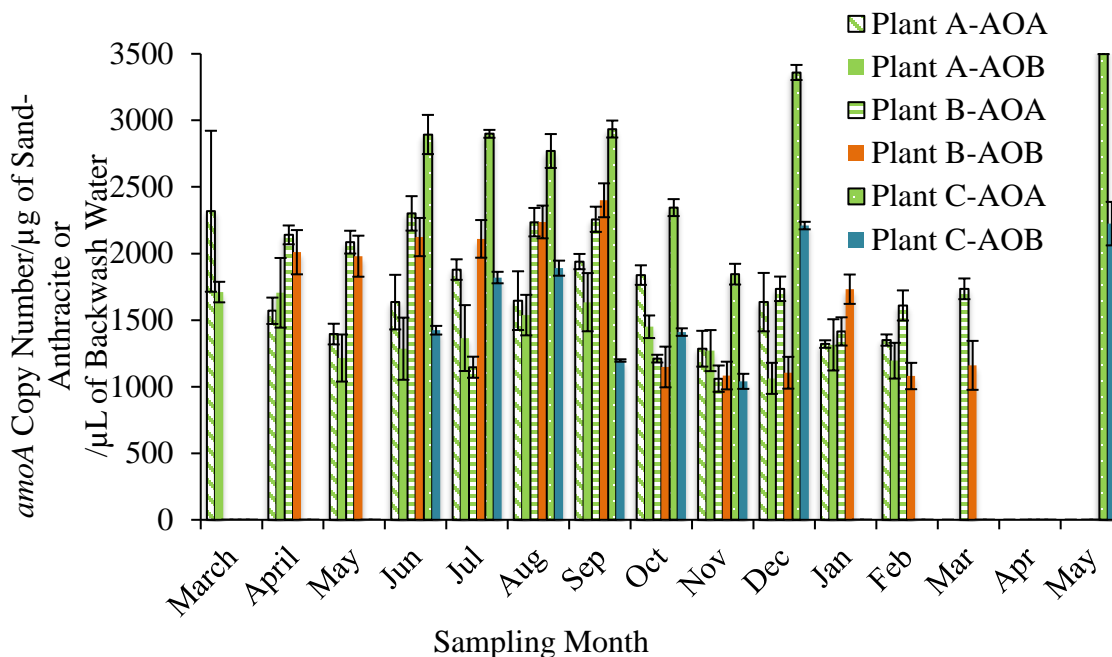
Figure A6. Abundance of *amoA* of AOA and AOB in sand/anthracite media biofilm samples from Plant A (March 2014-February 2015), Plant B (April 2014-March 2015), and in backwash water samples from Plant C (June 2014-May 2015 except January to April 2015 when sampling were not conducted due to plant maintenance).
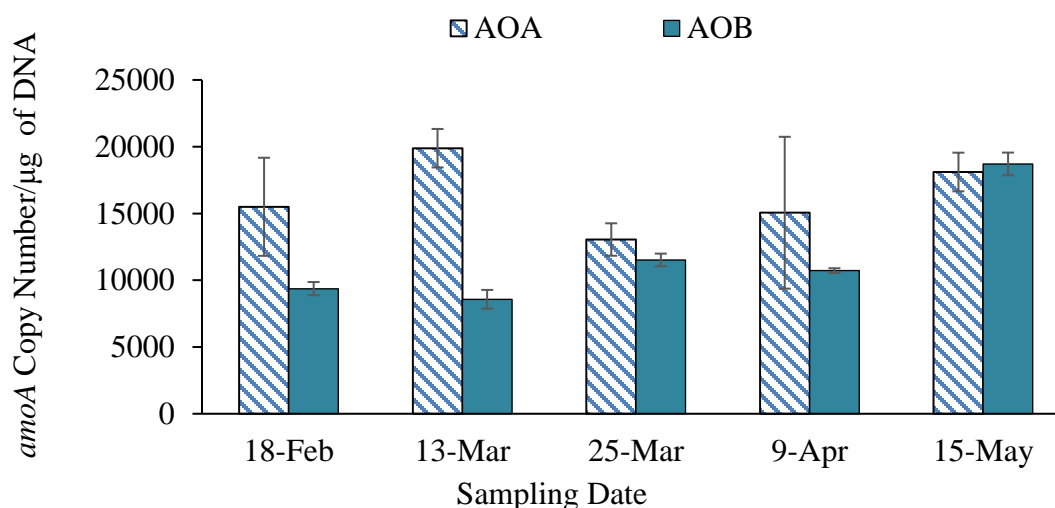

Figure A7. Abundance of *amoA* for AOA and *AOB* in biofilm in pipelines of Plant A (from water main break events). Samples were collected from February to May 2015.
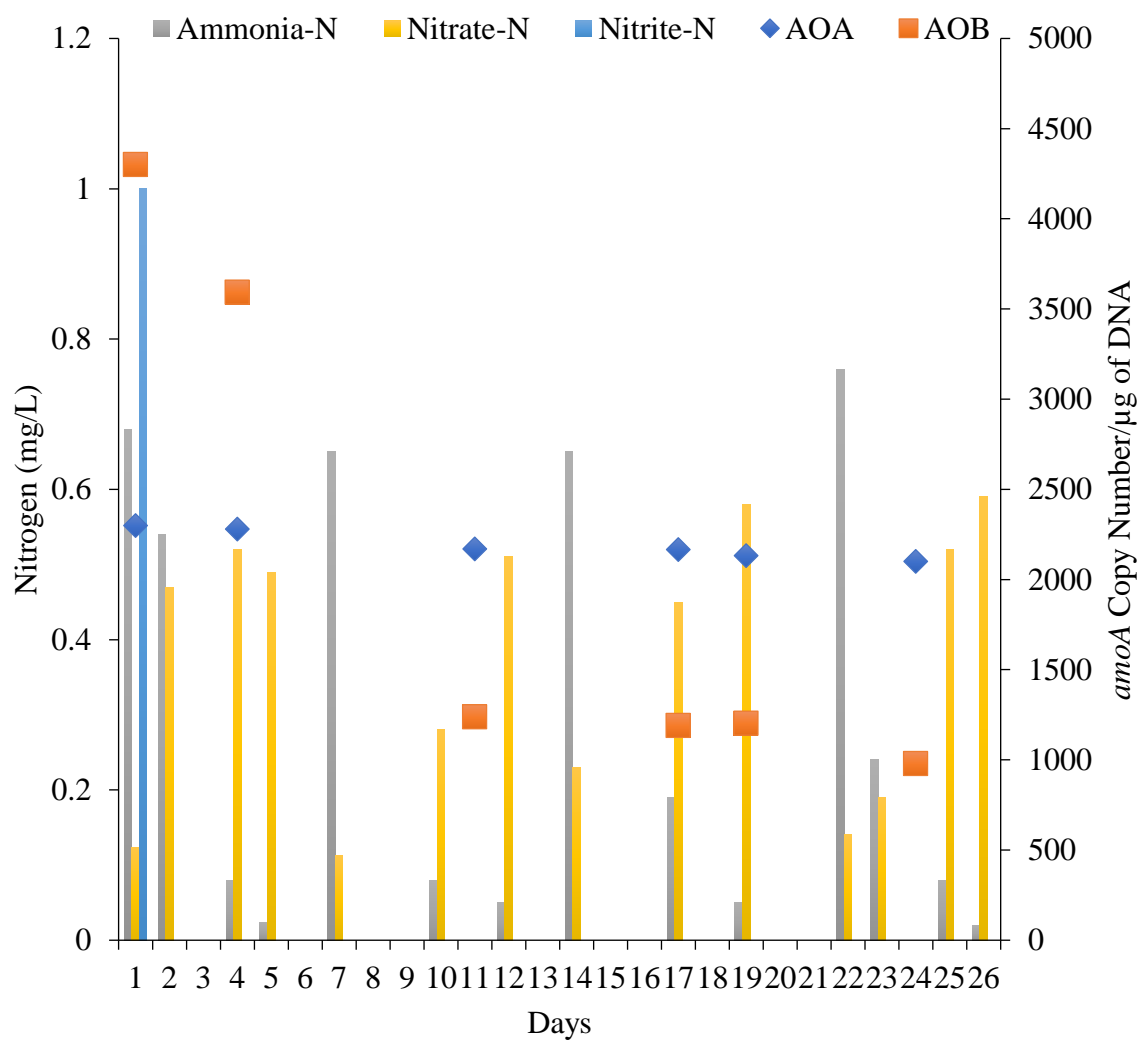
Figure A8. Ammonia-N, nitrite-N and nitrate-N concentrations and abundance of AOA and AOB in water phase in CDC reactor during initial biofilm development period.
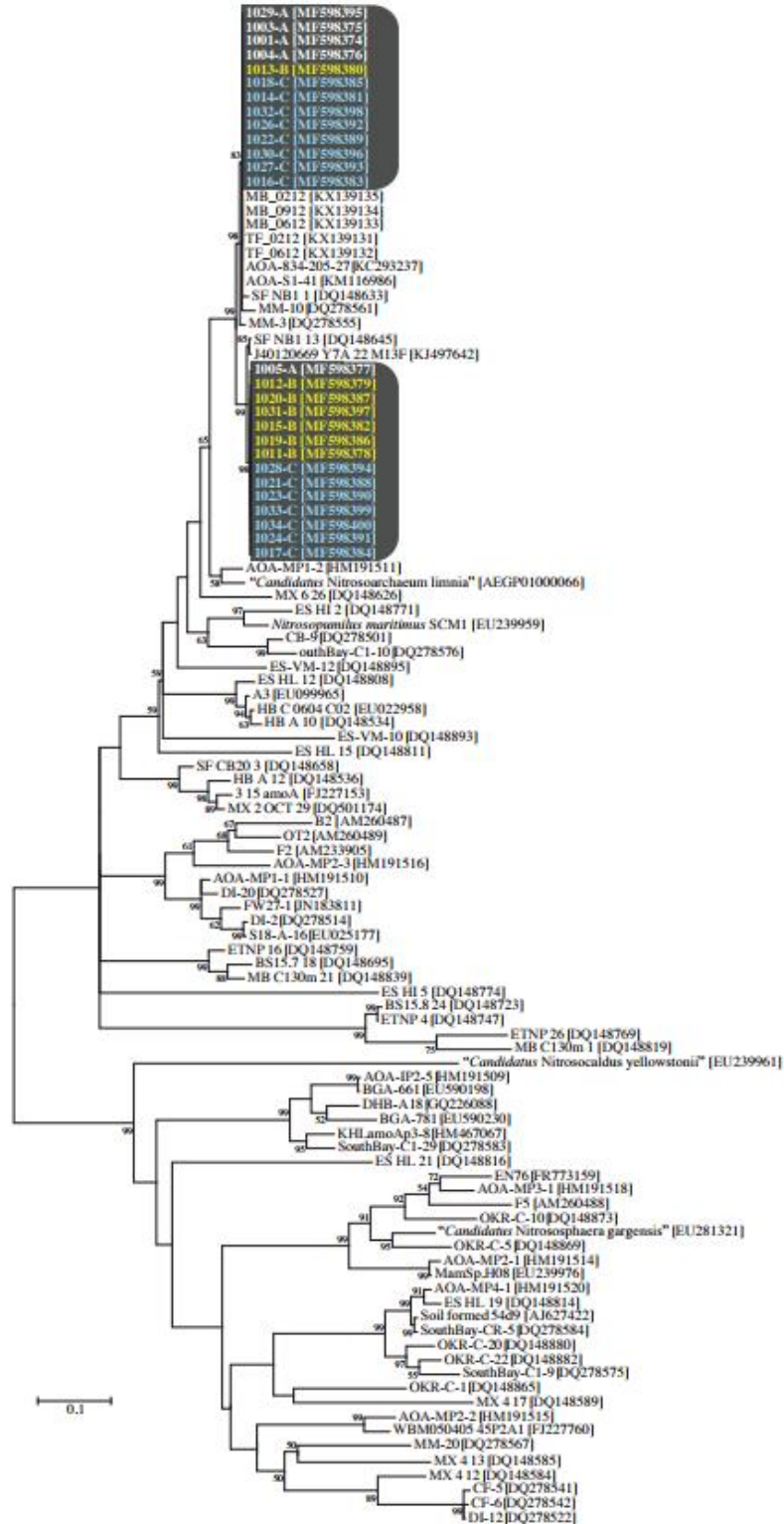
Figure A9. Molecular Phylogenetic analysis of *amoA* sequences by the Maximum Likelihood method