

MICROBIAL ECOLOGY OF NITRIFYING SIMULATED PREMISES PLUMBING

by

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DEDICATION

To my grandparents whose passion in teaching inspired my pursuit of learning.

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ABSTRACT

Because of the Stage 2 Disinfectants and Disinfection Rule limiting the concentration of disinfection by products in drinking water, the use of chloramine as an alternative to chlorine has been increasing. However, the ammonia introduced by chloramination can lead to nitrification which results in the production of nitrite and nitrate, leading to regulatory violations.

Nitrification in reactors with copper and polyvinyl chloride (PVC) surfaces was established by indigenous organisms from Bozeman tap water and has been stably maintained for more than 6 years.

Statistical analyses of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiles determined that the active bacterial populations were different in the two systems. The assemblage of the organisms was also different from the starting population (BAC influent) suggesting both material and ammonia/carbon source affect the population. No known ammonia oxidizing bacteria were detected suggesting the role of different group for ammonia oxidation.

Fluorescence *in situ* hybridization (FISH) detected archaea in the biofilm from both reactors. Archaeal 16S rRNA gene sequences were found to be phylogenetically affiliated with known archaeal ammonia oxidizers. Two archaeal *amoA* sequences were amplified from the system as determined by DGGE. We propose to provisionally classify a detected archaeon as *Candidatus Nitrosotenuis bozemanii*, based on its affinity to *Nitrosotenuis uzonensis* (Hatzenpichler et al., in preparation). Bacterial abundances were comparable in the two systems but archaeal abundances were higher in the PVC reactor suggesting material effect on the overall microbial population composition and density.

Enrichment in modified synthetic Crenarchaeota medium yielded a culture of archaea and bacteria that consistently oxidizes ammonia to nitrate. Attempts to isolate the archaeal component using antibiotics failed, suggesting the disruption of a possible beneficial relationship between the archaea and bacteria.

Genes involved in the transformation of nitrogen within the system were also investigated and *hao* distantly related to that of ammonia oxidizing bacteria was detected but its potential role remains unknown.

This study provides evidence of archaea associated with biofilms in drinking water and while further analysis is needed to definitively elucidate their role, results of this study prompts the reevaluation of the current concept of nitrification in drinking water.

CHAPTER 1

INTRODUCTION

Drinking Water Distribution Systems

Drinking water distribution systems carry water from a centralized treatment plant or well supplies to consumers' taps and are designed and operated to provide water of a quality acceptable for human consumption (NRC, 2006). Most regulatory mandates regarding drinking water focus on enforcing water quality standards at the treatment plant and not within the distribution system. However, water released into the distribution system becomes altered as it passes through pipes, open reservoirs, standpipes, and storage tanks and the issue of substantial changes to water quality remains to be addressed (NRC, 2006; Geldreich, 1996).

Premise or premises plumbing is defined as the portion of the distribution system associated with schools, hospitals, public and private housing, and other buildings connected via the service line to the main distribution system (NRC, 2006). Almost all problems in water mains can also occur in premises plumbing and conditions within it are favorable for microbial growth. Even so, no particular agency or utility is responsible for premises plumbing water quality except for what is provided for in the EPA regulation on lead and copper (Lead and Copper Rule) (NRC, 2006) and as such maintenance of water quality within the premises plumbing deserves critical evaluation.

Microorganisms in Drinking Water

The water treatment process is aimed at eliminating or killing bacteria, but public water supply has never been intended to be a sterile product (Brettar and Höfle, 2008; Geldreich, 1996). Microorganisms not affected by or introduced after treatment find their way to the water supply and as a consequence of their presence and or activity, cause a number of issues in drinking water distribution systems (DWDS) such as biofilm growth, nitrification, microbially-mediated corrosion and pathogen persistence (Berry et al., 2006). The issue of pathogen persistence is one of the most discussed and studied topic but along with this, the persistence and activity of “nuisance” organisms is also an important issue. These organisms cause adverse effects on taste, odor and color, ultimately causing poor water quality.

Along with survival, it is also important to investigate the ability of the organisms to increase and the factors that contribute to this within the system to determine potential control mechanisms. In chloraminated systems, ammonia oxidizing organisms increase from the proximal to the distal parts of the distribution system due to an increase in ammonia (Lipponen et al., 2002; Lipponen et al., 2004). On the other hand, in a system without a disinfectant residual, there was no significant difference in the number of ammonia oxidizing bacteria in the proximal and distal areas of the distribution system, while ammonia oxidizing archaea were found to be present in greater number in the distal portion compared to the proximal portion of the distribution system (van der Wielen et al., 2009).

Water Distribution System Pipe Material Type and Microbial Growth and Activity

Several types of pipe materials are used in drinking water distribution systems including metals (copper, cast iron, lead, brass, steel), plastics (PVC, PE, PB, latex), asbestos cement and concrete (Keinänen-Toivola, 2006). Pipe materials for service lines and building pipe networks (premises plumbing) are most often copper or plastic with the occasional black and galvanized iron (Geldreich, 1996). Numerous studies demonstrate that pipe material affects both microbial number and community structure in the system with effects ranging from stimulatory to inhibitory. Materials may provide a protective niche where growth can occur and some can provide nutrients to stimulate growth by releasing bioavailable forms of iron and phosphorus (Berry et al., 2006; Morton et al., 2005; Lehtola et al., 2004). On the other hand, some compounds released from pipe materials such as copper, can be toxic or inhibitory to microorganisms (Lehtola et al., 2004; van der Kooij et al., 2005). Pipe material can also modify the effectiveness of disinfectants (Lehtola et al., 2005). Physical characteristics of the material such as roughness of pipe surface have been found to influence density of biofilm growth (Pederson, 1990; Niquette et al., 2000; Lehtola et al., 2005). The interaction of pipe material with other factors affecting bacterial growth adds another level of complexity to the effect of material on bacterial regrowth. Camper et al. (2003) studied the interactions between distribution system materials, organics and disinfectants and their effect on bacterial regrowth and found that the extent of the effect of increased levels of carbon on bacterial regrowth depend on material providing more evidence in the implications of material choice in distribution systems.

Although specific groups of organisms are recognized to be important in water distribution system (i.e. pathogens and nitrifying organisms), knowledge on the whole community is important because the other organisms in the community affect the persistence and growth of these specific groups. With recent developments in molecular techniques to assess community composition, a few community-focused studies have demonstrated differences in microbial community composition brought about by pipe material differences. The microbial community on chlorinated polyvinyl chloride (c-PVC) was found to be the least diverse among several materials tested which included copper, polybutylene, polyethylene stainless steel and stainless steel coated with zinc (Yu et al., 2010), and c-PVC had the lowest biofilm formation potential. In another study, the microbial community on a stainless steel coupon was found to be least diverse compared to steel, copper and PVC (Jang et al., 2011). Stainless steel was also found to be superior based on its lower corrosion level and lower biofilm formation potential. Pipe material type can also be expected to strongly influence the occurrence of nitrification (Zhang et al., 2009). Because the copper which leaches from copper pipes is known to be toxic to bacteria (Trevors and Cotter, 1990) it is generally believed that nitrification does not occur in copper plumbing. However, the copper concentration that would inhibit nitrification varies based on nitrifier strain as well as growth conditions (Zhang et al., 2009; Rahman et al., 2011).

Microorganisms in Distribution Systems: Biofilm vs. Planktonic

Biofilms 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. Consequently, studies on the microbial ecology of drinking water distribution systems have mainly focused on biofilms. However, to be able to implement effective control strategies that will ensure safe and high quality drinking water, it is necessary to understand the microbial ecology of the whole distribution system (Berry et al., 2006). Large metabolic differences between attached and free-living bacteria can occur (Boe-Hansen et al., 2002). In low nutrient conditions, bulk phase growth accounted for a significant part of the total aftergrowth in the drinking systems (Boe-Hansen et al., 2002). Moreover, bulk water bacteria have been shown to dominate in portions of a distribution system that have low chlorine residual (Srinivasan et al., 2008) thus presenting a need to study both bulk phase and biofilm.

Approaches in the Study of Microbial Ecology

Although microorganisms, their interactions and their activities are important with regards to water quality, there is limited published information about the microbial ecology of distribution systems. Water distribution ecology as a whole is poorly understood and in addition, the relationship of the ecology of the main distribution system to that in premises plumbing is unknown (NRC, 2006). Historically, as with other environments, ecological studies in drinking water have mostly relied on culturing of microorganisms. However, because of inherent limitations of the culturing approach, disparity between culturable and *in situ* diversity exists (Nocker et al., 2007). As newer

methodological tools are developed for studying microbial diversity, it has been possible to analyze microbial communities independent of cultivation. Cellular biomarkers such as cell wall constituents, proteins, lipids, DNA and RNA allow for the monitoring of microbial communities without the need for culturing (Tzeneva et al., 2008). Identification of specific organisms and determining their distribution in a system without cultivation is possible with the use of fluorescence *in situ* hybridization (FISH). This technique involves the use of oligonucleotide probes labeled with a fluorescent dye that usually target the ribosomal RNA (Wagner et al., 2003). Although this method can be used to quantify organisms, it can be limited to the probes that are available and quantification may be complicated for samples wherein cells are in clusters/layers. Abundance can be determined by quantitative polymerase chain reaction (qPCR). qPCR is a method that combines the detection of a target template with quantification. This method uses PCR primers targeting a gene, as well a DNA dye and quantification is accomplished by measuring the fluorescent signal associated with product formation during each cycle in the PCR (Smith and Osborn, 2008).

Genetic fingerprinting approaches are among the most widely used methods in biodiversity studies in aquatic microbial communities (Dorigo et al., 2005). Using these techniques, a pattern or profile of the community diversity is obtained based on the physical separation of nucleic acid species (Muyzer, 1999). Denaturing Gradient Gel Electrophoresis (DGGE) is an example of a genetic fingerprinting technique. This technique involves the amplification of a target gene from the community (usually the 16S rRNA gene) which results in a mixture of amplicons coming from the different

members of the community. Separation of the DNA fragments follows based on their sequence-specific melting point in a polyacrylamide gel with a gradient of denaturing chemicals; fragments with different sequences will stop migration at different positions in the gel (Muyzer and Smalla, 1998). A number of studies on drinking water have successfully utilized this approach including studies on effect of pipe material on biofilm formation (Yu et al., 2010), effect of disinfectants on community (Roeder et al., 2010), and detection of pathogens and toxigenic organisms (Felföldi et al., 2010; Yen et al., 2012). Nocker et al (2007) mentions some of the advantages to this method as to its affordability, relative ease in interpreting results, as well as having the option to be able to identify phylotypes via sequencing of excised bands. In addition to this, statistical techniques can be applied in the validation of the interpretation of DGGE data as well as DGGE pattern correlation analysis against environmental variables (Fromin et al., 2002).

DNA-based methods such as PCR-DGGE allow for the study of microbial communities including yet to be cultured microbes but do not by themselves allow for the simultaneous distinction between the active and non active members of the community. One method to allow for the live/dead distinction of community members is the use of propidium monoazide (PMA). PMA is a membrane impermeable dye that intercalates DNA so that it cannot be amplified by PCR (Nocker et al., 2007). The basis for the distinction is the cell membrane integrity, wherein cells with intact cell membranes are considered “active” while those that have compromised cell membranes are considered “inactive”.

Nitrification

Being a major component of nucleic acids and proteins, nitrogen is an essential element of life. It is present in various oxidation states in the environment in a variety of chemical compounds (Martinez-Espinosa et al., 2011). Nitrification is a microbial process by which by which nitrogen compounds (primarily ammonia) are oxidized to nitrite and nitrate (USEPA, 2002). It is an essential link in the biogeochemical cycling of nitrogen in natural, industrial and agricultural systems (Ward et al., 2011). Nitrification also occurs in drinking water and has been described as early as 1935 (Faben, 1935). The nitrite and nitrate produced can have adverse health effects on humans including methemoglobinemia in infants and perhaps cancer (Fan and Steinberg, 1996, Aschebrook-Kilfoy et al., 2011). The USEPA considers nitrite and nitrate as drinking water contaminants and sets the maximum contaminant level (MCL) of 1 ppm for nitrite and 10 ppm for nitrate (USEPA, 2002). In addition, there are other changes in water quality parameters associated with nitrification such as decrease in chloramines residual, increase in heterotrophic plate count (HPC) and reduction in pH and alkalinity that can affect Lead and Copper Rule (LCR) compliance.

Recently, the formation of disinfection byproducts (DBPs) which arises when bromide or organics react with chlorine has been an important issue in drinking water because of the possible negative effects of DBPs on humans. The USEPA has promulgated the Stage2 Disinfectants/Disinfection By-Product Rule (USEPA, 2000) which limits the levels of DBPs in drinking water. As a result, the use of chloramine has been increasing as utilities use this alternative to chlorination to comply with the Stage 2

Disinfectant/Disinfection By-Product Rule. During chloramine formation and/or decay, ammonia is formed which can serve as the energy source for endogenous nitrifying organisms (Zhang et al., 2009).

Nitrifying Organisms

In the presence of oxygen, NH_4^+ is sequentially oxidized to NO_3^- ; organisms with the enzyme ammonia monooxygenase oxidize NH_4^+ to hydroxylamine, which is subsequently oxidized to NO_2^- by the enzyme hydroxylamine oxidoreductase and finally NO_2^- to NO_3^- by nitrite oxidoreductase (Canfield et al., 2010). In the traditional view, the process of nitrification was attributed to two groups of organisms: the ammonia oxidizing bacteria and the nitrite oxidizing bacteria and for over a century since their characterization, bacterial nitrifiers were assumed to be the only microbes capable of autotrophic nitrification (Ward, 2011).

Nitrifying bacteria are found in various environments including marine, estuarine and freshwater, soils and wastewater and other engineered systems (Norton, 2011). Ammonia oxidizing bacteria are affiliated with the Betaproteobacteria and Gammaproteobacteria while nitrite oxidizing bacteria are affiliated with the Alpha-, Delta and Gammaproteobacteria (Starkenburg et al., 2006). Because they were assumed to be the only microbes capable of autotrophic nitrification, cultivated nitrifying bacteria have been the basis for investigations into the physiology and biochemistry of nitrification for decades (Ward, 2011).

Recent developments in the molecular ecological tools enabled the discovery of a different group of ammonia oxidizing organisms that led to a major shift in the

understanding of nitrification. A homologue of the bacterial *amoA* (the gene that encodes for a subunit of the ammonia monooxygenase) was discovered in archaeal scaffolds of metagenomic libraries from the ocean, soil and other environments (Ward, 2011; Venter et al., 2004; Treusch et al., 2005; Francis et al., 2005). In some environments, ammonia oxidizing archaea have been found in greater abundance as compared to their bacterial counterpart. As such, it has been speculated that this group may often be more significant contributors to nitrification than the bacteria (Leininger et al., 2006; Prosser and Nicol, 2008; Urakawa et al., 2011).

The metabolism of ammonia oxidizing archaea is still to be completely elucidated. Like their bacterial counterparts, they possess the gene for ammonia monooxygenase that takes ammonia to hydroxylamine, however, they do not possess a hydroxylamine oxidoreductase which is the subsequent step taking hydroxylamine to nitrite. It is speculated that the archaea have another enzyme or may utilize an alternative pathway to produce nitrite (Urakawa et al., 2011). Details about the physiology, metabolic and biogeochemical functions, evolutionary history, and ecological niche partitioning of ammonia oxidizing archaea still remain to be determined (Blainey et al., 2011).

Data from both the cultivated and non-cultivated archaea have resulted in a better understanding of this group. From the analysis of the 16S rRNA genes from marine and terrestrial habitats, the phylogenetic affiliation of ammonia oxidizing archaea puts them to a new phylum Thaumarchaeota, instead of their previous phylum Crenarchaeota (Nicol et al., 2011). Cultivation into pure cultures of these organisms is a step towards

understanding their contribution to the nitrification process. To date, two mesophilic archaeal ammonia oxidizers have been isolated in pure culture: one from a tropical marine aquarium (Könneke et al., 2005) and from soil (Tourna et al., 2011). In addition, enrichments with ammonia oxidizing archaea have been obtained both from mesophilic and extreme environments.

Scope and Objectives of this Research

This project is focused on the characterization of the microbial population of nitrifying simulated premises plumbing. Modified CDC reactors with copper and PVC surfaces were constructed to simulate the surfaces on pipes with two of the most commonly used plumbing material in household plumbing. Operation conditions included periods of stagnation of flushing that are encountered in premises plumbing. Ammonia in the system simulates the amount of ammonia that would result from the decay of chloramines, if chloramination is used for secondary disinfection. The system has been nitrifying for more than 6 years as indicated by both ammonia loss and nitrate accumulation (Rahman et al., 2011).

The specific objectives of this project were to:

- Characterize and compare the bacterial populations in the copper, PVC and BAC (influent water) feed by PCR-DGGE and sequencing
- Compare replicate reactors and determine changes in the bacterial populations through two time points

- Determine difference of active and total bacterial populations by analyzing and comparing PMA and non PMA treated samples
- Characterize and quantify the archaeal ammonia oxidizers in the system by FISH, endpoint PCR, DGGE and quantitative PCR (qPCR)
- Enrich for ammonia oxidizing organisms in the system

Dissertation Structure

Chapter 2 and 3 will be submitted as two separate peer reviewed manuscripts. Chapter 4 is a separate discussion on the nitrogen cycle in the system and is not considered for independent publication. Chapter 5 provides the overall conclusions and recommendations. The paper by Rahman et al (2011) is presented in Appendix A as a background of the study.

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CHAPTER 2

BACTERIAL COMMUNITIES IN NITRIFYING
SIMULATED PREMISES PLUMBING

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Abstract

Nitrification was established using indigenous organisms from Bozeman tap water in modified CDC reactors containing copper and polyvinyl chloride (PVC) coupons to simulate pipe material surfaces. Reactors were operated to simulate periods of stagnation and have been actively nitrifying for more than 6 years. Previously, the copper and PVC systems were found to handle perturbations from the nitrification inhibitor chlorite differently as the PVC system was able to recover to its nitrifying state even before chlorite treatment was stopped whereas the copper system recovered approximately two months after chlorite treatment was stopped (Rahman et al., 2011). This observation suggested differences in the microbial communities in the two reactor types. The overall goal of this study was to determine a link between bacterial community composition and functional characteristics of the nitrifying systems.

The bacterial community in the system was analyzed using a molecular fingerprinting technique, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) targeting the 16S rRNA gene, in combination with a propidium monoazide (PMA) treatment that removes the DNA from cells with compromised cell membranes, in order to target the active population. Richness was not significantly different between the two types of materials or the influent water suggesting that the different materials can support the same number of types or ‘species’. Multivariate analysis of variance (MANOVA) and ordination analyses, principal coordinate analysis (PCO), and nonmetric multidimensional scaling method (NMDS) of DGGE profiles showed a significant effect of material on community structure. Reactor

bulk water samples formed a distinct cluster indicating that the community was unique from that of the biofilm and was not a mere result of biofilm detachment events. Bacterial 16S rRNA gene abundances showed that biofilm and bulk water from the copper and PVC reactors had comparable bacterial biomass. Results suggest that bacterial community composition was influenced by plumbing material, and that the population composition rather than density affected the function and recovery of the system from environmental perturbations. Shifts in the community structures including those of the biofilm were observed, suggesting a dynamic community composition even though nitrification was stable.

No known bacterial ammonia oxidizer was found by molecular methods in either the copper or PVC reactors. End point PCR with archaeal *amoA* primers detected archaea in the PVC reactor but not in the copper reactor.

Introduction

Many issues in drinking water distribution systems (DWDS) such as biofilm growth, nitrification, microbially-mediated corrosion and pathogen persistence are microbial in nature (Berry et al., 2006). Even with water treatment processes aimed at eliminating or killing bacteria, drinking water still contains a diverse microflora (Brettar and Höfle, 2008). Organisms that colonize pipe materials in distribution systems come from the system's source water or from the regrowth of biofilm cells already existing within the system (Camper, 1996). They may also be introduced during main repair or cross connection (Chauret et al., 2005) or through deficiencies of the infrastructure

(USEPA, 2002). Temperature, pipe surface, nutrient availability and concentration, disinfectant type and concentration, and the interaction of these factors influence the survival and regrowth of microorganisms in DWDS (Berry et al., 2006).

Among the factors affecting microbial growth and persistence in distribution systems and premises plumbing, pipe material type confers one of the most complex effects (Berry et al., 2006; Yu et al., 2010). Several types of pipe materials are used including metals (copper, cast iron, lead, brass, steel), plastics (PVC, PE, PB, latex), asbestos cement and concrete (Keinänen-Toivola, 2006). Numerous studies demonstrate that pipe material affects both microbial number and community structure in the system. Materials may provide a protective niche where growth can occur and some materials can provide nutrients to stimulate growth by releasing bioavailable forms of iron and phosphorus (Berry et al., 2006; Morton et al., 2005; Lehtola et al., 2004). On the other hand, some compounds released from pipe materials such as copper can be toxic or inhibitory to microorganisms (Lehtola et al., 2004; van der Kooij et al., 2005). Pipe material can also modify the effectiveness of disinfectants (Lehtola et al., 2005). Physical characteristics of the material, such as roughness of pipe surface, have been found to influence the density of biofilm growth (Pederson, 1990; Niquette et al., 2000; Lehtola et al., 2005). The interaction of pipe material with other factors affecting bacterial growth adds another level of complexity to the effect of material on bacterial regrowth. Camper et al. (2003) studied the interactions between distribution system materials, organics and disinfectants and their effect on bacterial regrowth and found that increased levels of organic carbon had a differential effect on bacterial regrowth depending on material.

As the effect of pipe material can range from stimulatory to inhibitory, pipe material can affect the bacterial community composition and the diversity as a whole. Although specific groups of organisms are recognized to be important in DWDS (e.g., pathogens and nitrifying organisms), knowledge of the whole community is important because other organisms in the community may affect the persistence and growth of these specific groups. With recent developments in molecular techniques to assess community composition, a few community-focused studies have demonstrated differences in microbial community composition brought about by pipe material differences. The microbial community on chlorinated polyvinyl chloride (c-PVC) was found to be the least diverse among several materials tested (including copper, polybutylene, polyethylene stainless steel and stainless steel coated with zinc) (Yu et al., 2010). c-PVC also had the lowest biofilm formation potential based upon measured ATP content of the biofilms. In another study, the microbial community on a stainless steel coupon was found to be the least diverse compared to steel, copper and PVC (Jang et al., 2011). Stainless steel was also found to be superior based on its lower corrosion level and lower biofilm formation potential. Pipe material type can also be expected to strongly influence the occurrence of nitrification (Zhang et al., 2009). Because the copper which leaches from copper pipes is known to be toxic to bacteria (Trevors and Cotter, 1990), it is generally believed that nitrification does not occur in copper plumbing. However, the copper concentration that would inhibit nitrification varies based on nitrifier strain as well as growth conditions (Zhang et al., 2009; Rahman et al., 2011).

Biofilms 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. Consequently, studies on the microbial ecology of DWDS have mainly focused on biofilms. However, to be able to implement effective control strategies that will ensure safe and high quality drinking water, it is necessary to understand the microbial ecology of the whole distribution system (Berry et al., 2006). Large metabolic differences between attached and free-living bacteria can occur (Boe-Hansen et al., 2002). In low nutrient conditions, bulk phase growth accounted for a significant part of the total aftergrowth in the drinking systems (Boe-Hansen et al., 2002). Moreover, bulk water bacteria have been shown to dominate in portions of a distribution system that have low chlorine residual (Srinivasan et al., 2008), thus presenting a need to study both bulk phase and biofilm.

Systems undergoing nitrification often have low or no disinfectant residual. Nitrification is a microbial process where ammonium is oxidized via nitrite to nitrate. This process has several adverse effects on water quality, including nitrite and nitrate accumulation, off taste, increases in heterotrophic plate counts (HPC), and loss of disinfectant residual (USEPA, 2002). Utilities have been switching from chlorine to chloramine as less of the potentially carcinogenic by-products of disinfection such as haloacetic acids (HAAs) and trihalomethanes (THMs) are produced with the use of the latter. However, through the formation of chloramines or through its decay, free ammonia can be made available in the water which can feed nitrification. A significant fraction of bacteria growing in the distribution systems are associated with biofilms (Camper, 1996)

and for nitrifiers, attachment to solid surfaces enhances their growth and provides more resistance to toxic substances (Wolfe et al., 1988). Premises plumbing, which is the portion of the distribution system from the property line through the inside of the home, school, hospital etc., provides more surface area for colonization of biofilms with its higher surface to volume ratios and greater length compared to water mains (NRC, 2006). In addition, other favorable conditions for nitrification such as long water age, low or no residual disinfectant and warmer temperature exist in premises plumbing and yet studies on nitrification in these systems are lacking compared to studies on water mains and treatment plants (NRC, 2006).

Previously, the use of chlorite as a nitrification control mechanism has been studied in the two types of simulated premises plumbing, copper and PVC. Although complete nitrification was established in both copper and PVC systems (as measured by complete utilization of ammonia), subsequent addition of chlorite had different effects on the two systems (Rahman et al., 2011). Specifically, nitrification was less inhibited in the PVC reactor by 20 ppm chlorite and recovered completely even with continued chlorite treatment. In contrast, in the copper reactor, 20 ppm chlorite caused complete loss of nitrification (no ammonia utilization). Furthermore, complete nitrification was not recovered until two months after the chlorite treatment ceased. These observations may indicate a difference in the microbial community between the two reactors, specifically in the nitrifying population, or a difference in the recolonization of the reactors.

The overall goal of this study was to determine a link between bacterial community composition and functional characteristics of the nitrifying systems. The

specific objectives of this study were 1) to determine differences in bacterial populations between the two types of reactors (copper and PVC), as well as among replicate reactors of the same material, 2) to investigate the community stability by analyzing changes in the communities between two timepoints, 3) to compare planktonic and biofilm communities, and 4) to characterize the nitrifying population in these systems.

The reactors used in this study were designed to approximate a premise system that is nitrifying with an ammonia level that would result from the decay of 4 mg/L of chloramine. The periods of use and stagnation that occur in household plumbing are simulated in the system under study using PVC and copper as pipe materials. The system has been nitrifying for 6 years as measured by ammonia loss and nitrate accumulation.

The approach taken in this study involved the use of molecular methods including PCR DGGE to obtain insight into the structure and taxonomic composition of the community. Since amplification of genes only implies presence and not activity, the culture- independent approach was coupled with the use of propidium monoazide which allowed for distinction between cells that have intact cell walls (active) and those that have compromised cell walls to provide a targeted analysis of the active bacterial community composition (Nocker et al., 2007).

Materials and Methods

Reactor Setup

Reactors simulating premises plumbing that have been actively nitrifying for six years were used in this study and have been reported previously (Rahman et al., 2011).

Reactors with either copper or polyvinyl chloride (PVC) surfaces, two of the most commonly used domestic pipe material, were assembled by modifying CDC reactors (Goeres et al., 2005). Modification involved adding parallel coupons to solid rods, adding a base plate, and changing the stir blades to either copper or PVC (Figure 2.1a). The surface area of the coupons, base plate and stir blades was equivalent to the surface to volume ratio of a six foot long $\frac{3}{4}$ " diameter domestic plumbing pipe. The coupons from which the biofilm samples were obtained had been in place for six years. Volume of the reactors was 120 mL. Each reactor type had four replicate reactors. Reactors were kept in the dark to simulate conditions within water distribution pipes.

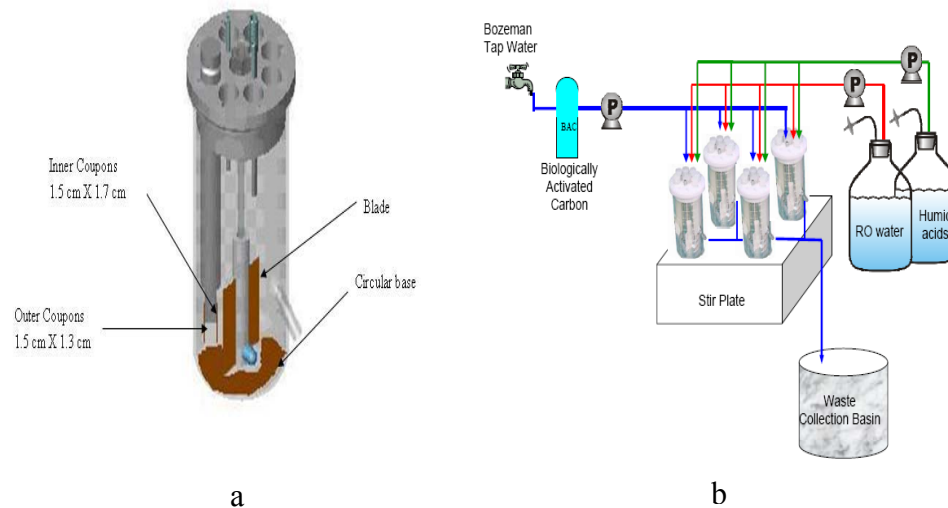


Figure 2.1. Schematic representation of the modified CDC reactor used in the study (a) and the model distribution system (b). (reactor image from Rahman (2008)).

To simulate premises plumbing, periods of flushing and stagnation were included in the operational conditions of the reactors (one reactor volume, three times daily). Reactors were flushed with peristaltic pumps with shear created by the stirplate for five

minutes followed by eight hr stagnation periods. The stirplates were set to create a rotational speed of the blade of 300 rpm, which was approximately equivalent to a velocity of 1m/s in the bulk water.

All reactors were fed with a combination of mineral amended reverse osmosis (RO) water, biologically treated Bozeman tap water, and a humic substances organic feed (Fig 1b). The RO water was amended to create an alkalinity of 35 mg/L as CaCO₃ and a stable pH of 8.15. Constituents of the RO water + mineral feed were MgSO₄ (39.6 mg/L), NaHCO₃ (59.6 mg/L), CaSO₄ 2H₂O (25 mg/L), Al₂(SO₄) 18H₂O (0.62 mg/L), CaCl₂ 2H₂O (20.80 mg/L), and Na₂SiO₃ 9H₂O (26 mg/L). Ammonium sulfate was added to provide a final concentration in the reactor of 0.71 mg/L as N which is equivalent to the amount of ammonia that would result from the decay of 4mg/L of chloramine. Dechlorinated Bozeman tap water (surface water source, no background ammonia, chlorinated) was treated biologically by passing through a granular activated carbon column followed by flow through a biologically active carbon (BAC) column and was provided as a separate, parallel influent. This served as the sole source of microorganisms, providing a continuous inoculum of indigenous organisms (10⁴ CFU/mL of heterotrophic plate count (HPC)) to the reactors. This influent water will be called BAC water from here on. Aside from providing the inoculum for the reactors, the BAC water contained sufficient background phosphate for microbial growth at the organic carbon level used. Organic carbon (4 mg/L in the reactor) was supplied in a third, separate influent in the form of soil-derived humic substances as described previously (Rahman et al., 2011). The RO water/ BAC water/organic carbon feed ratio

was 50:5:1. All reactors showed signs of stable, complete nitrification as measured by conversion of ammonia to nitrate.

Biofilm and bulk (liquid) phases of the reactors as well as the influent BAC water were used in the analyses. Sampling was done at the end of the 8 hr stagnation period prior to the flushing period.

Sample Preparation for Molecular Characterization

Samples were collected from each of the replicate reactors (biofilm and bulk water) as well as from the BAC influent water, with two sampling points one month apart (May and June) to provide a temporal dimension to the analysis. Samples were split into PMA-treated and -untreated subsamples as described below. Because the subsequent PMA treatment step required the samples to be resuspended in a liquid phase, bulk fluid for this purpose was collected from the respective reactor at the same time the biofilm and bulk samples were obtained and was filtered using a 0.2 micron pore size CORNING® sterile syringe filter (www.corning.com). Biofilm samples were obtained from the coupons (1.5 x 1.7 cm) by scraping with a rubber policeman. The biofilm was resuspended in the filtered bulk fluid (1 mL suspension). Following vortexing, the biofilm suspension was split (500 µL aliquots) between clear 1.5 mL microcentrifuge tubes (one for PMA treatment, one left untreated). One hundred mL bulk water from the each reactor was also collected and filtered through a 47 mm diameter 0.2 micron pore size polycarbonate membrane (www.millipore.com) to collect the biomass. The same method was used to collect biomass from the BAC influent water except that 500 mL was used. Membranes were aseptically cut in half and split between PMA-treated and -

untreated clear 1.5 mL centrifuge tubes. The half of the membrane to be PMA-treated was also shredded to facilitate light exposure during PMA treatment and 500 μ L of filtered bulk water was added.

PMA treatment was carried out using the method of Nocker et al (2007). One μ L of propidium monoazide (Biotium) (1 mg PMA in 97 μ L of 20% DMSO) was added to 0.5 mL of the sample in the 1.5 mL centrifuge tubes and the tubes and incubated at room temperature in the dark for 5 minutes. The tubes were vortex mixed in the start, middle and end of the incubation period. The tubes were laid flat on ice and subjected to light exposure for 2 minutes using a 650 W halogen light source (sealed beam lamp, FCW 120V, 3,200 K; GE Lighting, General Electric Co., Cleveland, OH). The samples were centrifuged at 5,000Xg for 5 minutes. DNA was extracted from PMA-treated and -untreated tubes using the FastDNA® SPIN Kit for Soil (MP Biomedicals, OH) according to the manufacturer's directions except for a modification that includes a step to remove humics by using guanidine thiocyanate (Burr et al., 2006). For controls, killed samples were prepared by autoclaving samples at 121°C for 20 minutes. Non PMA and PMA treated samples were prepared as described above.

Polymerase Chain Reaction (PCR)

All end point PCR amplifications were conducted in an Eppendorf Mastercycler® ep (Eppendorf North America, www.eppendorfna.com). Reactions were carried out in 25 μ L volumes which included 0.2 μ M primers, Go Taq® Green Master Mix (www.promega.com), DEPC treated water, Ultrapure BSA (Ambion) and 1 μ l of template DNA (approximately 5 ng). All oligonucleotide primers were synthesized by

IDT (Integrated DNA Technologies, www.idtdna.com). To characterize the bacterial community, the 16S ribosomal RNA gene was used in the molecular analysis. The primer pair 1070F (5'-ATGGCTGTCGTCAGCT-3') and 1392R+GC (5'-CGCCCGCCGCGCCCGCGCCCGGCCCCGCCGCCCGCCCCGCCCCACGGGCGGTGTGTAC-3') (Ferris et al., 1996) was used to target a portion of the 16S rRNA gene. Cycling conditions included the following: initial denaturation at 94°C for 2 min followed by 30 cycles of 45 s at 94°C, 45 s at 55°C and 45 s at 72°C and a final elongation at 72°C for 7 min. Products were visualized on a 0.8% agarose gel with ethidium bromide in 1XTBE buffer for 45 minutes at 40V. Gels were viewed using a FluorChemTM 8800 fluorescence imager (www.alphainnotech.com).

To detect the presence of ammonia oxidizing bacteria, the functional gene encoding for bacterial *amoA* was amplified using amoA1-F (5'-GGGGTTTCTACTGGTGGT-3') and amoA2-R (5'-CCCCTCKGSAAAGCCTTCTTC-3') (Rotthauwe et al., 1997). DNA from a known ammonia oxidizing bacterium, *Nitrosomonas europaea* Winogradsky (ATCC strain 25978) was used as a positive control. To detect ammonia oxidizing archaea, the functional gene *amoA* was amplified using arch-amoAF (5'-STAATGGTCTGGCTTAGACG-3') and arch-amoAR (5'-GCGGCCATCCATCTGTATGT-3') (Francis et al., 2005). Cycling conditions were as reported in the references. To verify the amplification product, gel purification, cloning and sequencing were performed essentially as described below (Cloning and sequencing of DGGE bands). Sequence data for the archaeal *amoA* PCR product was submitted to GenBank with accession number JQ406520. Amplified sequence was aligned with *amoA*

of known archaeal ammonia oxidizers using CLUSTAL W (<http://www.ch.embnet.org/software/ClustalW.html>; Thompson et al., 1994).

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE Marker. A DGGE marker was generated by pooling five unidentified 16S rRNA gene clones (in *E. coli*) (see following section) that were selected based upon their different migration distances in DGGE to span the entire length of the gel. 250 μ L from a broth of each of the five clones were mixed together. Plasmid DNA from the pooled clones was purified using the Wizard Plus SV Minipreps DNA Purification System (www.promega.com). Marker DNA was amplified using VectF (5'-AGTGTGCTGGAATTCGCC-3') and VectR+GC (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCCGCCCGCCCATATCTGCAGAA TTCGCC-3') primers, which target the plasmid vector immediately flanking the vector cloning site (Burr et al., 2006). PCR reactions (25 μ L) were prepared as described above and the amplification program included an initial denaturation of at 94°C for 60 s, followed by 35 cycles of 94°C for 60 s, 54°C for 60 s, and 72°C for 3 min, with a final extension step at 72°C for 10 min. PCR products were confirmed by agarose gel electrophoresis and staining with ethidium bromide prior to use in DGGE.

DGGE was performed using a DCodeTM system (www.biorad.com). Denaturing gels with denaturant concentration of 40%-60% from top to bottom were used for separating the PCR amplicon, where 100% denaturant is defined as 7 M urea and 40% formamide. Gels also contained a 8 to 12% polyacrylamide gradient from top to bottom.

Ten microliters of each sample was loaded per well. Electrophoresis was done at 60 V for 16 h. Gels were stained with Sybr®Gold (www.invitrogen.com) and visualized using a FluorChem™ 8800 fluorescence imager (www.alphainnotech.com). Marker lanes were included in each DGGE gel so that comparison between gels would be possible. Bands in DGGE images were identified using GelCompar II software (Version 6.1, Applied Maths) and confirmed visually. Pairwise correlation analysis of bands was done to determine if the occurrence of one band was correlated to another/other bands which may suggest that a single operational taxonomic unit was represented by more than one band. Phylotype richness of each sample was determined by counting the total number of distinct bands of each sample's DGGE profile. A binary matrix (band presence-absence data) was created from the normalized DGGE gels and saved as a Comma Delimited Format (CDL) file and used in the subsequent analysis in R v.2.11.1 (R Development Core Team, 2010).

The Dice coefficient of similarity/Sorensen's coefficient of similarity was computed using the following formula:

$$\text{Dice coefficient of Similarity/ Sorensen's coefficient} = \frac{2j}{a+b}$$

where j is the number of common bands between the two profiles being compared, a is the number of bands in DGGE profile 1 and b is number of bands in DGGE profile 2 (McCracken et al., 2001; Magurran, 1988). The computed Dice coefficient was then used for the following analyses: cluster analysis using flexible beta in the package cluster (Maechler et al., 2005), permutational multivariate analysis of variance (MANOVA)

using the function `adonis` in the package `vegan` (Oksanen et al., 2011), and ordination with principal coordinate analysis (PCO) and nonmetric multidimensional scaling method (NMDS) in the package `labdsv` (Roberts, 2010).

Cloning and Sequencing of DGGE Bands. In initial studies to separate members of total community, non PMA treated biofilm DNA from a representative copper and PVC reactor was extracted, followed by PCR-DGGE as described previously. Bands were cut from the lanes of the DGGE gel and resuspended in DEPC treated water. To extract the DNA from the gel, a freeze thaw cycle (3 cycles of 1 hr freezing and 1 hr at room temperature) was performed. One microliter of this was then used as template for PCR (1070F/1392R+GC). Resulting amplicons were run on DGGE to check for the position of the bands and to make sure that each amplicon produced only one band. PCR products were gel purified using QIAquick® Gel Extraction Kit (QIAGEN). Purified PCR products were cloned into plasmid vector pCR™4-TOPO® using the TOPO® TA Cloning kit (Invitrogen, www.invitrogen.com) following the manufacturer's protocol. Transformants were inoculated into 10 mL of Luria-Bertani (LB) broth plus 50 mg mL⁻¹ ampicillin. Cultures were incubated overnight at 37°C in a shaking incubator. Plasmid DNA was purified from individual clones using the Wizard Plus SV Minipreps DNA Purification System (www.promega.com) and quantified using the NanoDrop ND-1000 spectrophotometer (Nano Drop, Wilmington USA). Clones were sent to the Research Technology Support Facility (RTSF) at Michigan State University and were sequenced using the M13F (5'-TGTAACGACGGCCAGT-3') primer. Sequences were checked for chimeras using Chimera Slayer (Haas et al., 2011) or Bellerophon (Huber et al., 2004)

and were compared with known sequences in the GenBank database using the Basic Local Alignment Sequence Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were submitted to GenBank under the following accession numbers: JQ406488-JQ406516.

Quantitative PCR (qPCR)

Bacterial 16S rRNA gene and bacterial *amoA* gene abundances at one timepoint were determined in one copper and one PVC reactor using bacterial 16S rRNA gene primers 338F (5'-ACTCCT ACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') (Einen et al., 2008) and bacterial *amoA* primers *amoA1-F* and *amoA2-R* (Rotthauwe et al., 1997). The qPCR reaction mixture consisted of Power SYBR® Green PCR Master Mix (www.appliedbiosystems.com), 0.2 µM each of the forward and reverse primers, 1 µg/µL Ultrapure BSA (50 mg/mL, Ambion, www.ambion.com) and DEPC water. For each 25 µL reaction, 8 µL of template DNA was used.

Quantitative PCR Standards. 16S rRNA gene amplicons were cloned into pCR™4-TOPO® using the TOPO® TA Cloning Kit (Invitrogen, www.invitrogen.com). A purified plasmid was randomly chosen and was sequenced as described previously to verify a 16S rRNA gene insert. Plasmid concentration was determined by both a ND-100 spectrophotometer (Nano Drop, Wilmington USA) and by using a Sybr®Gold (www.invitrogen.com) assay with known concentrations of double stranded Lambda DNA (Promega) as standard for DNA quantification using an ND-3300

fluorospectrometer (Nano Drop, Wilmington USA). Copy number was calculated by considering the size of the plasmid (3890 bp) plus insert length (180 bp) and assuming a molecular mass of 660 Da for each basepair. qPCR standards were prepared by diluting the plasmid suspension ranging from 8.8×10^3 to 8.8×10^7 target gene copies/ μL (in ten fold dilutions). The same approach was done for the bacterial *amoA* qPCR standards but with the 491bp long PCR amplicon amplified from *Nitrosomonas europaea* Winogradsky (ATCC strain 25978). The bacterial 16S rRNA gene standard sequence was deposited in GenBank with the accession number JQ406518.

Sample DNA Dilution. To determine if there was PCR inhibition conferred by the samples, dilutions of sample DNA were prepared and used as template for the qPCR reaction along with standards. Ct values were plotted against the dilution and compared to that of the standards. The lowest dilution falling within the linear range that was parallel to that of the standards was chosen for analysis. To validate this, a spiked sample dilution was prepared by adding a known amount of standard template to the sample dilution. The Ct value of the spiked dilution was compared to that of a standard with an equal concentration of DNA.

Quantitative PCR Cycling Conditions. qPCR was performed in a Rotor-Gene 3000 real time PCR cycler (QIAGEN, www.qiagen.com) in a 72-well rotor using the program: 95°C for 15 min, 45 cycles of 94°C for 15 s, 61°C for 30 s, 72°C for 30 s. Data was acquired using the FAM/Sybr detection channel during the extension step. Standards and samples were prepared in duplicate and negative controls containing no template

DNA were included to ensure no contamination had occurred. Melt curve analysis was also performed after the amplification program to verify amplification of correct PCR products. Melt curve analysis was performed from 60-95°C in 0.1°C increments held for 5 s with an initial pre-melt hold for 90 s at the first step. Univariate analysis of variance (ANOVA) was performed on the gene copy abundance data with a general linear model using Minitab 16 (Minitab).

Biochemical Characterization of the Biofilms

Biofilm from each representative reactor type was scraped from the coupons using a sterile rubber policeman and resuspended in 500 µL of sterile distilled water for the biochemical characterization.

Carbohydrate. L-Cysteine sulphuric acid assay was used to determine the amount of carbohydrate in the biofilm (Chaplin, 1986). Two hundred µL of each sample and standard, and 1 mL of the reagent (700 mg/L cysteine in ice cold 86% v/v sulphuric acid) were mixed thoroughly in an ice bath. The mixture was heated at 100°C for 3 minutes in a glass-stoppered test tube. The mixture was rapidly cooled to room temperature and absorbance was determined at 415 nm.

Protein. Protein was measured using the Lowry method (Lowry et al., 1951) using serum albumin as the standard. To 0.15 mL sample and standards in screw capped test tubes, 0.15 mL of 0.2 N NaOH was added and the mixture was boiled for 15 minutes. To this, 5.0 mL of copper reagent was added and the mixture was incubated for 45 minutes at 39°C and then cooled to room temperature. One mL of the phenol reagent was

then added and the mixture was incubated for 30 minutes at room temperature. Absorbance was determined at 660 nm.

Results

Molecular Characterization

Because DNA samples may contain substances such as humic acids that may inhibit PMA treatment, the applicability of using PMA treatment to distinguish the active microbial population from the total population was first determined. PMA and non PMA treated killed samples were prepared and subsequently used as a template for PCR. Amplicons were obtained using the killed samples as template while no amplicons were obtained using the PMA treated killed samples, indicating that the PMA treatment effectively removed DNA from dead cells in the sample matrix. Two representative DGGE profiles (PMA and non PMA treated) from each sample were chosen for comparison. Dice Coefficient/Sorensen's pairwise similarity coefficient of the DGGE profiles of the samples were 84% for BAC, 98% for PVC biofilm, 96% for copper biofilm, 98% for PVC bulk and 90% for copper bulk. No profile for the same sample (PMA vs non PMA treated) matched 100% indicating the removal of members of the community that were dead and/or had compromised cell membranes. The remaining DGGE analyses were all done on the PMA treated samples.

Band Correlation and Richness

Figure 2.2 shows the normalized DGGE profiles of the samples. Figure 2.3 shows the percent occurrence of the bands in the samples. A total of 39 distinct

bands/operational taxonomic units (OTUs) were detected in the samples. There was no pairwise correlation greater than 0.75 in co-presence of any two bands suggesting each band is representative of a separate OTU.

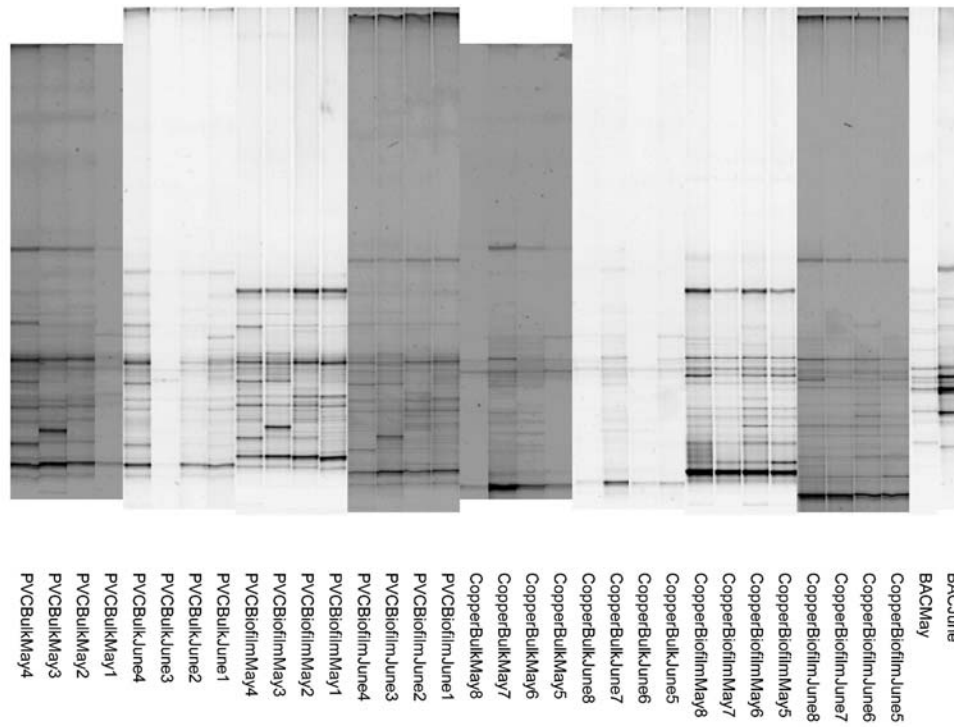


Figure 2.2. DGGE profiles of the samples from the PVC and copper reactors and the BAC influent as normalized by the GelCompar II v. 6.1 software (Applied Maths, Inc.). Number after the month corresponds to the reactor replicate (1-4, PVC and 5-8, copper).

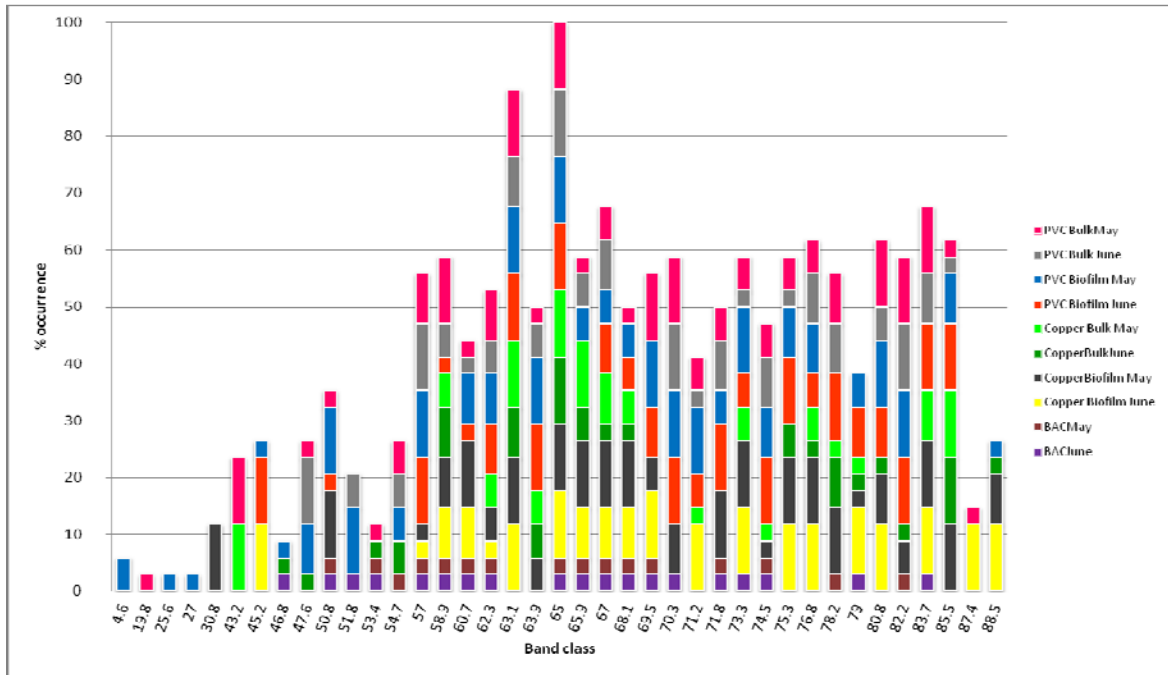


Figure 2.3. Occurrence of different bands in the samples. Band classes were assigned by the GelCompar II v. 6.1 software (Applied Maths, Inc.) and correspond to band migration distance. Percent occurrence corresponds to the frequency of band occurrence in the different samples (N=34) with each sample type having four replicates except for the BAC influent which has one per timepoint.

Table 2.1 shows the average richness of the samples across replicates and time points. Richness of BAC, copper and PVC samples were not significantly different from each other ($p \geq 0.58$) ranging from 5 to 27 bands. Only one band (65, Figure 2.3) was present across all samples. Three bands were found only in the PVC biofilm and one band was found only in a single PVC bulk sample. No band was unique to a particular phase or specific material.

Cluster Analysis

Figure 2.4 shows the result of the cluster analysis. BAC samples clustered together along with PVC samples. Biofilm samples from the PVC and from the copper

samples distinctly clustered separately from each other. Bulk phase samples of each type generally clustered with their respective biofilm counterparts and aggregated by date except for a few samples from both types that grouped in a third cluster. Although similarity is not 100% among replicates for the same reactor type, a general community profile is present across all replicates as indicated by their clustering together. Overall changes in community profiles were shared by the replicate reactors through the two time points in the study.

Table 2.1. Average richness/number of bands in the samples at the two timepoints and replicates. N=number of samples.

Sample	N	Richness
BAC	2	17.5
PVC Biofilm	8	19.4
PVC Bulk	8	10.5
Copper biofilm	8	21.4
Copper bulk	8	16.6

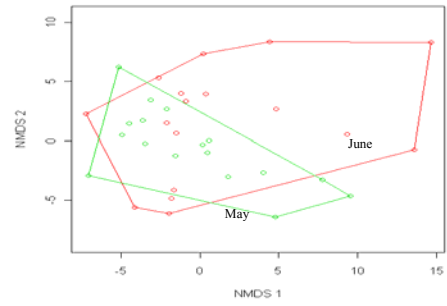
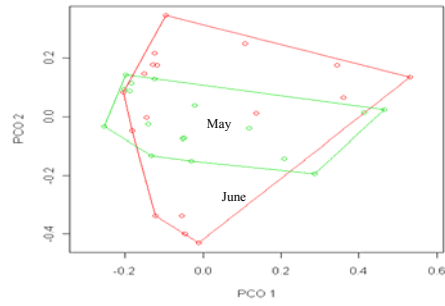


Figure 2.4. Cluster analysis using flexible beta (Lance and Williams, 1967) in the package cluster (Meachler et al., 2005) in R. Number after the month corresponds to reactor replicate (1-4, PVC and 5-8, copper).

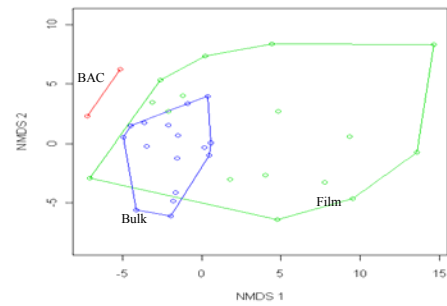
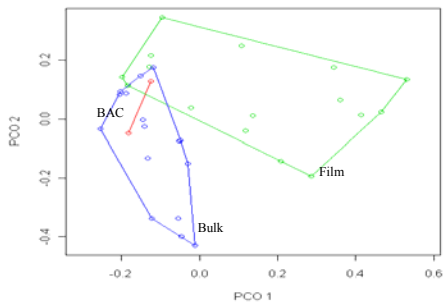
MANOVA, NMDS and PCO Analysis

The variables month (May and June), phase (BAC, bulk and biofilm) and type (BAC, copper and PVC) were converted into factors and analyzed by MANOVA in the *adonis* function of the *vegan* package of R. Both phase ($p=0.001$) and type ($p=0.001$) were significant factors in the clustering. Month by itself was not significant with $p=0.141$, however when combined with phase and type it was significant ($p=0.024$). This can be seen as the samples cluster according to type and are also generally clustered by month (Figure 2.4).

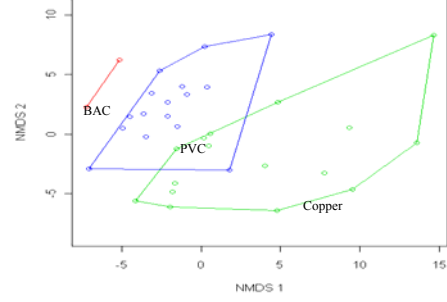
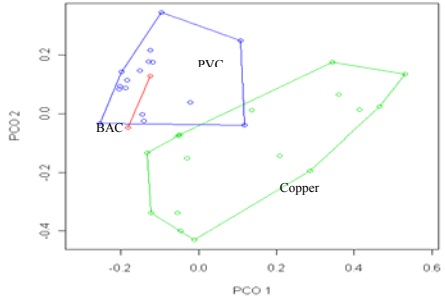
Figure 2.5 shows the PCO (2 dimensions) and NMDS (20 iterations, 2 dimensions) plots and clustering in convex hulls according to the factor type, phase and month. No distinct clustering was observed based on month (Figure 2.5a) as the samples from May and June overlapped. With NMDS, BAC samples clustered distinctly from all the other samples. On the other hand, this separation was not observed in the PCO where the BAC samples grouped with most of the PVC samples. For the grouping based on phase, overall bulk and biofilm samples clustered together in NNMDS while separation between these phases was observed in PCO. An almost distinct grouping was established based on type with NMDS as samples from copper and PVC reactors clustered distinctly from each other (Figure 2.5b and 2.5c). For the clustering based on both phase and type, both ordinations showed the separation of copper bulk and biofilm and the clustering together of PVC bulk and biofilm (Figure 2.5d).



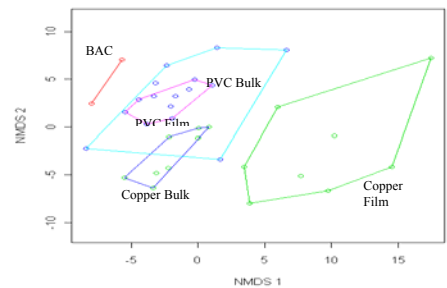
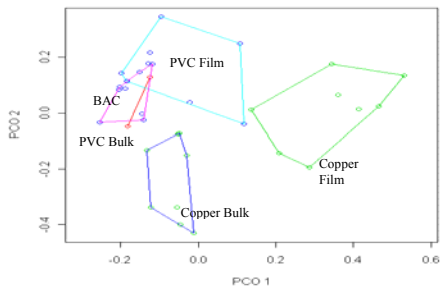
a



b



c



d

Figure 2.5. PCO and NMDS ordination plots with the chullord function for a) month, b) phase, c) type and d) type and phase in the package labdsv (Roberts, 2010) in R. First two axes of the PCO explain 35.8% of the variability; NMDS stress=19.14.

Sequence Analysis

For the sequence analysis, total DNA was used instead of PMA treated samples to obtain a picture of the community that included transient members. Tables 2.2 and 2.3 show the taxonomic identity of the bands which were excised from the DGGE profiles. Fifteen out of 16 bands from the biofilm from the PVC coupon and 14 out of 15 bands from the biofilm from the copper coupon sample were successfully reamplified to yield sufficient sequence information (>150bp). No known autotrophic ammonia oxidizer was detected using this method in either the biofilm from the copper or PVC. Nitrite oxidizing bacteria were detected in both reactor types belonging to the genus *Nitrospira*. To detect ammonia oxidizing bacteria, PCR targeting the functional gene *amoA* was attempted, however even with several attempts to optimize conditions (PCR conditions, PCR reaction component concentrations and template concentration), no amplicon was achieved from the samples while amplification was achieved for the control DNA. It was hypothesized that there are other microorganisms that might be responsible for the first step of nitrification in the systems. Using primers that target the archaeal *amoA* gene, an amplicon was obtained using DNA from the PVC reactor, while none was found from the copper reactor. The PCR product was purified, cloned and was sequenced and was found to be 79% similar to the archaeal *amoA* of *Nitrosopumilus maritimus* (Könneke et al., 2005) (Figure 2.6).

Table 2.2. Taxonomic identity of the bands from the DGGE profile of the biofilm on the PVC coupon.

Band number	Closest relative and GenBank accession number	Similarity (%)
1	Uncultured planctomycete FM 945332	98
2	Uncultured <i>Flavobacterium</i> GU230410	97
3	Uncultured <i>Nitrospira</i> GQ176326 /uncultured <i>Acidobacteria</i> JN 820186	98
4	Uncultured <i>Chloroflexi</i> FM 253630	93
5	Uncultured <i>Nitrospira</i> GQ176345	95
6	Uncultured Comamonadaceae JN125752	98
7	Uncultured Comamonadaceae JN125752	96
9	Uncultured <i>Desulfuromonadales</i> AM935314	97
10	Uncultured Chromatiaceae AJ698067	87
11	Uncultured Nitrospiraceae GQ302556	99
12	Uncultured <i>Nitrospira</i> GQ176345	100
13	Uncultured <i>Chloroflexi</i> JF707623	81
14	Uncultured Candidatus Entothenella AB683979	93
15	Uncultured Verrucomicrobiales FJ542937	87
16	Uncultured <i>Chloroflexi</i> EV403822	94

Table 2.3. Taxonomic identity of the bands from the DGGE profile of the biofilm on the copper coupon.

Band number	Closest relative and GenBank accession number	Similarity (%)
1	Uncultured Acidobacterium EU403975	99
2	<i>Sterolibacterium</i> AM990454	99
3	Uncultured planctomycete JF808985	94
5	<i>Gemmata</i> sp. GQ889433	95
6	Uncultured Acidobacterium EU403975	94
7	<i>Hylemonella gracilis</i> AB539995	98
8	Uncultured Nitrospirae AB252938	99
9	Uncultured Bacteroidetes EU633829/uncultured <i>Chloroflexi</i> EU633735	98
10	<i>Enhydrobacter aerosaccus</i> FR727223	98
11	Uncultured <i>Gemmatimonadetes</i> JN409149	95
12	<i>Reyranelia massiliensis</i> HM048834	91
13	Uncultured <i>Nitrospira</i> JN0388835	94
14	Uncultured planctomycete JN656908	83
15	Uncultured Bacteroidetes AB476710	75

```

putative_amoA      ACCCTGCTAACAAATTAACGCAGGAGACTACATCTTCTACACCGACTGGGCCTGGACTTCG
Cenarchaeum       ACCCTGTTGACGATCAATGCAGGAGATTACATCTTTTACACCGACTGGGCTTGGACGTCA
C.Nitrososphaera  ACACCTGCTAACCATCAACGCAGGTGACTACATCTTCTACACGGACTGGATGTGGACCTCC
Nitrosopumilus    ACACCTGTTAACAAATTAATGCAGGAGACTACATCTTCTACACTGACTGGGCTTGGACTTCG
** ** * * * * * ** * * * * * ** * * * * * ** * * * * * ** * * * * *

putative_amoA      TTTGTAATATTCTCAATATCACAATCGTTGATGTTGGTAGTTGGCGCATGCTACTATCTC
Cenarchaeum       TTCACGGTATTCTCTATATCGCAGACGCTCATGCTGGTTGTCGGTGCAACGCTACTACCTG
C.Nitrososphaera  TTCGTAGTATTCTCGATCTCCAGTCTACAATGCTCGTAGTGGGCGGATATACTACATG
Nitrosopumilus    TACACGGTATTCTCAATATCGCAAACGTTGATGCTTATTGTAGGTGCAACATATTACCTA
*      * * * * * * * * * * * * * * * * * * * * * * * * * * * *

putative_amoA      ACATTTACGGGAGTCCAGGAACAGCAACGTATTATGCGTTGATAATGACCGTCTACACA
Cenarchaeum       ACGTTTACAGGCGTACCCGGAACCCGCGACATACTATGCCCTCATCATGACGGTGTATGTA
C.Nitrososphaera  CTCTTACAGGAGTACCAGGAACGGCCACATACTATGCAACAATCATGACAATCTACACC
Nitrosopumilus    ACATTTACAGGCGTTCAGGCACAGCAACGTACTACGCTCTGATTATGACAGTTTACACA
** * * * * * * * * * * * * * * * * * * * * * * * * * * *

putative_amoA      TGGGTAGCAAAGGTGCATGGTTGCACTCGGATATCCATATGACTTCATTGTAACACC
Cenarchaeum       TGGATAGCAAAGGGCGCATGGTTCGCCCTTGGCTACCCGTATGACTTCATCGCGACCCCG
C.Nitrososphaera  TGGGTTGCAAAGGGCGCATGGTTCGCACTCGGCTACCCGTACGACTTCATCGTGGTGCCT
Nitrosopumilus    TGGATTGCAAAGGCAGCTGGTTCCTACTAGGTTATCCATATGACTTCATTGTAACACCA
*** * * * * * * * * * * * * * * * * * * * * * * * * * * *

putative_amoA      GTTGGCTGCCGTGAGCAATGTTGCTTGACCTGGCATACTGGGCGACAAGAAGAACAAG
Cenarchaeum       ATATGGCTTCCATCAGCAATGCTGTTGGATCTGGCATACTGGGCCACGAAGCGCAACAAG
C.Nitrososphaera  GTGTGGATACCTTACGAATGCTGTTGGATCTCACATACTGGGCAACAAGAAGAACAAG
Nitrosopumilus    GTTGGTTACCATCAGCAATGCTGTTGGACTTAGTCTACTGGGCAACAAGAAGAACAAG
* * * * * * * * * * * * * * * * * * * * * * * * * * *

putative_amoA      CACTACTGAT-ACTATTCGGTGGTGTGATGTGTGGAATGTCATTGCCGTTGTTAACAT
Cenarchaeum       CACTCGCTGAT-ATTGTTGCGCGGTGCTTGGTGGGCATGTATTACCGCTGTTCAACAT
C.Nitrososphaera  CACGCTGCAATCATTAT-CGGTGGAACTGGTGGCCTCTCGTTCGCCGCTGTTCAACAT
Nitrosopumilus    CACTCCTTGAT-ACTGTTGCGCGGTGACTGGTAGGAATGTCATTACCATTGTTCAACAT
*** * * * * * * * * * * * * * * * * * * * * * * * * * * *

putative_amoA      GGTCAACCTCATCACTGTGGTGATCCATTAGAGACTGCATTCAAATATCCAAGACCAAC
Cenarchaeum       GGTAAACCTGATCACAGTAGCAGATCCGCTGGAGACGGCCTTCAAGTATCCGAGGCCAC
C.Nitrososphaera  GGTCAACCTGCTGCTCGTCAGAGACCCGCTCGAGGTGGCATTCAAGTATCCAAGGCCTAC
Nitrosopumilus    GGTAAACCTGATAACAGTAGCAGCCACTAGAAACGGCATTCAAGTATCCAAGACCAAC
*** * * * * * * * * * * * * * * * * * * * * * * * * * * *

putative_amoA      GCTCGCTCCATATATGACACCGATAGAACCTGCGGTAGGCAAGTTCTATAACAGTCCGGT
Cenarchaeum       GCTGCCACCCTACATGACTCCAATAGAACCTCAAGTTGGAAGTTCTATAACAGTCCGGT
C.Nitrososphaera  GCTGCCTGCATACATGACGCCAATCGAACCTCAGGTGGGCAAGTTCTACAACAGTCCGTG
Nitrosopumilus    ATTGCCACCATACATGACACCGATAGAACCCCAAGTAGGTAAGTTCTATAACAGCCAGT
* * * * * * * * * * * * * * * * * * * * * * * * * * *

putative_amoA      CGCATTGGGCGCAGGTGCAGGTGCTGTATTATCAGTAACCATGGCCCGCTAGGATGTAA
Cenarchaeum       GGCGCTAGGGGCTGGGGCTGGGGCCGCTTATCGGTGACGTTGCGCGGCTTGGGTGTCAA
C.Nitrososphaera  AGCCTTGGGTTGAGGAGCAGGAGCAGTGCTGTCAGTTCCCATTGCTGCATTGGGTGCGAA
Nitrosopumilus    TGCTCTCGGTGCAAGTGCAGTGCAGTTTGGGATGTACATTTGCAGCGTTAGGTTGTAA
** * * * * * * * * * * * * * * * * * * * * * * * * * * *

putative_amoA      ACTGAACACGTGG
Cenarchaeum       GCTCAATACGTGG
C.Nitrososphaera  GCTTAACACGTGG
Nitrosopumilus    ATTGAATACATGG
* * * * *

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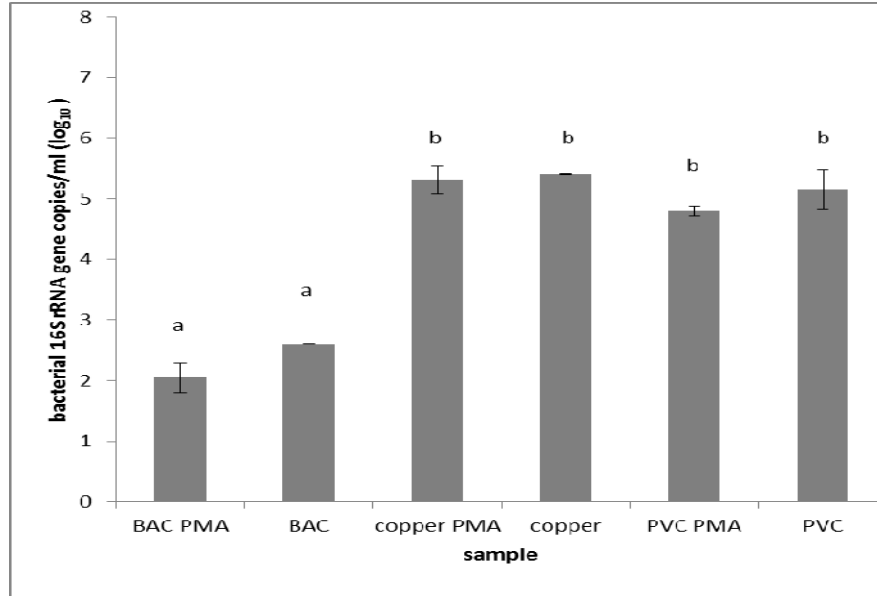
Figure 2.6. Alignment of the amplified putative archaeal *amoA* with the *amoA* from known ammonia oxidizing archaea using CLUSTALW. GenBank accession numbers of sequences are as follows: *Cenarchaeum symbiosum* (DQ397580), *Candidatus Nitrososphaera gargensis* (EU281321) and *Nitrosopumilus maritimus* (EU2399959).

Quantitative PCR

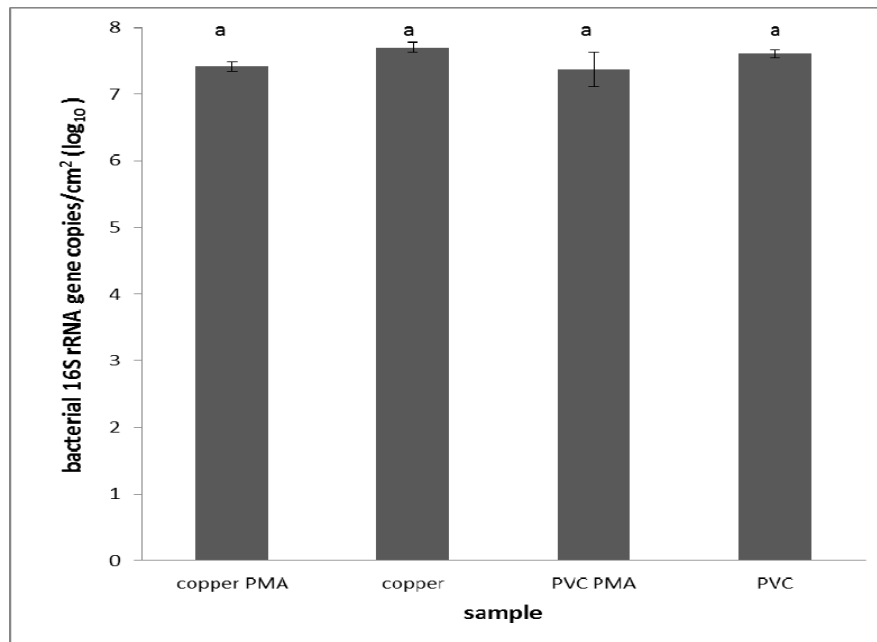
Ten-fold dilution of samples was used for the subsequent qPCR as this dilution fell within the linear range of the standards (Figures S1 and S2). qPCR results are shown in Fig 7 a and b. PMA treated vs PMA non treated samples in both bulk and biofilm were not significantly different ($p \geq 0.067$). BAC influent copy number was significantly less than copper and PVC copy numbers ($p \leq 0.0004$). For both bulk and biofilm phases, the bacterial 16S rRNA gene copy number of the copper reactor are not significantly different from that of PVC ($p \geq 0.4411$).

Biochemical Characterization

Protein and carbohydrate content of the biofilm on the copper coupon were determined at $0.38 \pm 0.04 \mu\text{g}/\mu\text{L}$ and $0.09 \pm .01 \mu\text{g}/\mu\text{L}$ respectively giving a carbohydrate to protein ratio of 0.24, while for the biofilm on the PVC coupon, protein and carbohydrate content were determined at $0.134 \pm 0.01 \mu\text{g}/\mu\text{L}$ and $0.01 \pm 0.002 \mu\text{g}/\mu\text{L}$ respectively giving a carbohydrate to protein ratio of 0.07.



a



b

Figure 2.7. Average (N=2) bacterial 16S rRNA gene copies/mL of the bulk water and BAC samples (a) and copies/cm² of the biofilm samples (b). Bars with same lowercase letter are not significantly different ($p < 0.05$). Error bars represent standard deviation.

Discussion

Nitrifying reactors simulating premises plumbing with copper and PVC surfaces were previously used for nitrification studies (Rahman et al., 2011). Results showed that there was complete ammonia utilization over the 8 hr stagnation time in the reactors with two different surfaces. Aside from similarity in ammonia utilization, the two systems were also similar in heterotrophic plate counts (HPC), numbers of autotrophic ammonia oxidizing organisms, and numbers of autotrophic nitrite oxidizing organisms numbers as follows: HPC were 1.2×10^6 CFU/cm² (PVC) and 1.8×10^5 (copper), autotrophic ammonia oxidizing organisms were 2.4×10^3 MPN/cm² (PVC) and 1.1×10^3 MPN/cm² (copper) and autotrophic nitrite oxidizing organisms were 2.8×10^3 MPN/cm² (PVC) and 8.2×10^2 MPN/cm² (copper). However, the two systems displayed a difference in recovery to full nitrification when nitrification inhibitors were introduced to the system. Nitrification was only slightly inhibited in the PVC system with a decrease in its ammonia utilization and a rebound to complete ammonia utilization even in the presence of chlorite. On the other hand, the copper system's ammonia utilization dropped to zero and it took almost two months after the chlorite treatment was stopped for the system to return to its nitrifying state. This difference suggests a difference in the community and how it reacts to environmental perturbation.

In the current study, to focus on the active members of the community, samples were subjected to PMA treatment to remove DNA from cells that have compromised cell membranes. Distinct profiles of PMA and non PMA treated samples were achieved. Microbial communities, especially biofilm communities are comprised of members that

may be dead and or metabolically inactive (Fagerlind et al., 2012). However, to make accurate conclusions on genetic fingerprinting studies, it is necessary to limit the analysis on the live members of the community (Nocker et al., 2007). Although the total community was different from the active community based on DGGE analysis, the total biomass deduced from qPCR data was not significantly different between PMA and non PMA treated samples. This may mean that these membrane-compromised or “inactive” cells do not contribute significantly to the biomass of the system as their removal does not significantly alter total 16S rRNA gene DNA mass.

The 16S rRNA gene sequencing approach in identifying the members of the community showed members that are commonly found in oligotrophic conditions. Planctomycetes, acidobacteria, verrucomicrobia, *Cytophaga* and *Gemmatimonas* have been detected in drinking water or in portions of the water treatment or distribution system (Martiny et al., 2005; Kwon et al., 2011; Yu et al., 2010). *Commamononas* and *Flavobacterium*, though not directly detected in drinking water, have been found in mineral water (Bischofberger et al., 1990; Vachée et al., 1997). No pathogenic organism was detected in either PVC or copper reactors, however sulfur reducing bacteria such as members of Desulfuromonadales and sulfur oxidizing organisms, such as Chloroflexaceae, whose products of metabolism may adversely affect water quality (Egboka et al., 1989) were detected. In both reactor types (PVC and copper), nitrite oxidizing bacteria were detected. However, only one sequence (PVC biofilm band 10, Table 2.2) was found to be distantly related to a family to which a known ammonia oxidizing bacterium, *Nitrosococcus*, belongs (Campbell et al., 2011), but only with 87%

similarity. Thus, organisms that are known to catalyze the first step of ammonia oxidation (ammonia oxidizing bacteria) were not conclusively found. Similarly, even with the use of a more targeted approach with the amplification of the genes encoding for one of the subunits of the ammonia monooxygenase enzyme (*amoA*), no ammonia oxidizing bacteria were detected in either the PVC or copper reactor biofilm or in bulk water by either end point PCR or qPCR. If ammonia oxidizing bacteria are present, they may be novel so that the methods employed in the study to target bacteria failed to detect them, or possibly another group of organisms may be responsible for the ammonia oxidation in the system. Failure to detect ammonia oxidizing bacteria has been reported previously in other nitrifying systems (Hovanec and De Long, 1996; van der Wielen et al., 2009) and the activity has been attributed to other yet to be characterized organisms. Recently, attention has been focused on the group Thaumarchaeota belonging to the domain Archaea. These organisms have been detected using molecular methods in numerous environments, sometimes in even greater number than their bacterial counterparts (Leininger et al., 2006; Francis et al., 2005; Pester et al., 2011). To date, two mesophilic Thaumarchaeota have been isolated in pure culture (Könneke et al., 2005; Tourna et al., 2011), the analysis of which will further prove their contribution to ammonia oxidation. The detection of this group of microorganisms in a nitrifying premises plumbing system prompts the need to study their contribution in this specific system and as well as a reassessment of the control mechanisms for nitrification in water distribution systems that have for years solely focused on nitrifying bacteria.

A shift in community composition for all samples over time indicates a dynamic microbial community in the reactors. A shift was observed in the BAC water, which serves as the inoculum for the system, between the two time points one month apart. Although functional stability has been established (in terms of consistent ammonia oxidation into nitrate), community composition was dynamic. As a shift in the population of the BAC itself was observed, a dynamic community from the source water can be inferred. “New” organisms are then introduced into the system and some of these “new” organisms can be integrated into the biofilms while sloughing of current species occurs. Stable population states in biofilms have been observed at 500 days however the biofilm may continue to evolve (Martiny et al., 2003). The biofilms in these reactors are at least six years old (2190 days) and the temporal changes demonstrate continuous change in the stable biofilms. The link between community composition and function is not absolute. Because different microorganisms have varied metabolisms, changes in the community profile are expected to coincide with changes in functionality. However, the diversity of bacteria may also reflect functional redundancy among species. The systems in study have been consistently nitrifying and thus even with possible community reorganization implied by the shifts in community composition, the established function is maintained.

Using a common inoculum (BAC water feed) and amendment conditions, distinct communities developed on the copper and PVC coupons. Based on the qPCR data, bacterial abundance in both reactors significantly increased from the source (BAC). This can be explained by the availability of more carbon in the form of humics (4mg/L) and nitrogen in the form of ammonia (0.71mg/L) in the reactors.

Because plumbing material confers one of the most complex effects on microbial growth and persistence in distribution systems (Berry et al., 2006; Yu et al., 2010), evaluating different materials has been the subject of numerous studies in relation to biofilm formation. In this study, two materials, copper, which is the most commonly used household plumbing material in the US (Oskarsson and Norrgren, 1998) and PVC, another commonly used plumbing material (NSF, 2012) were evaluated. Species richness, one of the measures used to evaluate plumbing material, was not significantly different among the copper, PVC and BAC samples. This suggests that a similar number of types or ‘species’ can be supported by the two materials.

Plumbing materials are also evaluated in the context of nitrification. Previously, nitrification kinetics in simulated premises plumbing with copper and PVC surfaces were evaluated (Rahman, 2008). In the 8hr stagnation period, ammonia was completely utilized in both, however, ammonia was undetectable in the PVC system at hr 3 while it took 4 hr in the copper system for ammonia to be undetectable. Rahman et al. (2011) also reported a difference in how each handled perturbations. The copper system was affected to a greater extent than the PVC system upon the addition of chlorite and took longer to recover to its nitrifying state while the PVC system was more greatly affected by the introduction of combined chlorine (Rahman et al., 2011). These differences between copper and PVC systems might suggest a difference in the density of the microorganisms in the two systems but they were found to have similar levels of HPC as well as ammonia oxidizing and nitrite oxidizing organisms (Rahman et al., 2011). In the current study, no significant difference in bacterial numbers between the two types of

systems was found based on qPCR data. Toxicity resulting from the exposure to the copper leached from the copper surfaces does not appear to be a significant limiting factor for bacterial growth in both biofilm and bulk phases. While it was shown previously that the two types of nitrifying systems showed differences in nitrification kinetics as well as in how they reacted to nitrification inhibitors, results of the current study show that there is no difference between copper and PVC in terms of bacterial richness and density. This indicates that the difference in the community composition rather than density may play a bigger role in nitrification kinetics and how the system recovers from disturbances, specifically the application of nitrification inhibitors.

The bacterial community structures in the simulated premises plumbing systems were studied using the molecular fingerprinting technique PCR-DGGE. To analyze the fingerprints, three exploratory multivariate analysis methods namely cluster analysis, PCO and NMDS were used (Ramette, 2007). PCO failed to discriminate the BAC from the PVC samples and similarly, the BAC samples clustered with the PVC biofilm samples in the cluster analysis. However, the three methods generally concurred in the clustering of the samples. Even for the same data set, different results may be obtained using different methods. For example, Okubo and Sugiyama (2009) used PCO and NMDS in their molecular fingerprinting data to discriminate between fungal communities in four land use types and found that PCO showed a clearer separation of land use types than NMDS. The same similarity index of the samples (Dice Coefficient/Sorensen's Coefficient) was used in all the methods and the differences in the results reflect the inherent differences in algorithms used in each. Based on the MANOVA and NMDS

results, phase is an important factor in the similarities of these communities. The results showed that the biofilm as well as the bulk phase communities clustered separately. The belief that biofilm dominates in distribution systems is not true in all conditions (Srinivasan et al., 2008), however, most studies on the microbial ecology of drinking water have focused on the biofilm phase while the bulk phase community has been overlooked. From the results of the current study, the bulk phase community was unique and distinct. The distinction of bulk phase from biofilm communities has been reported previously (Martiny et al., 2005). Bulk phase organisms may cause problems that may adversely affect water quality such as regrowth. Since the assemblage of microorganisms in the bulk phase is distinct from its biofilm counterpart, this implies that limiting the analysis to the biofilm community leaves out details about the distribution system that may be integral to the full understanding of the processes that occur in them. Generally, a tighter clustering was observed for the bulk samples (among replicates and in the two timepoints) as compared to the biofilm samples indicating that there is less diversity in the communities that can persist in the bulk phase. At the same time, the organisms that persist do so consistently and independently of factors such as the protective mechanisms conferred by a biofilm lifestyle.

Looking at the bulk and biofilm communities of the copper and PVC reactors separately, the biofilm on the copper coupons was distinct from its bulk counterpart. On the other hand, the biofilm on the PVC coupons clustered with its bulk counterpart. This finding may be interpreted as the inability of some microbes to persist in the biofilm phase of the copper reactor in close contact with the copper. A distinct group of

organisms that can tolerate the close proximity to copper therefore may have developed, distinct from the copper bulk organisms that cluster with the rest of the samples. This finding is supported by the result of the biochemical analyses showing a difference in the carbohydrate: protein ratio of the PVC and copper biofilms. Proteins and carbohydrates are two of the major macromolecules that are found in a prokaryotic cell (Madigan and Martinko, 2006) and macromolecules such as these, influence the formation of biofilms (Andrews et al., 2010). Some microorganisms produce large amounts of copper complexing exopolymers to protect themselves from the toxic effects of Cu^{2+} (Geesey et al., 1986). It can be hypothesized that all microbes in the biofilm associated with the copper surface have a level of tolerance to copper and at the same time, these organisms are benefited by the protective exopolymers that are synthesized by some or all of the members of the biofilm.

The difference in the BAC water for the two time points would indicate that the influent introduced new species into the system that may or may not have been integrated. The extent to which each existing community was affected was different. Thus it can be deduced that the persistence or dispersal of the existing members of the biofilm community was a greater influence than the newly introduced species and that the newly introduced species persistence and integration depended not only on their inherent characteristics but on the characteristics of the community as a whole. This view of biofilm community is important to studies involving the survival or reintroduction of nitrifying organisms to drinking water when control mechanisms for nitrification are implemented.

Conclusion

Community profiles of the total and the active populations were found to be different, however, there was no significant difference in their biomass. Bacteria were enriched from the BAC as they were significantly higher in number in both the PVC and copper reactors. No significant difference in terms of microbial number and total microbial species was seen between copper and PVC. However, community analysis showed that the community in the copper was unique from that of the PVC, and both were different from that of the BAC. From this, it can be inferred that the difference in the community structure of the copper and PVC systems is a possible explanation for the difference in how each system recovered from the nitrification inhibitors. In these nitrifying premises plumbing systems that have been shown to consistently utilize ammonia to nitrate (Rahman et al., 2011), nitrite oxidizing bacteria were detected in both copper and PVC systems while no ammonia oxidizing bacteria were found using both end point PCR and qPCR approaches. Ammonia monooxygenase gene-carrying archaea were detected in the PVC system while none were detected in the copper system. Ammonia oxidizing bacteria are traditionally assumed to carry out the first step of nitrification. Consequently, control of nitrification in drinking water distribution systems have been aimed at the ammonia oxidizing bacteria and nitrite oxidizing bacteria. However, results of this study suggest that the ammonia oxidation in the simulated premises plumbing may be attributable to a group of ammonia oxidizers other than the ammonia oxidizing bacteria. The results of this study highlight the need to reassess the view of nitrification and its control in water distribution systems.

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CHAPTER 3

DETECTION AND ENRICHMENT OF A NITRIFYING COMMUNITY
CONTAINING A NOVEL ARCHAEON FROM
SIMULATED PREMISES PLUMBING

Contribution of Authors and Co-authors

Manuscript in Chapter 3

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Contributions: Experimental design, laboratory experiments, data analysis, manuscript writing

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Contributions: Experimental design and manuscript editing

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Abstract

Nitrification in premises plumbing was simulated using reactors with copper and PVC surfaces that have been nitrifying for more than 6 years. Ammonia oxidizing archaea were detected in the absence of known bacterial ammonia oxidizers and a possible role for archaea is hypothesized. The study determined the presence and abundance of archaea using fluorescence in situ hybridization (FISH) and qPCR with and without propidium monoazide (PMA) treatment. Archaeal distribution relative to bacteria was determined to provide insight on the overall microbial ecology including assessment of plumbing material effect on the populations. The study also aimed to enrich the autotrophic ammonia oxidizing organisms that putatively includes an ammonia oxidizing archaeon.

Archaea were detected along with bacteria in the biofilms from both copper and PVC systems by FISH using domain specific probes. DGGE analysis of amplified archaeal *amoA* sequences indicate the presence of at least two phylotypes that are both present in the copper but not in the PVC. Archaeal 16S rRNA and archaeal *amoA* gene sequencing reveal phylogenetic affinity of the organisms to known ammonia oxidizing archaea. Quantitative PCR (qPCR) data show that although bacterial abundances are comparable in the copper and PVC systems, archaeal abundance was greater in PVC suggesting a domain-specific material effect. A nitrifying enrichment was obtained containing both archaea and bacteria. Attempts to isolate the archaea with the use of three bacteria-specific antibiotics failed.

The presence of ammonia monooxygenase gene-carrying archaea in the absence of their bacterial counterpart is evidence for the archaeal role in premises plumbing nitrification.

Introduction

Nitrogen compounds (primarily ammonia) are oxidized by microorganisms to nitrite and nitrate in a process called nitrification that occurs in natural, industrial and agricultural systems (Ward et al., 2011). Although nitrification is an important aspect of the biogeochemical cycle of nitrogen, there can be negative effects when it takes place in drinking water. The USEPA considers nitrite and nitrate to be drinking water contaminants and sets the maximum contaminant level (MCL) in distribution systems of 1 ppm for nitrite and 10 ppm for nitrate (USEPA, 2002) because adverse health effects on humans that include methemoglobinemia in infants and perhaps cancer (Fan and Steinberg, 2002, Aschebrook-Kilfoy et al., 2011) can occur. In drinking water, the ammonia that serves as the energy source for nitrifying organisms can be naturally occurring in the source water or introduced during chloramine formation and/or decay (Zhang et al., 2009). The use of chloramine has been increasing as utilities use this alternative to chlorination to comply with the Stage 2 Disinfectant/Disinfection By-Product Rule which limits the levels of DBPs in drinking water (USEPA, 2000). Disinfection by products (DBPs) arise when bromide or organics react with chlorine and has been an important issue in drinking water because of the possible negative effects of DBPs on humans.

In the traditional view, the two-step process of nitrification is attributed to two groups of organisms: the ammonia oxidizing bacteria and the nitrite oxidizing bacteria. The first step in nitrification in drinking water is attributed to bacterial ammonia oxidizers. Their presence and abundance are well studied and pure cultures of these organisms are routinely used in disinfection studies (Chauret et al., 2005; Wahman et al., 2009; Regan et al., 2003). Previous work conducted to investigate nitrification in premises plumbing also initially assumed that ammonia oxidizing bacteria were responsible for nitrification (Rahman et al., 2011). In this study, glass reactors simulating premises plumbing containing copper or polyvinyl chloride (PVC) surfaces had been nitrifying for several years and were then subjected to potential control mechanisms including the addition of chlorite. The use of chlorite has been proposed as a control mechanism for nitrification because ammonia oxidation by ammonia oxidizing bacteria (AOB) is inhibited by chlorite (Hynes and Knowles, 1983). Low levels of chlorite have also been shown to reduce the culturability of AOB (McGuire et al., 1999), although nitrification control ineffectiveness using chlorite in the field have been reported (Karim and LeChevallier, 2006; McGuire et al., 2006). Surprisingly, an unrealistic high dose of 20 ppm chlorite (20 times the EPA allowed concentration in drinking water (USEPA, 2000)), had limited effect on either the copper or PVC system. Both systems recovered their ability to nitrify after the chlorite treatment was stopped. Subsequent investigations found that ammonia oxidizing bacteria were not detected in either the copper or PVC system (Chapter 2). This work also determined that archaea carrying ammonia monooxygenase genes were present in the reactors.

Archaea have long been known to be extremophiles and as such, they were believed to play only a marginal role in most global element cycles (Nicol et al., 2011). With the development of molecular methods for the detection of microorganisms, they have been discovered in non-extreme environments and their contribution to global element cycles has been elucidated. In recent years, mesophilic archaea with a predicted capability to oxidize ammonia based on their ammonia monooxygenase gene have been discovered (Urakawa et al., 2011). The apparent great abundance of mesophilic ammonia oxidizers in marine, terrestrial and even engineered environments (Urakawa et al., 2011) leads to the conjecture of their possible dominance over their ammonia oxidizing bacteria counterparts in said environments. Abundance has been used as evidence for a greater role for archaea in nitrification (Prosser and Nicol, 2008). The cultivation in pure culture and enrichment of ammonia oxidizing archaea related to ones detected in the oceans (Könneke et al., 2005) and from soil (Tourna et al., 2011; Jung et al., 2011) has provided stronger evidence for the role of this group in ammonia oxidation. Thus, a shift into a more contemporary view of nitrification that involves another group of organisms has resulted.

The reactors used in the study were designed to simulate conditions within a nitrifying premises plumbing with an ammonia level equivalent to that resulting from the decay of 4mg/L chloramine. The reactors have copper and PVC surfaces, two of the most commonly used pipe material in premises plumbing. This system has been nitrifying for more than 6 years as measured by ammonia loss and nitrate accumulation. As ammonia oxidizing archaea were detected in the absence of known bacterial ammonia oxidizers

(Chapter 2), a possible role for archaea is hypothesized. This study was conducted to determine the presence, diversity and abundance of archaea in the reactor systems. Because studies on this group of organisms are limited in the context of water distribution systems, this study also compared their distribution relative to bacteria to provide insight into the overall microbial ecology including assessment of possible plumbing material effect on the populations. The culture independent approach was coupled with the use of propidium monoazide to be able to analyze both total and active populations (Nocker et al., 2007). Because enrichments are important tools in assessing the composition and function of the microbial community, the study also aimed to enrich the autotrophic ammonia oxidizing organisms that putatively includes an ammonia oxidizing archaeon.

The specific objectives of the study were 1) to determine and compare the presence and distribution of the archaea to bacteria by fluorescence in situ hybridization (FISH) using domain specific probes on biofilms formed in nitrifying simulated premises plumbing, 2) to compare the diversity of organisms with archaeal *amoA* gene using DGGE in the copper and PVC reactors and the BAC influent, 3) to determine the identity of the archaea in the system by amplifying and sequencing of the 16S rRNA gene and the *amoA* gene and comparing these with known ammonia oxidizing archaea, 4) to characterize the microbial community by determining and comparing the abundance of archaea and bacteria using 16S rRNA and *amoA* gene copies, and to compare abundance in the source and the reactors, and 5) to enrich for autotrophic ammonia oxidizing archaea and determine ammonia utilization and nitrite production.

We report the presence of organisms that are phylogenetically affiliated with ammonia oxidizing archaea in nitrifying reactors that simulate premises plumbing. The presence of these archaea in greater numbers compared to their known bacterial counterparts can be taken as a preliminary evidence for their contribution to nitrification in the described system. While further studies are needed to confirm the archaeal contribution to drinking water distribution nitrification, results emphasize the need to reevaluate the impact of the composition of the indigenous microbial flora on nitrification. Acknowledging the ecology of drinking water microbial communities involved in nitrification can then influence decisions on drinking water treatment, such as choices of disinfectants, plumbing materials and nitrification prevention/control mechanisms.

Materials and Methods

Reactor Setup

Reactors simulating nitrifying premises plumbing were operated as described previously (Rahman et al., 2011; Chapter 2). Modified CDC reactors (Goeres et al., 2005) were assembled with coupons attached to solid rods and contained a base plate and stir blades made of copper or PVC to simulate surfaces in premises plumbing (Figure 3.1). The surface area of the coupons, base plate and stir blades was equivalent to the surface to volume ratio of a six foot long $\frac{3}{4}$ " diameter domestic plumbing pipe. Volume of the reactors is 120 ml. Each reactor type had four replicate reactors.

Reactors were flushed with peristaltic pumps with shear created by the stirplate for five minutes followed by eight hr stagnation periods thrice daily to simulate flushing and stagnation periods in premises plumbing. The stirplates were set to create a rotational speed of the blade of 300 rpm, which was approximately equivalent to a velocity of 3 ft/s in the bulk water. Reactors were kept in the dark to simulate conditions within distribution pipes.

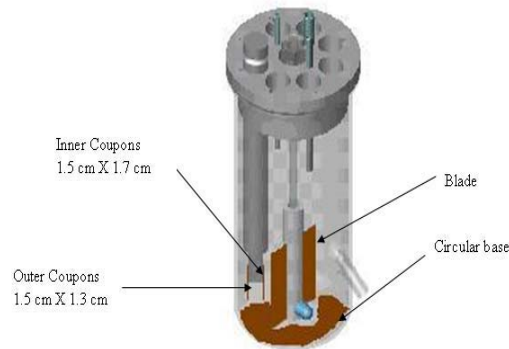


Figure 3.1. Schematic representation of the modified CDC reactor used in the study (Rahman, 2008).

To simulate water quality in premises plumbing, reactors were fed with a combination of mineral amended reverse osmosis (RO) water, biologically treated Bozeman tap water, and a humic substances organic feed as previously described (Rahman et al., 2011; Chapter 2). Ammonium sulfate was added with the RO water (0.71 mgNH₃N/L reactor volume) equivalent to the amount of ammonia that would result from the decay of 4mg/L of chloramine. Bozeman tap water (surface water source, no background ammonia, chlorinated) was treated biologically by passage through a

granular activated carbon column followed by flow through a biologically active carbon (BAC) column and was provided as a separate, parallel influent (BAC water). This served as the sole source of microorganisms, providing a continuous inoculum of indigenous organisms (10^4 CFU/ml of heterotrophic plate count (HPC)) to the reactors. Aside from providing the inoculum for the reactors, the BAC water contained sufficient background phosphate for microbial growth at the organic carbon level used. Organic carbon was supplied to the reactors in a third, separate feed in the form of soil-derived humic substances at a final concentration of 4mg/L carbon as described previously (Rahman et al., 2011). The RO water/ BAC water/organic carbon feed ratio was 50:5:1. All reactors showed signs of stable, complete nitrification as measured by conversion of ammonia to nitrate and had been in operation for over six years at the time of this study.

Biofilm and bulk (liquid) phases of the reactors as well as the influent BAC water were used in the analyses. Sampling was done at the end of the 8 hr stagnation period prior to the flushing period. Biofilm samples for the Fluorescence in situ hybridization (FISH) were obtained at one time point from a representative copper or PVC reactor. Bulk and biofilm samples for the abundance study and end point PCR were obtained from the same representative copper or PVC reactor at another time point, within the same month of the collection of the sample for FISH. PMA treatment was performed to be able to compare total and active populations in the system (Nocker et al., 2007). Inoculum for the enrichment was bulk fluid from the PVC reactor.

Detection and Quantification of Ammonia-oxidizing Organisms

Fluorescence *In Situ* Hybridization (FISH). FISH Probes used in the study (Table 3.1) were obtained from Thermo Scientific Custom Biopolymers (Ulm, Germany). Biofilm was scraped from a 1.5 x 1.7 cm coupon and resuspended in 1ml phosphate-buffered saline (PBS) (0.8%NaCl in 10mM phosphate, pH 7.2). Five hundred µl of resuspended biofilm were mixed with ice cold 4% paraformaldehyde (PFA) solution in PBS. Samples were incubated at 4°C for 4 hrs. A centrifugation (14,000 x g, 5 min) and supernatant removal step was repeated 3 times to remove the paraformaldehyde. Samples were then resuspended in 1 volume of ice cold PBS and 1 volume of ice cold 96% (v/v) ethanol.

Table 3.1. FISH probes used in the study.

Probe	Target	Sequence (5'-3')	Label	Reference
DOPE-EUB338	Bacteria	GCT GCC TCC CGT AGG AGT	Double labeled (DOPE) with one Cy3 on each end	Stoecker et al., 2010; Amann et al., 1990
ARCH915	Archaea	GTG CTC CCC CGC CAA TTC CT	5'Cy5	Stahl and Amann, 1991
DOPE-NONEUB	Control non EUB	ACT CCT ACG GGA GGC AGC	Double labeled with Cy3	Wallner et al., 1993
Control NONSENSE	Control nonsense	AGA GAG AGA GAG AGA GAG	5'Cy5	Hatzenpichler et al., 2008

Five µl of fixed sample was deposited on a Teflon coated slide and air dried for 10 min at 46°C. Samples were dehydrated by dipping the slide for 3 minutes in an

increasing ethanol concentration series of 50, 80 and 100%. Ten μ l of the hybridization buffer (0.9 M NaCl, 20 mM Tris HCl, 35% formamide, 0.01%(w/v) sodium dodecyl sulfate and 0.3ng of each probe) was added to the samples on the slide followed by incubation at 46°C for 3 hrs. After hybridization, the slide was transferred to a 50 ml tube containing the washing buffer (0.07 M NaCl, 0.02 mM Tris HCl, 5 mM EDTA) at 48°C for 10 minutes. After the wash step, the slide was dipped in ice cold water and dried. Samples were mounted with Citifluor AFI antifadent (Citifluor Ltd, Leicester,UK).

A Leica TCS-SPZ AOBS Laser scanning confocal microscope was used for imaging. A 561 nm laser was used to excite Cy3 and a 633 nm laser to excite Cy5. Fluorescence was collected from 568-618 for Cy3 and from 660-800 nm for Cy5. Cy3 fluorescence was false colored red and Cy5 green. Samples were imaged using a HCX PL APO CS100x1.4NA oil objective.

DNA Extraction. For endpoint PCR of 16S rRNA, biofilm samples were obtained from one copper and PVC reactor. The template used for the amplification of bacterial and archaeal 16S rRNA and archaeal *amoA* qPCR standards was from the PVC reactor. Biofilm samples were obtained from the coupons (1.5 x 1.7 cm) by scraping with a rubber policeman. Bulk fluid was collected from the respective reactor at the time the biofilm sample was obtained and filtered through a 0.2 micron CORNING® sterile syringe filter (www.corning.com). The filtered water was used to resuspend the biofilm (500 μ l suspension). For qPCR reactions, one hundred ml bulk water from each representative copper or PVC reactor was collected and filtered through a 0.2 micron polycarbonate membrane (Millipore, www.millipore.com) to collect the biomass.

Biomass from the BAC influent was collected using the same method except 500 ml volume was used. The membrane was cut and resuspended in filtered bulk fluid. DNA extraction followed using the FastDNA® SPIN Kit for Soil (MP Biomedicals, OH) according to the manufacturer's directions except for a modification that includes a step to remove humics by using guanidine thiocyanate (Burr et al., 2006).

PMA Treatment. PMA treatment was carried out using the method of Nocker et al (2007). One μ l of propidium monoazide (Biotium) (1mg PMA in 97 μ l of 20%DMSO) was added to 0.5 ml of the sample in clear 1.5 ml centrifuge tubes and incubated at room temperature in the dark for 5 minutes. The tubes were vortex mixed in the start, middle and end of the incubation period. The tubes were laid flat on ice and subjected to light exposure for 2 minutes using a 650 W halogen light source (sealed beam lamp, FCW 120V, 3,200 K; GE Lighting, General Electric Co., Cleveland, OH). The samples were centrifuged at 5,000Xg for 5 minutes. DNA extraction was performed as described previously. For controls, killed samples were prepared by autoclaving samples at 121°C for 20 minutes. PMA treatment and DNA extraction were then performed as previously described.

Polymerase Chain Reaction (PCR). End point PCR was carried out to determine the identity of archaea in the system as well as to generate standards for the quantitative PCR. DNA extraction from biofilm, bulk phase and BAC samples as well as PMA treatment were carried out as previously described (Chapter 2). All end point PCR amplifications were conducted in an Eppendorf Mastercycler® ep (Eppendorf North

America, www.eppendorfna.com). Reactions were carried out in 25µl volumes which included 0.2µM primers, Go Taq® Green Master Mix (www.promega.com), DEPC treated water, Ultrapure BSA (Ambion) and 1µl of template DNA (approximately 5 ng). To amplify the archaeal 16SrRNA gene from the copper sample, an endpoint PCR was carried out and the resulting PCR product was diluted 1:10 and then used as a template for another round of PCR. All oligonucleotide primers were synthesized by IDT (Integrated DNA Technologies, www.idtdna.com). Table 3.2 shows the primers used in the study and the cycling conditions used for each are shown in Table 3.3. To verify the amplification product, gel purification, cloning and sequencing were performed as described in the subsequent portion. Products were visualized by running on a 0.8% agarose gel with ethidium bromide in 1XTBE buffer for 45 minutes at 40V. Gels were viewed using a FluorChem™ 8800 fluorescence imager (www.alphainnotech.com).

Table 3.2. Target genes and the PCR primers used in the study.

Target gene	Application	Primer	Sequence (5'-3')	Amplicon length (bp)	reference
Archaeal 16SrRNA	End point PCR	21F	TTCCGGTTGATCCYGCCGGA	1471	DeLong, 1992
		1492R	GGTTACCTTGTTACGACTT		
Archaeal 16S rRNA	DGGE screening of clones	ARC344F-GC	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G ACG GGG YGC AGC AGG CGC GA	611	Casamayor et al., 2000 and references herein
		ARC915R	GTG CTC CCC CGC CAA TTC CT		Casamayor et al., 2000 and references herein
Bacterial 16S rRNA	qPCR	338F	ACT CCT ACG GGA GGC AGC AG	180	Einen et al., 2008
		518r	ATT ACC GCG GCT GCT GG		
Archaeal 16S rRNA	qPCR	931F	AGG AAT TGG CGG GGG AGC A	169	Einen et al., 2008
		m1100R	BGG GTC TCG CTC		

Table 3.2- Continued

			GTT RCC		
bacterial <i>amoA</i>	qPCR	amoA-1F	GGGGTTTCTACTGGT GGT	491	Rotthauwe et al., 1997
		amoA-2R	CCCCTCKGSAAAGCC TTCTTC		
archaeal <i>amoA</i>	qPCR	Arch- amoA-for	CTG AYT GGG CYT GGA CAT C	256	Wuchter et al., 2006
		Arch- amoA-rev	TTC TTC TTT GTT GCC CAG TA		
GC clamp	DGGE of archaeal <i>amoA</i>	Attached to Arch- amoA-rev	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C	-	Ferris et al., 1996

Table 3.3. Cycling conditions used to amplify the target genes.

Target gene	Primer pair	Cycling conditions
Archaeal 16S rRNA	21F/1492R	Initial denaturation at 95° C for 1.5 min, annealing at 55° C for 1.5 min, and extension at 72° C for 1.5 min for a total of 30 cycles
Archaeal 16S rRNA (DGGE screening)	ARC344FGC/915R	94°C for 5 min and then 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 9 min
Bacterial 16S rRNA (qPCR)	338F/518R	95 °C for 15 min, 45 cycles of 15 s at 94° C, 30 s at 61°C, 30 s at 72 °C
Archaeal 16S rRNA (qPCR)	931F/m1100R	95 °C for 15 min, 45 cycles of 15 s at 94 °C, 30 s at 64 1C, extension 30 s at 72 °C
Bacterial <i>amoA</i> (qPCR)	amoA-1F/amoA-2R	Initial hold at 50°C for 2 min and denaturation for 10 min at 95°C, followed by 40 cycles of: 95°C for 60 s, 50°C for 60 s, and 72°C for 60 s
Archaeal <i>amoA</i> (DGGE and qPCR)	Arch-amoA-for/Arch-amoA-rev with GC/ Arch-amoA-for/Arch-amoA-rev	Initial hold at 95°C for 10 min, followed by 50 cycles of 95°C for 20 s, 53°C for 45 s, and 72°C for 60 s

Denaturing Gradient Gel Electrophoresis (DGGE). Archaeal *amoA* sequences were amplified using Arch-amoA-for and Arch-amoA-rev with a GC clamp and the sequences were run on a denaturing gel to investigate diversity. DGGE was performed using a DCode™ system (www.biorad.com) using a gel with denaturant concentration of 40%-60% from top to bottom, where 100% denaturant is defined as 7 M urea and 40% formamide. Gels also contained an 8 to 12% polyacrylamide gradient from top to bottom. Ten microliters of each sample were loaded per well and DGGE markers were also run alongside samples as previously described (Chapter 2). Electrophoresis was done at 60 V for 16 h. Gels were stained with Sybr®Gold (www.invitrogen.com) and visualized using a FluorChem™ 8800 fluorescence imager (www.alphainnotech.com).

Cloning and Sequencing of DGGE Bands. Bands were cut from the lanes of the DGGE gel and resuspended in DEPC treated water. To extract the DNA from the gel, a freeze thaw cycle (3 cycles of 1 hr freezing and 1 hr at room temperature) was performed. One microliter of this was then used as template for PCR (using archaeal *amoA* primers without the GC clamp) and PCR products were then purified, cloned and sequenced as described in the following section.

Cloning and Sequencing of PCR Amplicons. PCR products were gel purified using QIAquick® Gel Extraction Kit (QIAGEN). Purified PCR products were cloned into the plasmid vector pCR™4-TOPO® using the TOPO® TA Cloning kit (Invitrogen, www.invitrogen.com) following the manufacturer's protocol. Transformants were inoculated into 10 mL of Luria-Bertani (LB) broth plus 50 mg mL⁻¹ ampicillin. Cultures

were incubated overnight at 37°C in a shaking incubator. Plasmid DNA was purified from individual clones using the Wizard Plus SV Minipreps DNA Purification System (www.promega.com) and quantified using the NanoDrop ND-1000 spectrophotometer (Nano Drop, Wilmington USA). For the sequencing of the almost full length archaeal 16S rRNA gene, clones were screened by checking for insert using a modified method of Burr et al. (2006). One hundred clones from each cloning reaction were screened by pooling 25 clones and checking for differences in migration pattern on a DGGE gel to determine if the inserts have different sequences. Twenty five clones were pooled by mixing 10µl of each clone then using 1µl of this mixture as a template to amplify a portion of the archaeal 16S rRNA gene internal to cloned fragment. PCR was carried out using ARC344F-GC/ARC915R (Casamayor et al., 2000). Profiles of each of the pools were verified for position of bands.

Clones were sent to the Research Technology Support Facility (RTSF) at Michigan State University and were sequenced using the M13F primer. Sequences were analyzed for chimeras using Chimera Slayer (Haas et al., 2011) or Bellerophon (Huber et al., 2004) and were compared with known sequences in the GenBank database using the Basic Local Alignment Sequence Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were submitted to GenBank under the following accession numbers: JQ717287-JQ717299.

Phylogenetic Analysis. The archaeal 16S rRNA sequence amplified from the biofilms from copper and PVC coupons, as well as the amplified archaeal *amoA* from the biofilms from the copper and PVC coupons were analyzed by alignment with sequences

of known archaeal ammonia oxidizers using CLUSTAL W (Thompson et al., 1994) at <http://www.ch.embnet.org/software/ClustalW.html> and their phylogenetic relationships were determined in MEGA4 (Tamura et al., 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). 16S rRNA sequences used in the analyses are as follows: *Nitrosopumilus maritimus* (CP000866), *Candidatus Nitrososphaera gargensis* (GU797786), *Candidatus Nitrosocaldus yellowstonii* (EU239960), *Cenarchaeum symbiosum* (AF083071), and *Nitrosoarchaeum limnia* (Blainey et al., 2011). *amoA* sequences used in the analysis were as follows: *N. maritimus* (EU2399959), *Candidatus N. gargensis* (EU281321), *Candidatus N. yellowstonii* (EU23996), *C. symbiosum* (DQ397580), and *N. limnia* (Blainey et al., 2011). All sequences were obtained from GenBank except for *N. limnia* which was obtained from the supplementary material of Blainey et al. (2011).

Quantitative PCR (qPCR). Bacterial 16SrRNA, bacterial *amoA*, archaeal 16S rRNA and archaeal *amoA* gene abundances were determined. Primers used are given in Table 3.2 and cycling conditions are given in Table 3.3.

qPCR standards for bacterial 16S rRNA gene, archaeal 16S rRNA and archaeal *amoA* genes were obtained by amplifying DNA from the biofilm from the PVC coupon. qPCR standard for bacterial *amoA* gene was obtained by amplifying DNA from *Nitrosomonas europaea* Winogradsky (ATCC strain 25978). Amplicons for each gene were cloned into pCRTM4-TOPO[®] using the TOPO[®] TA Cloning Kit (Invitrogen,

www.invitrogen.com). A purified plasmid was randomly chosen and was sequenced as described previously to verify the gene insert. Plasmid concentration was determined by both a ND-100 spectrophotometer (Nano Drop, Wilmington USA) and by using a Sybr®Gold (www.invitrogen.com) assay with known concentrations of double stranded Lambda DNA (Promega) as the standard for DNA quantification using an ND-3300 fluorospectrometer (Nano Drop, Wilmington USA). Copy number was calculated by considering the size of the plasmid (3890bp) plus insert length (180bp) and assuming a molecular mass of 660 Da for each basepair. qPCR standards were prepared by diluting the plasmid suspension ranging from 8.8×10^3 to 8.8×10^7 target gene copies/ μl (in ten fold dilutions). Standard sequences were deposited in GenBank with the accession numbers: JQ406517-JQ406519.

DNA was extracted as previously described (Chapter 2). Dilutions of sample DNA were prepared and used as template for the qPCR reaction along with standards to determine if samples contained PCR inhibitors. Ct values were plotted against the dilution and compared to that of the standards. The lowest dilution falling within the linear range that was parallel to that of the standards was chosen for analysis. To validate this, a spiked sample dilution was prepared by adding a known amount of standard template to the sample dilution. The Ct value of the spiked dilution was compared to that of a standard with an equal concentration of DNA.

The qPCR reaction mixture consisted of Power SYBR® Green PCR Master Mix (www.appliedbiosystems.com), 0.2 μM each of the forward and reverse primers, 1 $\mu\text{g}/\mu\text{l}$ Ultrapure BSA (50 mg/mL, Ambion, www.ambion.com) and DEPC water. For each 25

µl reaction, 8 µL of template DNA was used. qPCR was performed in a Rotor-Gene 3000 real time PCR cycler (QIAGEN, www.qiagen.com) in a 72-well rotor using the programs specified in Table 3 for each target gene. Data was acquired using the FAM/Sybr detection channel during the extension step. Standards and samples were prepared in duplicate and negative controls containing no template DNA were included to ensure no contamination had occurred. Melt curve analysis was also performed after the amplification program to verify amplification of correct PCR products. Melt curve analysis was performed from 60-95°C in 0.1°C increments held for 5 s with an initial pre-melt hold for 90 s at the first step. To enable direct comparison of gene abundance values among samples, values for the BAC samples were adjusted according to the reactor feed ratio. Cells/ml (BAC, copper and PVC bulk phases) or cells/cm² (biofilm) were computed by dividing the 16S rRNA gene copy values by the average 16S rRNA gene copy number per cell for Bacteria (4.17) and Archaea (1.72) based on the ribosomal RNA operon copy number database (rrnDB) (<http://rrndb.mmg.msu.edu>) (Klappenbach et al., 2001; Lee et al., 2009) accessed December 2011. Univariate analysis of variance (ANOVA) was performed on the gene copy abundance data with a general linear model using Minitab 16 (Minitab).

Enrichment from PVC Reactor

To enrich for autotrophic nitrifiers, bulk fluid from the PVC reactor was serially diluted up to 10⁻⁷ and inoculated at 10% inoculum into synthetic Crenarcheota medium (SCM) (Könneke et al., 2005) modified by reducing the concentration of NaCl to 1g/L and adding Al₂(SO₄) 18H₂O (0.62 mg/L) and Na₂SiO₃ 9H₂O (26 mg/L). Cultures were

incubated at ambient temperature with shaking in the dark. Enrichment with the undiluted bulk fluid was transferred into fresh media (10%) bimonthly and enrichment cultures consistently produced nitrite.

To determine the sensitivity to light of the nitrifying community, nitrite production was determined in cultures kept in the dark by covering the culture bottle and those maintained in non covered bottles. Ten ml of the enrichment was inoculated into 50 ml fresh medium in triplicates in covered and uncovered culture bottles. Nitrite production was determined as described in the subsequent portion.

To characterize the ammonia oxidation in the enrichments, 100 ml of the enrichment was inoculated into 1L fresh medium in quadruplicate. Ammonia was measured by the phenate method (Eaton et al., 2005) using a Synergy™ HT Multi-detection microplate reader (BioTek Instruments, Winooski, VT). Nitrite was measured using the Griess Ilosvay method (Alexander and Clark, 1965) with NO_2N standards 1, 0.5, 0.2, 0.1, 0.01 and 0 ppm, and nitrate was measured by ion chromatography using a Dionex ion chromatography system with a CD20 conductivity detector and GP40 gradient pump unit. An AS4A column and DS3 detection stabilizer was also used in this method. Calibration was done using the following sodium nitrate standards: 1, 0.5, 0.2, 0.1, and 0 ppm $\text{NO}_3\text{-N}$. To minimize experimental error, after every seven measurements a standard solution of nitrate was measured to check the accuracy of the measurement, making sure that the measured standard was within 90-110% of the standard value otherwise calibration was repeated and the sample was measured again (Eaton et al., 2005).

Samples for DNA extraction were taken at day 0, 7 and 27. One replicate was used at each sampling point to minimize any effect that withdrawing the culture medium might have on the cultures. DNA was extracted as described in the previous section. Total bacterial and archaeal 16SrRNA gene abundances were determined by qPCR as described in the previous section. Archaeal *amoA* was amplified from the DNA extracted from the enrichments as described in the previous section but with modified PCR cycling conditions that included a touchdown step with the annealing temperature starting at 60 °C, decreasing by 1 °C after every two cycles up to 56 °C and followed by 30 cycles with annealing at 53 °C.

To provide selective pressure favoring the growth of archaea, antibiotics that inhibit bacteria but not archaea were used. Transfers of the enrichment to fresh medium with 50µg/ml of one of the following antibiotics streptomycin, kanamycin and ampicillin was done and nitrite production was monitored at least every 5 days and a final measurement at day 21 of incubation. Presence of heterotrophic bacteria was determined by plating on R2A medium, with and without the combination of the three antibiotics. Because heterotrophic lifestyle is possible for archaea, heterotrophic growth was verified by picking resulting colonies resuspending these in 50 µl of DEPC water. One µl of this was used as a template for PCR amplifying the archaeal 16S rRNA gene as previously described.

Results

Detection and Identification of Archaea in the Nitrifying System

FISH. Using domain specific probes, archaea were detected along with bacteria in the biofilm from copper and PVC coupons (Figure 3.2). Nonspecific binding was not observed using the control probes indicating specificity of binding. Archaea appear to be more abundant in the PVC sample as compared to the copper.

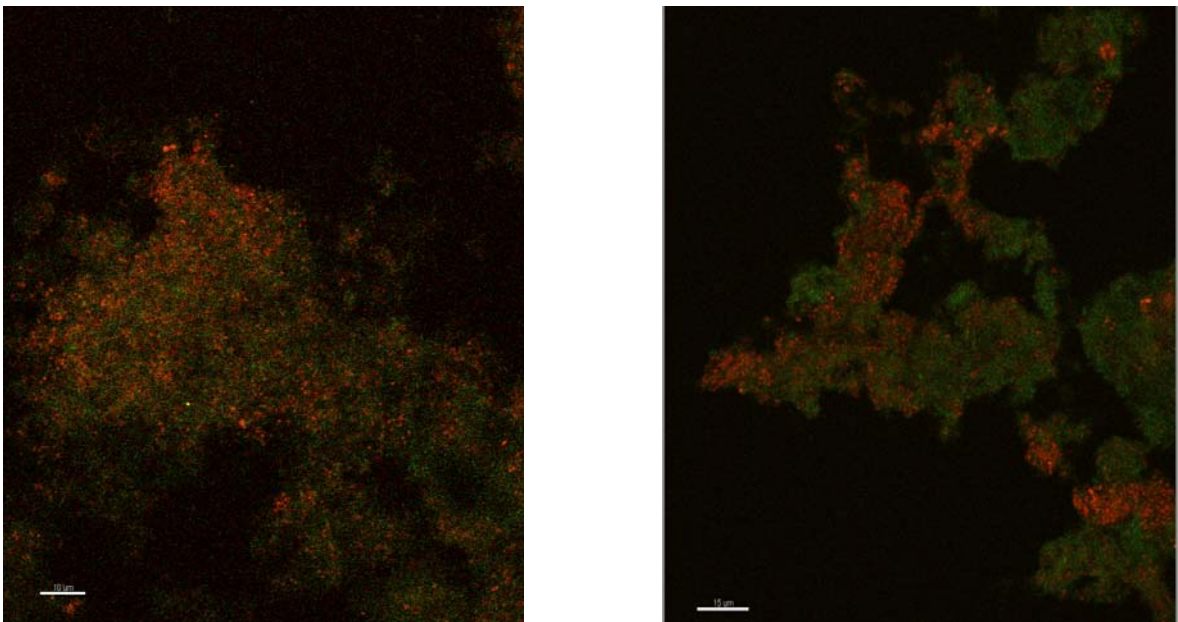


Figure 3.2. FISH microscopy images of biofilms from copper (left) and PVC (right) coupons showing bacteria (red) and archaea (green).

DGGE. Analysis of the archaeal *amoA* amplicons indicate the presence of at least two phylotypes which were both detected in the BAC influent as well as the biofilm from the copper coupon (Figure 3.3). Only one of the two phylotypes was detected in the PVC

system. The PMA treated BAC influent showed both bands while the lower band, although present, was very faint in the non PMA treated BAC sample. This may be because of the removal of DNA from the other phylotype that allows for the amplification of the other phylotype.

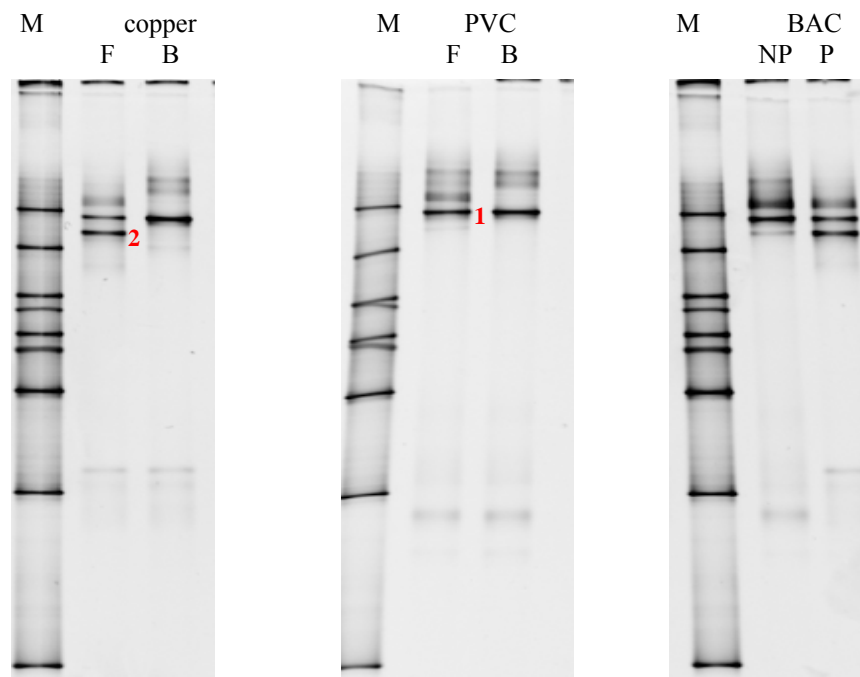


Figure 3.3. DGGE profile of the archaeal *amoA* genes amplified from copper, PVC and BAC influent water. (M=DGGE marker, F=biofilm, B=bulk, NP=non PMA treated, P=PMA treated). The upper (1) and lower (2) bands were excised for sequencing.

Phylogenetic Analysis. An almost full length archaeal 16S rRNA gene fragment was amplified from both the biofilm from the PVC coupon and the copper coupon. However, a two step PCR was needed to be able to amplify the 16S rRNA gene from the copper sample, using the diluted amplicon from the first round of PCR as template for a

second round. Screening of the archaeal 16S rRNA gene clones showed that all clones had the same insert as revealed by their identical migration on the DGGE gel. Identical sequences were amplified from both PVC and copper and were found to be similar to *Nitrosotenuis uzonensis* (98.5%), *Nitrosoarchaeum limnia* (94%) and *Nitrosopumilus maritamus* (94%), all of which are known ammonia oxidizing archaea (Figure 3.4). Figure 3.5 shows the phylogenetic relationship of the 16S rRNA sequence amplified from the nitrifying reactors.

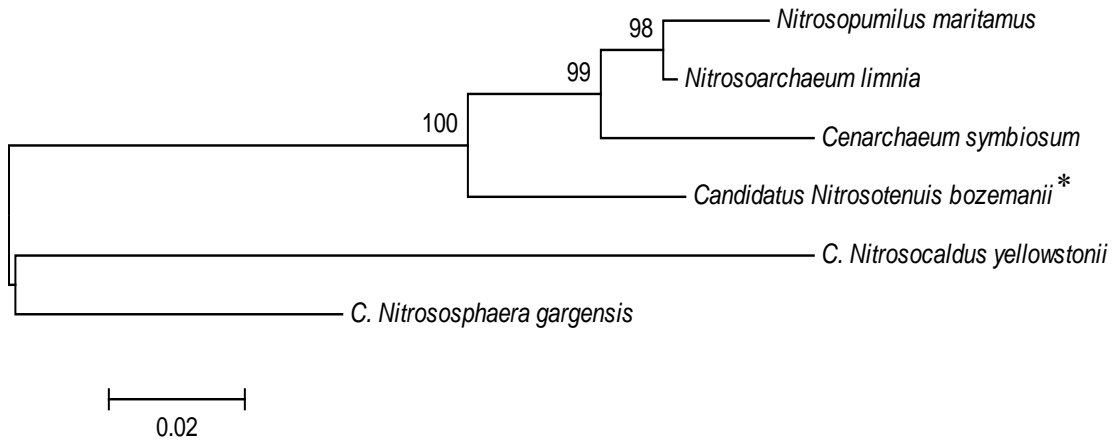


Figure 3.4. Evolutionary relationships of the amplified archaeal 16S rRNA sequence indicated by asterisk (*Candidatus Nitrosotenuis bozemanii*) with five known archaeal ammonia oxidizers. Phylogenetic analysis was done in MEGA4 (Tamura et al., 2007).

```

C.Nitrosotenuis -----TTTCCCTTGTCACTGACCTCAAGCTCGATAATGCCAACAGACACTA
Nitrosopumilus -----TTTCCCTTGTCAAGAACCTCAAGTTGCGATAACGCCAATCAGACGTCA
C.Nitrosocaldus -----TCTCCCCCTCAGCGGGTTACGTTCCGGTGATACCAACATGCCACCA
C.Nitrososphaera CCTTGTACGACTTCTCCCCCGTGTGCTGACCCAGGTTGCGATAAGCCCAATAGGGCCCA
Cenarchaeum -----TTTCCCTTGTGCGCTTACCTCAAGTTGCGATAACGCCAATTAGACGTCA
Nitrosoarchaeum -----TTTCCCTTGTCACTTACCTCAAGTTGCGATAACGCCAATCAGACGTCA
          * * * * *

C.Nitrosotenuis CCTCACTAAAAGCCAACTTCAATGAAACGACGGGGCGGTGTGTGCAAGGAGCAGGGGACGTA
Nitrosopumilus CCTCGCTAAAAGCTCACTTCAATGAAACGACGGGGCGGTGTGTGCAAGGAGCAGGGGACGTA
C.Nitrosocaldus CCTCACGTAACCCACCTCGGGTGAAGCGACGGGGCGGTGTGTGCAAGGAGCAGGGGACGTA
C.Nitrososphaera CCTCGCAAGAACCCGACTTTCGGTGAAGCGACGGGGCGGTGTGTGCAAGGAGCAGGGGACGTA
Cenarchaeum CCTCACTAAAAGCAAACCTTCAATGAAACGACGGGGCGGTGTGTGCAAGGAGCAGGGGACGTA
Nitrosoarchaeum CCTCGCTAAAAGCAAACCTTCAATGAAACGACGGGGCGGTGTGTGCAAGGAGCAGGGGACGTA
          * * * * *

C.Nitrosotenuis TTACCCGCGCGGTAGTGACGCGCAATTACTAGGGATTCCATATTCATGAGGGCGAGTTAC
Nitrosopumilus TTACCCGCGCGATAATGACACCGCAGTTACTAGGGATTCCATATTCATGAGGGCGAGTTGC
C.Nitrosocaldus TTACCCGCGCGATGTTGACACCGCAGTTACTAGGGATTCCAGATTCCAGGGCGGGTTGC
C.Nitrososphaera TTACCCGCGCGATGTTGACACCGCAGTTACTAGGGATTCCATATTCATGAGGGCGAGTTGC
Cenarchaeum TTCACTCGCGGTAATGACGCGCGTACTAGGGATTCCAGATTCCATGAGGGCGAGTTGC
Nitrosoarchaeum TTACCCGCGCGATAATGACACCGCAGTTACTAGGGATTCCATATTCATGAGGGCGAGTTGC
          * * * * *

C.Nitrosotenuis AGCCCTCAATCATAACTGAGGTATTGTTGATG-ATTGCCTCCTCTTCCGGATTCCGAA
Nitrosopumilus AGCCCTCAGTCATAACTGTGGTAACTGTTGAGG-ATTACCTCCTCTTCCGGATTCCGAAA
C.Nitrosocaldus AGCCCTCAGTCATAACTGAGGTAACTGTTGAGG-ATTACCTCCTCTTCCGGATTCCGAA
C.Nitrososphaera AGCCCTCAATCATAACTGAGGTAACTGTTGAGG-ATTACCTCCTCTTCCGGATTCCGAA
Cenarchaeum AGCCCTCAGTCATAACTGTGGTAACTGTTGAGG-ATTACCTCCTCTTCCGGATTCCGAA
Nitrosoarchaeum AGCCCTCAGTCATAACTGTGGTAACTGTTGAGG-ATTACCTCCTCTTCCGGATTCCGAA
          * * * * *

C.Nitrosotenuis CACATTGTCAATACCATTGACGCCCCGCTGTGGCCCCAGAGTTTCCGGGCATACTGACCT
Nitrosopumilus CCATTCAGTATCACTGACGCGCGGTGTGGCCAGAGTTTCCGGGCATACTGACCT
C.Nitrosocaldus CCATTCAGTATCACTGACGCGCGGTGTGGCCAGAGTTTCCGGGCATACTGACCT
C.Nitrososphaera CCATTCAGTATCACTGACGCGCGGTGTGGCCAGAGTTTCCGGGCATACTGACCT
Cenarchaeum CCATTCAGTATCACTGACGCGCGGTGTGGCCAGAGTTTCCGGGCATACTGACCT
Nitrosoarchaeum CCATTCAGTATCACTGACGCGCGGTGTGGCCAGAGTTTCCGGGCATACTGACCT
          * * * * *

C.Nitrosotenuis GCGGTGCGCCCTTCCCTCCCGCATTAACTGCGGGCGTCTACTAATTCCGTCGCGTAC
Nitrosopumilus GCGGTGCGCCCTTCCCTCCCGCATTAACTGCGGGCGTCTACTAATTCCGTCGCGTAC
C.Nitrosocaldus GCGGTGCGCCCTTCCCTCCCGCATTAACTGCGGGCGTCTACTAATTCCGTCGCGTAC
C.Nitrososphaera GCGGTGCGCCCTTCCCTCCCGCATTAACTGCGGGCGTCTACTAATTCCGTCGCGTAC
Cenarchaeum GCGGTGCGCCCTTCCCTCCCGCATTAACTGCGGGCGTCTACTAATTCCGTCGCGTAC
Nitrosoarchaeum GCGGTGCGCCCTTCCCTCCCGCATTAACTGCGGGCGTCTACTAATTCCGTCGCGTAC
          * * * * *

C.Nitrosotenuis TCCAGAAAGTAACGGTAGCAACTAGGAGCAGGGGCTCGCTCGTTACCTGACTTAACAGG
Nitrosopumilus TCCAGAAAGTAACGGTAGCAACTAGGAGCAGGGGCTCGCTCGTTACCTGACTTAACAGG
C.Nitrosocaldus TCCAAAGAGTAATGGTAGCAACTAGGAGCAGGGGCTCGCTCGTTACCTGACTTAACAGG
C.Nitrososphaera TCCAGAAAGTAACGGTAGCAACTAGGAGCAGGGGCTCGCTCGTTACCTGACTTAACAGG
Cenarchaeum TCCAGAAAGTAACGGTAGCAACTAGGAGCAGGGGCTCGCTCGTTACCTGACTTAACAGG
Nitrosoarchaeum TCCAGAAAGTAACGGTAGCAACTAGGAGCAGGGGCTCGCTCGTTACCTGACTTAACAGG
          * * * * *

C.Nitrosotenuis ACATCTCAGGCACGAGCTGGCGACGGCCATGCACCACCTCTCAGCTTGTCTGGTAAAGT
Nitrosopumilus ACATCTCAGGCACGAGCTGGCGACGGCCATGCACCACCTCTCAGCTTGTCTGGTAAAGT
C.Nitrosocaldus ACACCTCAGGCACGAGCTGGCGACGGCCATGCACCACCTCTCAGCTTGTCTGGTAAAGT
C.Nitrososphaera ACATCTCAGGCACGAGCTGGCGACGGCCATGCACCACCTCTCAGCTTGTCTGGTAAAGT
Cenarchaeum ACATCTCAGGCACGAGCTGGCGACGGCCATGCACCACCTCTCAGCTTGTCTGGTAAAGT
Nitrosoarchaeum ACATCTCAGGCACGAGCTGGCGACGGCCATGCACCACCTCTCAGCTTGTCTGGTAAAGT
          * * * * *

C.Nitrosotenuis CTTACGCTTGACCTTCACTTCTGCTGTGCTCGCGGTAAGATTCTGGCGTGGACTCCAAT
Nitrosopumilus CTTACGCTTGACCTTCACTTCTGCTGTGCTCGCGGTAAGATTCTGGCGTGGACTCCAAT
C.Nitrosocaldus CTTACGCTTGACCTTCACTTCTGCTGTGCTCGCGGTAAGATTCTGGCGTGGACTCCAAT
C.Nitrososphaera CTTACGCTTGACCTTCACTTCTGCTGTGCTCGCGGTAAGATTCTGGCGTGGACTCCAAT
Cenarchaeum CTTACGCTTGACCTTCACTTCTGCTGTGCTCGCGGTAAGATTCTGGCGTGGACTCCAAT
Nitrosoarchaeum CTTACGCTTGACCTTCACTTCTGCTGTGCTCGCGGTAAGATTCTGGCGTGGACTCCAAT
          * * * * *

C.Nitrosotenuis TGAACCCGAGGCTTACCCCTTGTGGTGTCCCGCGCAATTCCCTTAAAGTTTCATACTT
Nitrosopumilus TGAACCCGAGGCTTACCCCTTGTGGTGTCCCGCGCAATTCCCTTAAAGTTTCATACTT
C.Nitrosocaldus TGAACCCGAGGCTTACCCCTTGTGGTGTCCCGCGCAATTCCCTTAAAGTTTCATACTT
C.Nitrososphaera TGAACCCGAGGCTTACCCCTTGTGGTGTCCCGCGCAATTCCCTTAAAGTTTCATACTT
Cenarchaeum TGAACCCGAGGCTTACCCCTTGTGGTGTCCCGCGCAATTCCCTTAAAGTTTCATACTT
Nitrosoarchaeum TGAACCCGAGGCTTACCCCTTGTGGTGTCCCGCGCAATTCCCTTAAAGTTTCATACTT
          * * * * *

C.Nitrosotenuis GCGTACGTACTTCCCAGGGCGGCAAACTTAAACGGCTTCCCTGCGGCACTGCATTGGC-CAC
Nitrosopumilus GCGTACGTACTTCCCAGGGCGGCAAACTTAAACGGCTTCCCTGCGGCACTGCATTGGC-CAC
C.Nitrosocaldus GCGGCGGTACTTCCCAGGGCGGTAAGGTTAAACGGCTTCCCTGCGGCACTGCATTGGC-CAC
C.Nitrososphaera GCGACCGTACTTCCCAGGGCGGCAAACTTAAACGGCTTCCCTGCGGCACTGGGTGGCGCG-
Cenarchaeum GCGTACGTACTTCCCAGGGCGGCAAACTTAAACGGCTTCCCTGCGGCACTGCATTGGC-CAC
Nitrosoarchaeum GCGTACGTACTTCCCAGGGCGGCAAACTTAAACGGCTTCCCTGCGGCACTGCATTGGC-CAC
          * * * * *

C.Nitrosotenuis AAGCCAATGCATCAGGAGTTTGCATCGTTTACAGCTGGGACTACCCGGGTATCTAATCC
Nitrosopumilus AAGCCAATGCATCAGGAGTTTGCATCGTTTACAGCTGGGACTACCCGGGTATCTAATCC
C.Nitrosocaldus GGGT-AACCCAACTCAGGCTCATCGTTTACAGCTGGGACTACCCGGGTATCTAATCC
C.Nitrososphaera AAGCCAATGCATCAGGAGTTTGCATCGTTTACAGCTGGGACTACCCGGGTATCTAATCC
Cenarchaeum AAGCCAATGCATCAGGAGTTTGCATCGTTTACAGCTGGGACTACCCGGGTATCTAATCC
Nitrosoarchaeum AAGCCAATGCATCAGGAGTTTGCATCGTTTACAGCTGGGACTACCCGGGTATCTAATCC
          * * * * *

C.Nitrosotenuis GGTT-
Nitrosopumilus GGTTT
C.Nitrosocaldus GGTT-
C.Nitrososphaera -----
Cenarchaeum GGTT-
Nitrosoarchaeum GGTTT
    
```

Figure 3.5. Alignment using CLUSTAL W of the amplified archaeal 16S rRNA gene from the nitrifying reactors with the 16S rRNA gene of known archaeal ammonia oxidizers.

Sequence analysis of the two *amoA* bands from the denaturing gel revealed a 97% similarity between the two (Figure 3.6). Figure 3.7 shows the evolutionary relationship of the amplified *amoA* sequences with *amoA* from known archaeal ammonia oxidizers.

```

upperband      ACTGGGCCTGGACATCGTTTGAATATTCTCAATATCA--CAATCGTTGATGTTGGTAGT
lowerband      ACTGGGCCTGGACATCGTTTGAATATTCTCAATATCA--CAATCGTTGATGTTGGTAGT
Cenarchaeum    ACTGGGCCTGGACCTCCTTCTAGTATTCTCGATCTC--CCAGTCTACAATGCTCGTAGT
Nitrososphaera ACTGGGCCTGGACTTCGTACACGGTATTCTCAATATCG--CAAACGTTGATGCTTATGTF
Nitrosopumilus ACTGGATGTGGACATCCTTTGTAATATTC--AACCTTAGTCAAGCAACAATGCTGTAGT
Nitrosocaldus  ACTGGGCTTGGACATCCTTTGTTGATTTTCAATATCA--CAACATGATGCTCGCGGT
Nitrosoarchaeum *****

upperband      CGGCGCATGCTACTATCTCACATTTACGGGAGTCCCAGGAACAGCAACGTATTATGCGTTF
lowerband      TGGCGCATGCTACTATCTCACATTTACGGGAGTCCCAGGAACAGCAACGTATTATGCGTTF
Cenarchaeum    CGGTGCAACGTAACCTGACGTTTACAGGCGTACCCAGGAACCGGCACATACTATGCCCTT
Nitrososphaera GGGCGCATATACTACATGCTCTTACAGGAGTACCAGGAACCGGCACATACTATGCAAC
Nitrosopumilus AGGTGCAACATATTACCTAACATTTACAGGCGTTCAGGCAACAGCAACGTACTACGCTCT
Nitrosocaldus  AGGCACAATCTATTACCTACTCTTACAGGTGTTCCGTTACAGCAACATGCTAC
Nitrosoarchaeum AGGTGCAACATACTACCTGACATTTACAGGTGTTCCAGGAACAGCGAGTATTACGCCCT
*****

upperband      GATAATGACCGTCTACACATGGGTAGCAAAGGTGCATGGTTTGCA--CTCGGATATCC
lowerband      GATAATGACCGTCTACACATGGGTAGCAAAGGTGCATGGTTTGCA--CTCGGATATCC
Cenarchaeum    CATCATGACCGTGTATGATGGTAGCAAAGGGCGCATGGTTCGCC--CTTGGCTACCC
Nitrososphaera AATCATGACAATCTACACCTGGGTGCAAAGGGCGCATGGTTCGCC--CTCGGCTACCC
Nitrosopumilus GATFATGACAGTTTACACATGGATTGCAAAGGCGAGCCTGGTTCCTCA--CTAGGTTATCC
Nitrosocaldus  AATAGCCACAATCTATACATGGGTGCAAAGTGGGCATGGATAGGAGGCTTAGGATACCC
Nitrosoarchaeum AATTATGACAGTATACATGGGTAGCAAAGGTGCATGGTTTGCA--CTAGGATATCC
*****

upperband      ATATGACTTCATTGTAACACCAGTTTGGCTACCGTCCGCAATGTTGCTCGACTTGGCGTA
lowerband      ATATGACTTCATTGTAACACCAGTTTGGCTACCGTCCGCAATGTTGCTCGACTTGGCGTA
Cenarchaeum    GTATGACTTCATTGTAACACCAGTTTGGCTACCGTCCGCAATGTTGCTCGACTTGGCGTA
Nitrososphaera GTACGACTTCATTGTAACACCAGTTTGGCTACCGTCCGCAATGTTGCTCGACTTGGCGTA
Nitrosopumilus ATATGACTTCATTGTAACACCAGTTTGGTTACCATCAGCAATGCTGTTGGACTTAGTCTTA
Nitrosocaldus  ATATGACTTCATTGATAGTCCAGTATGGATCCAAAGTGGCAATGCTGTTAGATCTAGCATA
Nitrosoarchaeum ATATGACTTCATTGTAACACCAGTTTGGTTACCATCAGCAATGTTGCTTGTATTAGCCTTA
*****

upperband      CTGGGCAACAAGAA---
lowerband      CTGGGCAACAAGAA---
Cenarchaeum    CTGGGCCACGAAGCG---
Nitrososphaera CTGGGCAACAAGAA---
Nitrosopumilus CTGGGCAACAAGAA---
Nitrosocaldus  CTGGGCTACAAGAG---
Nitrosoarchaeum TTGGGCCACAAGAA---
*****
    
```

Figure 3.6. Alignment using CLUSTAL W of the archaeal *amoA* sequences from the reactors with the *amoA* from known archaeal ammonia oxidizers.

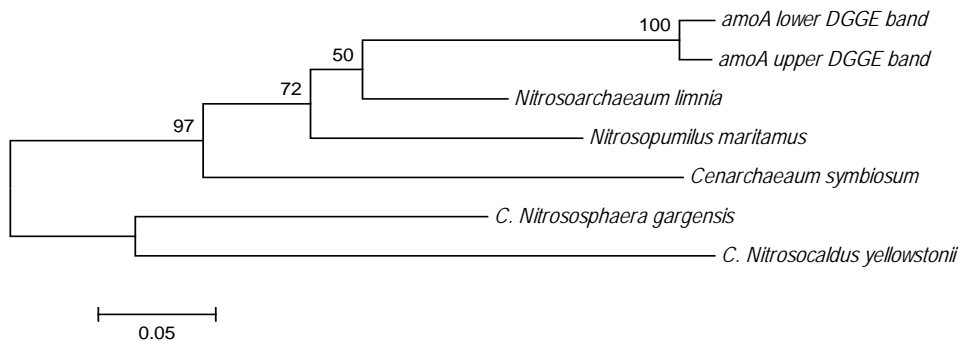


Figure 3.7. Evolutionary relationships of the two *amoA* sequences with the *amoA* gene sequences of five known archaeal ammonia oxidizers. Phylogenetic analysis was done in MEGA4 (Tamura et al., 2007).

QPCR.

Active (PMA treated) (Nocker et al., 2007) and total abundance of bacteria and archaea were determined in the BAC and bulk and biofilm phases of the copper and PVC reactors. Because the average 16S rRNA copies per cell is different between Bacteria and archaea, cells/ml or cells/cm² were reported. Values were computed using the copies/ml or copies/cm² values as previously described to be able to make a more direct comparison of cell number abundances. For the *amoA* gene qPCR data, results are reported as copies/ml or copies/cm² because bacterial *amoA* was not detected in any of the samples thus no direct comparison is needed.

Abundance in Bulk Phase. Figure 3.8 shows the cells/ml of the BAC (influent), copper reactor bulk water and PVC reactor bulk water. Overall, PMA treated samples are not significantly different from non-PMA treated samples ($p \geq 0.1257$). Bacterial abundance is greater in copper reactor bulk water and PVC reactor bulk water compared to the BAC by almost 3 logs ($p \leq 0.004$). Bacterial abundance in copper reactor bulk water and PVC reactor bulk water are not significantly different at $p \geq 0.8862$.

Archaeal abundance is not significantly different between BAC and PMA treated copper reactor bulk water ($p = 0.5091$). Non PMA treated copper reactor bulk water is not significantly different from PMA treated PVC reactor bulk water ($p = 0.2757$) but there are 2 logs less archaea in the PMA treated copper reactor bulk water compared to PVC reactor bulk water ($p \leq 0.0017$).

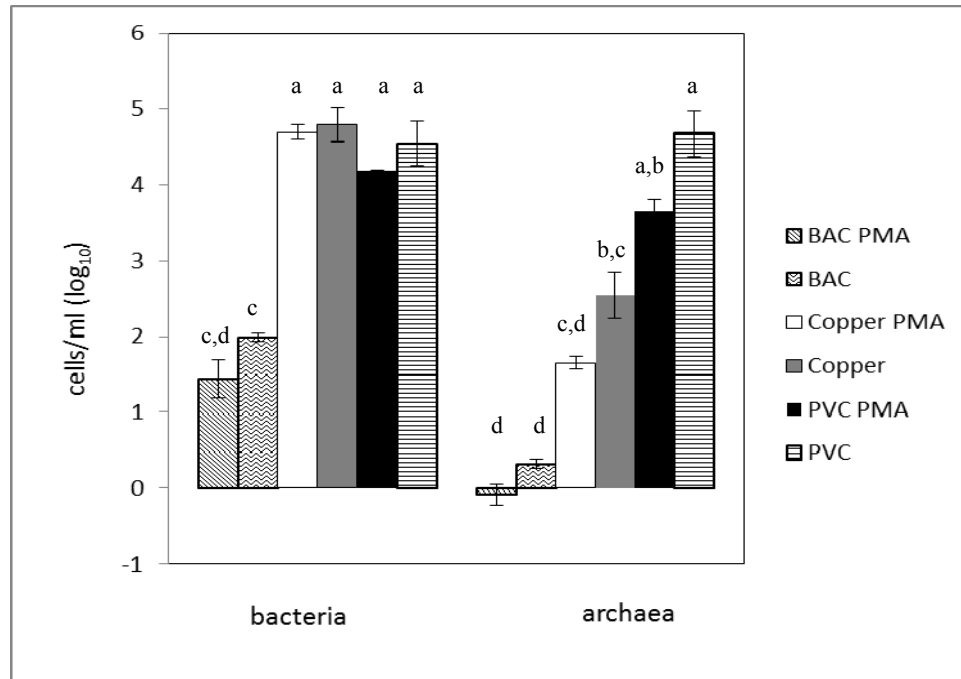


Figure 3.8. Cells/mL of bacteria and archaea in the bulk phase of the copper and PVC reactors and in the BAC influent as determined by qPCR (N=2). Bars that share the same lowercase letter are not significantly different ($p > 0.05$).

Comparing the abundance of bacteria and archaea from reactors with each material, there are almost 2 logs more bacteria than archaea in BAC (influent) ($p = 0.0059$). Similarly, there are more bacteria than archaea in copper reactor bulk water with more than 2 logs difference ($p \leq 0.0045$). On the other hand, there is no significant difference between bacteria and archaea in PVC reactor bulk water ($p \geq 0.5139$).

For the archaeal *amoA* gene (Figure 3.9), PMA treated BAC and PVC reactor bulk water samples are significantly less than their non PMA treated counterparts by approximately half a log ($p = 0.0416$ and $p = 0.0323$). BAC is not significantly different from copper reactor bulk water ($p > 0.1410$) while there are less archaeal *amoA* genes in

copper reactor bulk water than in PVC reactor bulk water with more than a 2 log difference ($p=0.000$).

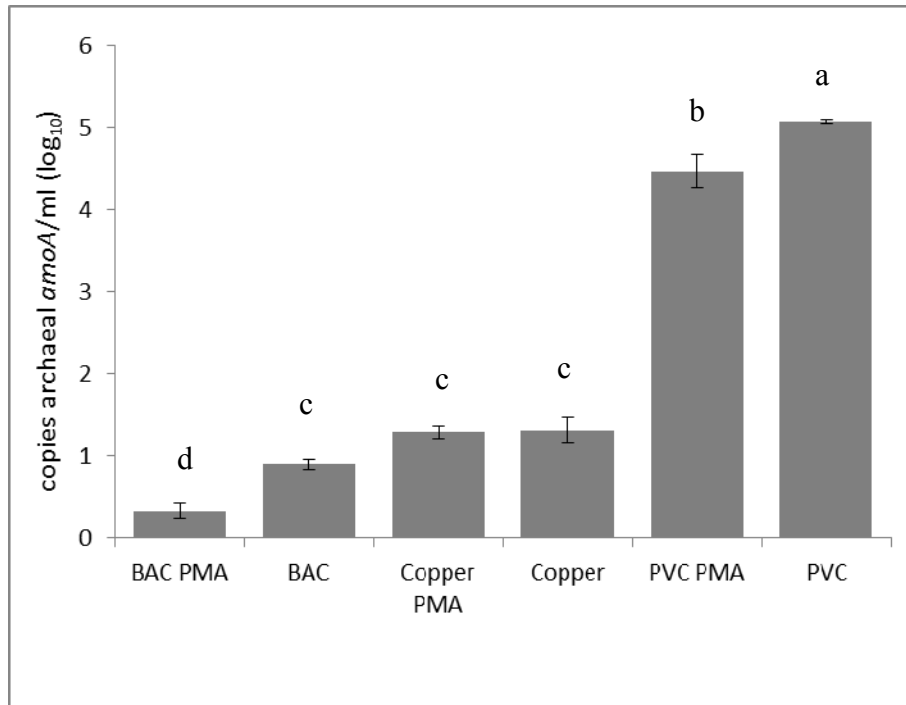


Figure 3.9. Copies archaeal *amoA* gene/mL (N=2). Bars that share the same lowercase letter are not significantly different ($p>0.05$).

Abundance in Biofilms. Figure 3.10 shows the cells/cm² of bacteria and archaea on copper and PVC coupons. PMA treated samples were not significantly different from their non PMA treated counterparts. Bacteria on the biofilm from the copper and PVC coupons were not significantly different ($p>0.9270$) while there are significantly more archaea on the biofilm from the PVC coupon than the biofilm on the copper coupon with a 3 log difference ($p<0.0004$). There are significantly more bacteria than archaea on the biofilm from the copper coupon at a 4 log difference ($p=0.000$). Bacteria and archaea cell

numbers are the same for the non PMA treated biofilm from the PVC coupons ($p>0.4872$). However, in the PMA treated biofilm sample from the PVC coupon, there are fewer archaea than bacteria with a difference of approximately 1.5 logs ($p<0.0117$).

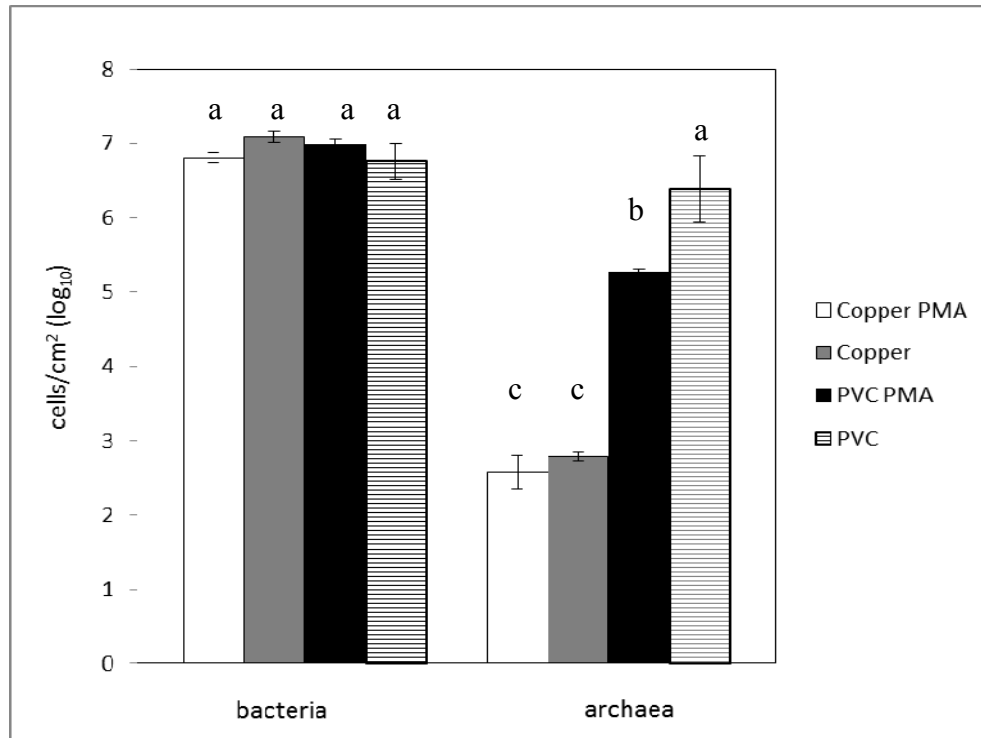


Figure 3.10. Cells/cm² of bacteria and archaea on the copper and PVC coupon (N=2). Bars that share the same lowercase letter are not significantly different ($p>0.05$).

Figure 3.11 shows the copies of archaeal *amoA* on the biofilm from the copper and PVC coupons. The PMA treated samples are not significantly different from each other ($p>0.3407$). *amoA* copies are fewer by almost 4 logs on the biofilm from the copper coupon than that of from the PVC ($p<0.0083$).

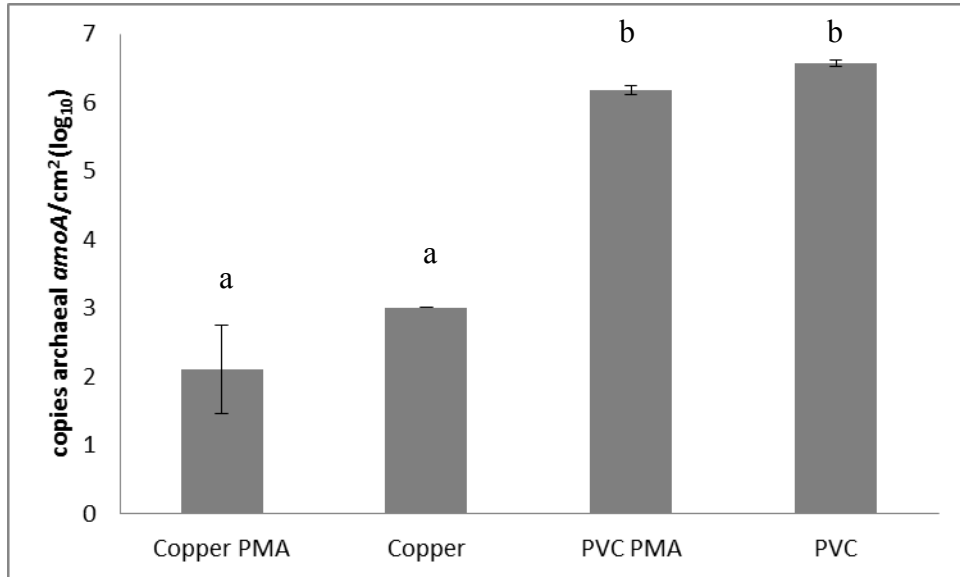


Figure 3.11. Copies of archaeal *amoA*/cm² of copper and PVC coupons (N=2). Bars that share the same lowercase letter are not significantly different ($p > 0.05$).

Enrichment of Nitrifying Community

A culture that consistently produced nitrite was obtained by inoculating undiluted bulk fluid from the PVC reactor with transfers into fresh media (10%) bimonthly. Because archaeal ammonia oxidation like bacterial ammonia oxidation is known to be photosensitive (Merbt et al., 2012), photosensitivity of the culture was determined by incubating both in the presence and absence of light (Figure S3). Rate of nitrite production was found to be higher in the cultures incubated in the dark (0.06 ppm/day) compared to the cultures exposed to light (0.02 ppm/day) suggesting inhibition of ammonia oxidation by light exposure. Upon this determination, all cultures were incubated in the dark.

Figure 3.12 shows the ammonia loss with corresponding nitrite and nitrate production in the enrichments. Both nitrite and nitrate level off at around day 35. Using the points where slope was maximum, rate of ammonia loss was determined to be 0.5ppm/day, lower than the rate of ammonia loss in the reactors where the inoculum was for the enrichments was taken (~2-3ppm/day). Rate of nitrite accumulation was determined to be 0.07ppm NO₂-N/day. Since nitrification proceeded to nitrate, rate of nitrate+ nitrite was determined to be 0.41ppm/day. A consortium of bacteria and archaea were enriched using the medium and culture conditions employed as found by amplification of both archaeal and bacterial 16S rRNA genes. Archaeal *amoA* was also successfully amplified from the enrichments. The presence of a consortium and not a pure culture may explain the slight differences in the rates of nitrite production in the various cultures described in this study as a more reproducible result can be expected from pure cultures. There was an initial increase in both bacteria and archaea but a decrease in archaea coincided with a decrease in pH from an initial pH of 8.15 to 6. Attempts to produce pure cultures of archaea involved serial dilution and the failure to successfully do so may be partly due to the presence of an almost equal number of bacteria and archaea in the system (Figures 3.8 and 3.10).

The effect of antibiotics that are known to inhibit bacteria and not archaea was determined (Figure S4). Nitrite was observed in the enrichment with the antibiotic ampicillin at 0.03ppm NO₂-N/day which was lower than the control at 0.06ppm NO₂-N/day, suggesting a slight inhibition. No increase in nitrite was observed in the enrichments with kanamycin and streptomycin suggesting a total inhibition of ammonia

oxidation. An endpoint measurement determined nitrate in both the control and enrichment with ampicillin at 8.68 ppm and 8.32 ppm respectively, while it was not detected in the enrichments with kanamycin or streptomycin. Heterotrophic members of the consortium were detected when the enrichment culture was inoculated into a heterotrophic medium (R2A) with and without antibiotics. Presence of heterotrophs that are resistant to the antibiotics used in the study was determined, therefore the selective pressures applied (media without heterotrophic carbon source and antibiotics) to select for ammonia oxidizing archaea were not successful.

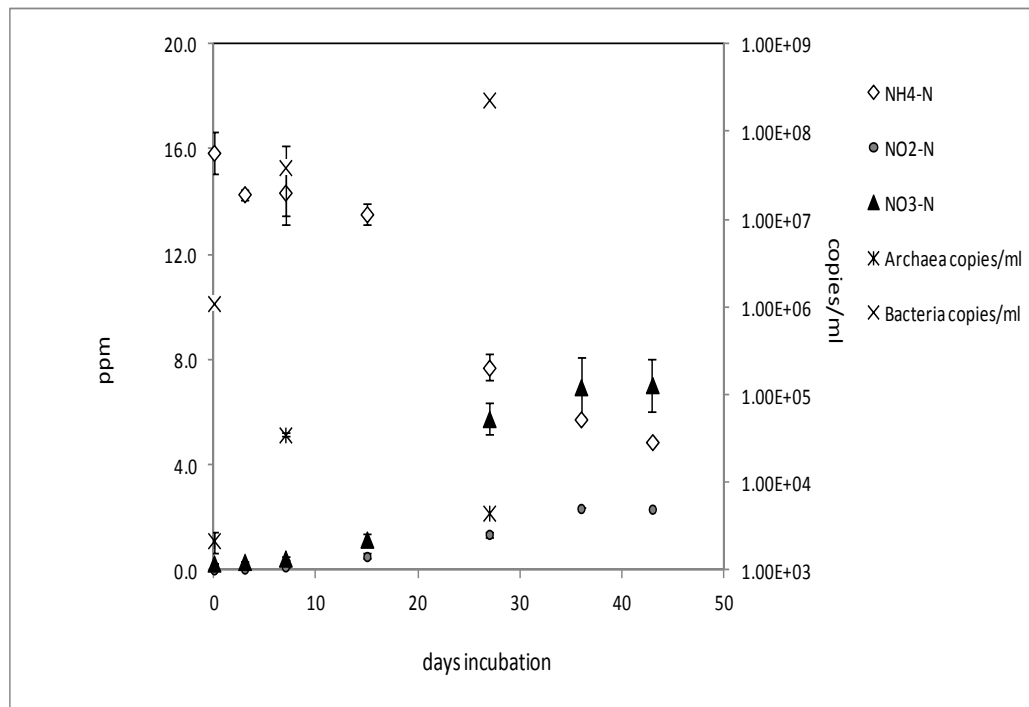


Figure 3.12. Ammonia, nitrite and nitrate concentration and 16S rRNA gene copy numbers of bacteria and archaea in the enrichment.

Discussion

Previously, the bacterial population of nitrifying simulated premises plumbing that has been actively nitrifying in terms of ammonia loss and nitrate accumulation was characterized (Chapter 2). Repeated efforts demonstrated that ammonia oxidizing bacteria, which are the organisms that are traditionally considered to dominate ammonia oxidation in these types of systems, were not detected. This prompted further investigation and archaeal ammonia monooxygenase gene (*amoA*) was detected by end point PCR in the PVC system but not in the copper system (Chapter 2). Since ammonia loss was occurring in both reactors, it was apparent that some component of the microbial community was responsible for ammonia oxidation and the efforts of this study centered on the contribution of the archaea.

Using FISH, archaea were detected in biofilm from both copper and PVC coupons, prompting a reevaluation of the previously observed absence of *amoA* in the copper system. Presence of PCR inhibitors had been observed in the DNA samples and a dilution step was included prior to amplification. Following dilution and an additional PCR step, an amplicon was obtained from the copper system. With the FISH results and this endpoint PCR result, the target gene is present albeit at a low number. Since no bacterial ammonia oxidizers were found, these results strongly suggest that the ammonia oxidizing archaea dominate nitrification in these reactors.

The archaea detected in the system were found to be phylogenetically affiliated with known ammonia oxidizing archaea. DGGE revealed the presence of at least two *amoA* phylotypes in the system with a differential distribution depending on the material.

However, with the screening method used for the 16S rRNA clones, only one phylotype was detected. The presence of at least two strains whose 16S rRNA sequences are very similar is inferred which needs to be further verified.

To obtain evidence for their potential contribution to nitrification, archaeal abundance was determined. To distinguish the active from the total population, a PMA treatment step was performed prior to DNA extraction. In general, cell numbers of PMA treated samples were not significantly different from their non PMA treated counterparts for both bacteria and archaea. PMA selectively enters cells with compromised membranes, intercalates with the DNA, and upon exposure to intense light, the binding of the dye to the DNA results to inhibition of PCR amplification (Nocker et al., 2007). Although the use of PMA treatment has not been directly tested on pure cultures of archaea, the use of propidium iodide which is structurally identical to propidium monoazide (Nocker et al., 2007) has been evaluated in archaea (Leuko et al., 2004).

Archaea are present in influent BAC, as well as copper and PVC reactors as reflected by amplification of archaeal 16S rRNA and archaeal *amoA* sequences in varying numbers. Archaeal cell numbers and archaeal *amoA* copies (at 1 copy per cell based on cultivated ammonia oxidizing archaea) are comparable with an almost one to one correspondence. Adding to this, the difference between copper and PVC for both archaeal 16S rRNA gene and archaeal *amoA* in both bulk and biofilm phases is consistent. The results suggest that each archaeal cell detected in the system carries one copy of the *amoA* gene which is consistent with what is currently known about the archaeal ammonia oxidizers (Mincer et al., 2007).

Abundance data for archaea relative to bacteria were analyzed to gain insight into the overall microbial ecology in the context of the simulated premises plumbing. An important consideration was the change in populations and numbers of organisms after the stagnation period in the reactors relative to that of the BAC influent. Results of this study show that bacteria increase in numbers through the stagnation period in both the copper and PVC reactors. This increase in suspended counts could be attributed to either growth of planktonic cells or biofilm detachment (Srinivasan et al., 2008; van der Wende et al., 1989). In our system, the bulk fluid of the reactors contains an organic carbon source in the form of humics that may support heterotrophic growth and proliferation.

With nitrification, a corresponding increase in heterotrophic bacteria has often been observed which may be attributed to either the loss of disinfectant (chloramine decay) or the increased availability of organic carbon released by nitrifiers (Zhang et al., 2009). Similarly, most probable number of ammonia oxidizing organisms has been found to increase toward the distal part of the distribution system suggesting either planktonic growth or biofilm detachment (Lipponen et al., 2002; Lipponen et al., 2004). Van der Wielen et al (2009) found that water systems without disinfectant residuals dominated by ammonia oxidizing archaea (AOA) had significant increases in AOA numbers as the water traveled through the distribution system. In contrast, distribution systems dominated by ammonia oxidizing bacteria (AOB) showed no significant difference from influent to further reaches of the system. This is similar to our result wherein archaea are enriched as their numbers increase from the influent BAC water to the PVC reactor bulk water. This result coincides with that of the archaeal *amoA* abundance as this gene was

enriched in the PVC reactor. Because of an almost one to one correspondence of the 16S rRNA gene and *amoA* copy number, we hypothesize that all archaea in the PVC system are potentially oxidizing ammonia. However, the data indicates a preferential enrichment of the archaea on the PVC over the copper.

Similar to the data from the bulk phase, the number of bacteria was not significantly different between biofilms from copper and PVC coupons suggesting that material effect on bacterial total biomass is negligible. Copper is reported to be inhibitory to bacteria however it has been shown in this system to have no effect on heterotrophic plate count (Rahman et al., 2011).

The trend in archaeal abundance in the bulk phases is also observed in the biofilm phases, wherein more archaea was found in the biofilm from the PVC coupon compared to that from the copper as implied by both the 16S rRNA gene and archaeal *amoA* gene. This suggests that the copper surfaces/copper itself may be inhibitory to the archaea in the system. Although the results are qualitative, the same trend was observed with the FISH results. Previously, copper added as CuSO_4 was found to have no effect on the ammonia oxidizing organisms in the same PVC system (Rahman et al., 2011) however the method employed to quantify the organisms was the MPN method instead of a molecular approach. Our results therefore present a probable scenario for a distribution system that has naturally occurring archaea either in the source water or in the surrounding environment, and how the choice of material might affect the population of organisms that might potentially nitrify.

A nitrifying community was enriched from the PVC reactor that showed ammonia loss coupled with nitrite and nitrate accumulation. Photoinhibition of ammonia oxidation was observed as nitrite production was lesser in the cultures exposed to light as compared to those kept in the dark. The photosensitivity of archaeal ammonia oxidation was recently demonstrated by Merbt et al (2011) wherein archaeal growth was shown to be even more sensitive than their bacterial counterparts at lower light intensities. In a water distribution system, specifically within premises plumbing, an environment devoid of light is a very permitting niche for such organisms.

In the enrichments, archaea increased initially, however, a decrease was observed that coincided with a drop in pH. Cultivated ammonia oxidizing archaea grow optimally at neutral pH (Gubry-Rangin et al., 2011) and thus archaea in the enrichment might be adversely affected by the decrease in pH. In the reactors that were the source of the inoculum for the enrichments, pH is maintained at around pH 8. Also, the flushing after the 8hr stagnation period ensures that the pH is brought up to that which simulates water in premises plumbing. This could potentially account for the differences in rates between the enrichments and reactors. In addition, the medium used for the enrichments does not contain any organic C sources thus selecting for autotrophs. While ammonia oxidizing archaea are autotrophic, heterotrophs and nitrifiers are known to have synergistic effects on each other (Zhang et al., 2009) and the exclusion of some or a particular heterotroph might have caused the observed difference in nitrification rate.

Three types of antibiotics were tested on their effect on ammonia oxidation. Archaea are known to be resistant to the antibiotics used however it is only with

ampicillin that nitrite was detected. Other pure culture attempts for ammonia oxidizing archaea with the use of antibiotics has not been successful (Park et al., 2010; Santoro and Casciotti, 2011; Hatzenpichler et al., 2008; dela Torre et al., 2008). Even with their resistance to the antibiotics, the archaea may be dependent on other organisms in the system for growth factors and with these organisms' sensitivity to the antibiotics used, the beneficial relationship is not maintained and the archaea do not survive or maintain activity.

16S rRNA sequencing revealed that the archaea in the system are related to known ammonia oxidizing archaea with a 98.5% similar to *Nitrosotenuis uzonensis* (Hatzenpichler et al., in preparation). With the current 98.7%-99.0% sequence similarity threshold between species (Stackebrandt and Ebers, 2006), we propose to provisionally classify this archaeon as *Candidatus Nitrosotenuis bozemanii*; *Nitrosotenuis* as it is closely related to the described genus, and the specific epithet *bozemanii* for the place where it was first described. An *amoA* sequence amplified from the system and described previously (Chapter 2) reveals affinity to *amoA* sequences of known ammonia oxidizing archaea such as *Nitrosotenuis uzonensis* and *Nitrosopumilus maritimus*. Although a screening was done for the 16S rRNA sequences cloned from the system, the possibility of other ammonia oxidizing archaea is still possible and it would be interesting to evaluate archaeal diversity and their overall contribution to the processes in the nitrifying system.

Conclusion

Archaea affiliated with ammonia oxidizing archaea, as well as the gene for archaeal ammonia monooxygenase were detected in nitrifying premises plumbing wherein ammonia oxidizing bacteria were not found suggesting a possible role for this newly described group in drinking water distribution nitrification. qPCR results showed that although bacterial populations are not significantly different between copper and PVC systems, archaeal populations are. This implies that if the archaea are significantly involved in the nitrification in the system, then the use of copper might significantly affect their presence and activity. To date, no study has described the presence and possible contribution of archaea phylogenetically affiliated with ammonia oxidizers in biofilms within drinking water distribution systems.

Nitrifying enrichments show the initial increase in archaea as well as bacteria followed by the decrease of archaea coinciding with a decrease in pH. Nitrite production was not observed in enrichments with kanamycin and streptomycin suggesting a possible relationship between bacteria and archaea that is disrupted as the bacteria are inhibited by the antibiotics. These results merit further study in the enrichment and cultivation of these potentially nitrifying organisms as pure cultures will lead to the improvement in the understanding of the ammonia oxidation process in archaea. Results of this study provides evidence of the presence of archaea affiliated with known archaeal ammonia oxidizers in a system where known ammonia oxidizing bacteria have not been detected. Although further analysis is needed to assess their contribution in nitrification in the system, this study provides evidence of archaea that are associated with biofilms in

drinking water distribution systems, prompting a reevaluation of the models used in nitrification control in drinking water distribution systems.

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CHAPTER 4

SURVEY OF GENES INVOLVED IN THE NITROGEN CYCLE IN NITRIFYING
SIMULATED PREMISES PLUMBINGAbstract

In drinking water, nitrification is the most studied process of the nitrogen cycle as its products nitrite and nitrate can cause serious health issues. Other transformations within the nitrogen cycle have not been investigated in relation to drinking water. Recognizing the diversity of microorganisms harbored in biofilms and bulk fluid, we hypothesize the existence of the genetic capacity that can potentially transform the harmful products of nitrification to ones that are less harmful in the context of drinking water. Using functional gene targets, the presence of archaeal *amoA* and *nxr* from at least two groups of organisms was determined. Genes for denitrification and annamox were not detected which may indicate the absence of organisms that carry out these processes, or the inadequacy of the method used. Interestingly, an *hao* distantly related to that of ammonia oxidizing bacteria (75% identity) was detected where the non- detection of these groups have been previously reported. Further study is needed to elucidate the presence and role of this *hao* in the nitrogen cycle of these reactors.

Introduction

Nitrogen Cycle

Nitrogen is an essential element for life being a major component of proteins and nucleic acids (Ward, 2011). It is present in the environment in various oxidation states as a variety of chemical compounds including organic nitrogen, ammonium, hydroxylamine, nitrate, nitric oxide, nitrous oxide and dinitrogen gas (Martínez-Espinosa, et al., 2011; Richardson and Watmough, 1999). The earth's atmosphere is an abundant source of nitrogen in the form of dinitrogen, however, this form of nitrogen is virtually inert (Canfield et al., 2011) and it is the fixed nitrogen, the ionic and organic forms that are most important to life (Ward, 2011). The interconversions of nitrogen compounds in the environment or the nitrogen cycle are mostly dependent on the reduction-oxidation reactions primarily mediated by microorganisms (Canfield et al., 2011).

In the recent years, discovery of new links as well as players in the nitrogen cycle has increased the understanding of this biogeochemical cycling. It is recognized that much of the basic knowledge on the nitrogen cycle is based on data obtained using cultivated isolates (Zehr and Ward, 2002). The advent of molecular tools led to the discovery and a better understanding of the microbes involved in the nitrogen cycle. Figure 4.1 shows the transformations and the genes of microbes.

Catabolic Processes in the Nitrogen Cycle

Nitrification. Nitrification is the sequential oxidation of NH_4^+ to NO_3^- in the presence of oxygen; organisms with the enzyme ammonia monooxygenase (Amo)

oxidize NH_4^+ to hydroxylamine, which is subsequently oxidized to NO_2^- by the enzyme hydroxylamine oxidoreductase (Hao) and finally NO_2^- to NO_3^- by nitrite oxidoreductase (Nxr) (Canfield et al., 2010). Autotrophic nitrifying bacteria were discovered near the end of the 19th century and until recently have been considered the major contributor to nitrification. Ammonia oxidizing bacteria (AOB) are affiliated with the Beta- and Gammaproteobacteria while nitrite oxidizing bacteria (NOB) are affiliated with the Alpha-, Delta- and Gammaproteobacteria (Starkenburg et al., 2006). In recent years, it has been discovered that archaea (AOA) rather than bacteria can dominate ammonia oxidation in many environments such as oceans and soils (Nicol and Schleper, 2006). These discoveries, together with data from pure cultures of ammonia oxidizing archaeal isolates, have changed the view on nitrification.

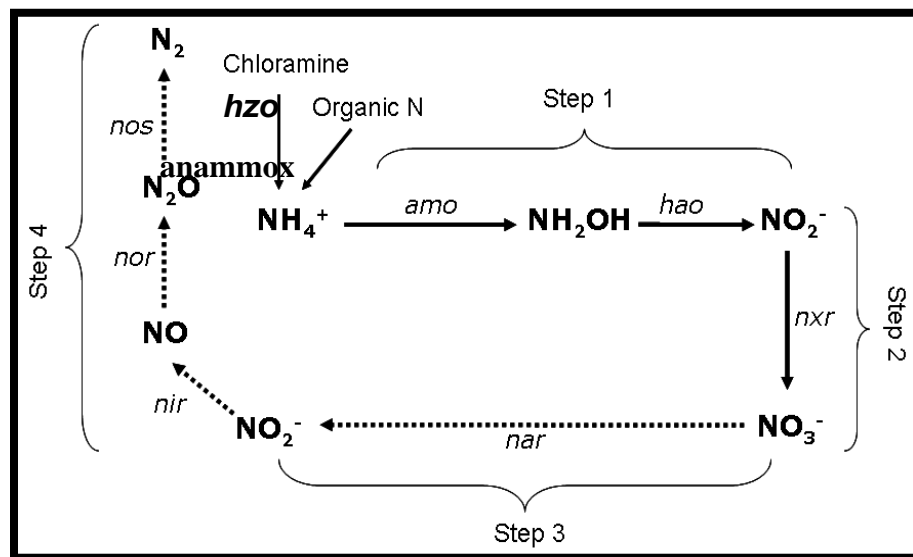


Figure 4.1. The major transformations in the nitrogen cycle: ammonia oxidation (step 1), nitrite oxidation (nitrite oxidation), nitrate reduction (step 3) denitrification (step 4) and anaerobic ammonia oxidation (anammox) and the genes encoding the enzymes that conduct these transformations. Sources of ammonia in drinking water are also indicated.

In the study of nitrifying organisms, the use of a 16S rRNA gene based method is the most traditionally used however a more targeted approach involves the use of functional gene based methods. AOB and AOA first oxidize ammonia by the enzyme Amo encoded for by the genes that belong to an operon with the structure *amoCAB* and it is a portion of the gene *amoA* that is frequently used in distribution and diversity studies (Junier et al., 2010; Rotthauwe et al., 1997). Another suitable target for use with AOB is the gene *hao* that encodes for Hao as it is highly conserved across species (Junier et al., 2010; Klotz et al., 2008). Unlike *amo*, *hao* does not appear to have any homologous gene in the genome of AOA (Urakawa et al., 2011).

Compared to AOB, NOB are much less studied in the environment (Ward, 2011). Nitrite oxidizing bacteria oxidize nitrite to nitrate as catalyzed by their enzyme nitrite oxidoreductase (Nxr). Even though NOB are phylogenetically heterogeneous (Teske et al., 1994; Lücker et al., 2010), studies have been focused on the *Nitrobacter* species (Meincke, et al., 1992; Vanparrys et al., 2007; Wertz et al., 2008). All known Nxr belong to the same enzyme super family with the mature holoenzyme composed of at least three subunits (alpha, beta, gamma) however genomic differences exist among the Nxr of nitrite oxidizing bacteria (Maixner, 2009). PCR primers that target the genes encoding for subunits of the Nxr are available for specific genera.

Denitrification. Denitrification is a respiratory process in which nitrate is reduced stepwise to dinitrogen (Kraft et al., 2011). Organisms capable of denitrification are found among bacteria, archaea and eukaryotes. Denitrification is not a trait that is shared by one particular clade thus environmental studies on their distribution rely on functional genes

rather than the 16S rRNA genes (Kraft et al., 2011; Jones et al., 2008). The nitrite reductase genes, *nirS* and *nirK*, are frequently used as marker genes for denitrification. They encode for the enzyme nitrite reductase that catalyzes the reduction of NO_2^- to nitric oxide which is an intermediate step in denitrification. These are periplasmic enzymes found in gram negative bacteria (Kraft et al., 2011). They are evolutionary unrelated and differ in that NirK is a homotrimeric copper-containing enzyme while NirS is a homodimeric cytochrome *cd₁* nitrite reductase (Kraft et al., 2011; Moura and Moura, 2001).

Orthologues to *nirK* have been described for several AOB and NOB (Cantera and Stein, 2007; Casciotti and Ward, 2001; Chain et al., 2003; Klotz et al., 2006), with diverse sequences and regulation among nitrifiers. Similarly, the presence of *nirK*-like genes in AOA have also been reported (Urakawa et al., 2011). The presence of a putative *nirS* has also been reported in bacteria that can oxidize ammonia anaerobically (described in the following section) as the enzyme is hypothesized to participate in the oxidation of nitrite to nitric oxide, and then together with ammonium forms hydrazine as catalyzed by hydrazine hydrolase (Strous et al., 2006; Junier et al., 2010).

Nitrification/Anammox. Anammox or anaerobic ammonia oxidation is a process wherein the oxidation of ammonia is coupled to the reduction of nitrite to produce dinitrogen in anoxic or suboxic environments (Klotz and Stein, 2008). The hypothesized anammox pathway involves at least three redox reactions including the reduction of nitrite to NO, the condensation of NO and ammonia producing hydrazine and the oxidation of hydrazine to dinitrogen gas (Strous et al., 2006; Jetten et al., 2009). This

newly elucidated pathway of ammonia oxidation was discovered in wastewater treatment plants and has since been determined to occur in marine sediments (Zehr and Kudela, 2011). The reaction is carried out by specific members of the order Brocadiales, in the phylum Planctomycetes. Four of the five “Candidatus” anammox genera were enriched from activated sludge and one is frequently detected in natural habitats such as marine sediments and oxygen minimum zones (Jetten et al., 2009 and references herein).

Because anammox bacteria are monophyletic, the use of specific 16S rRNA gene PCR primers in combination with universal primers is possible (Jetten et al., 2009). A more functional approach is also possible to target the genes that encode for hydroxylamine/hydrazine oxidoreductase (Hzo). Interestingly, Hao and Hzo can oxidize the alternate substrate; Hao can oxidize hydrazine, and Hzo can oxidize hydroxylamine (Junier et al., 2009).

Objectives of the Study

In drinking water, nitrification is the most studied process of the nitrogen cycle. Ammonia that may be naturally occurring in the source water or introduced as chloramine decays is oxidized to nitrite and nitrate which can cause serious health issues. However, other transformations within the nitrogen cycle have not been studied within this system. Because nitrate (or in the case of incomplete nitrification, nitrite) is produced during nitrification, it would be interesting to determine the genetic capacity that in certain microniches within the distribution system may permit the further transformations of these nitrification products to those that are considered less harmful in the context of drinking water.

This study aims to determine if the nitrifying reactors contain genes to carry out the processes in the nitrogen cycle. The specific objectives of this study were to 1) determine the presence of key genes involved in the nitrogen cycle specifically: the genes in nitrification (*amoA*, *hao*, *nxr*), denitrification (*nar*, *nir*, *nrf*) and anammox (*hzs*) and, 2) to compare the presence and absence of these genes in the two types of reactors with copper and PVC surfaces.

Materials and Methods

Reactor Setup

Reactors simulating nitrifying premises plumbing were operated as described previously (Rahman et al., 2011; Chapter 2). CDC reactors (Goeres et al., 2005) were modified by putting coupons attached to solid rods and a base plate and stir blades made of copper or PVC to simulate surfaces in premises plumbing (Figure 4.2). The surface area of the coupons, base plate and stir blades was equivalent to the surface to volume ratio of a six foot long $\frac{3}{4}$ " diameter domestic plumbing pipe. Volume of the reactors is 120 ml.

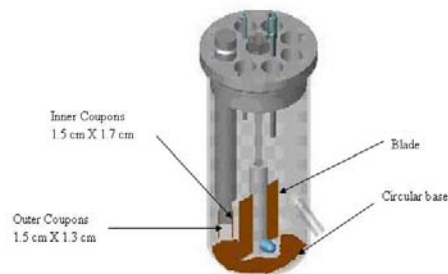


Figure 4.2. Schematic representation of the modified CDC reactor used in the study (Rahman, 2008).

To simulate periods of stagnation and flushing in premises plumbing, reactors were flushed with peristaltic pumps with shear created by the stirplate for five minutes followed by eight hr stagnation periods thrice daily. The stirplates were set to create a rotational speed of the blade of 300 rpm, which was approximately equivalent to a velocity of 3 ft/s in the bulk water.

Reactors were fed with a combination of mineral amended reverse osmosis (RO) water, biologically treated Bozeman tap water, and a humic substances organic feed as previously described (Rahman et al., 2011; Chapter 2) to simulate water quality in a nitrifying premises plumbing. The RO water contained 0.71 mgNH₃N/L reactor volume which is equivalent to the amount of ammonia that would result from the decay of 4mg/L of chloramine. Biologically treated Bozeman tap water (surface water source, no background ammonia, chlorinated) was provided as a separate and parallel influent, serving as the sole, continuous source of microorganisms with sufficient background phosphate for microbial growth at the organic carbon level used. Organic carbon was supplied to the reactors in a third, separate feed in the form of soil-derived humic substances at a final concentration of 4mg/L carbon as described previously (Rahman et al., 2011). The RO water/ BAC water/organic carbon feed ratio was 50:5:1. All reactors showed signs of stable, complete nitrification as measured by conversion of ammonia to nitrate and had been in operation for over six years at the time of this study.

DNA Extraction

For a one time point analysis, biofilm samples were obtained from a coupon (1.5 x 1.7 cm) of a representative copper and PVC reactor by scraping with a rubber

policeman. DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, OH) according to the manufacturer's directions except for a modification that includes a step to remove humics by using guanidine thiocyanate (Burr et al., 2006).

Polymerase Chain Reaction (PCR)

All PCR amplifications were conducted in an Eppendorf Mastercycler® ep (Eppendorf North America, www.eppendorfna.com). Reactions were carried out in 25µL volumes which included 0.2µM primers, Go Taq® Green Master Mix (www.promega.com), DEPC treated water, Ultrapure BSA (Ambion) and 1µL of template DNA (approximately 5 ng). All oligonucleotide primers were synthesized by IDT (Integrated DNA Technologies, www.idtdna.com). Table 4.1 shows the primers used in the study and the cycling conditions used for each. To verify the amplification product, gel purification, cloning and sequencing were performed as described in the previous chapter. Products were visualized by running on a 0.8% agarose gel with ethidium bromide in 1XTBE buffer for 45 minutes at 40V. Gels were viewed using a FluorChem™ 8800 fluorescence imager (www.alphainnotech.com).

Cloning and Sequencing of *hao* Amplicons

PCR products were gel purified using QIAquick® Gel Extraction Kit (QIAGEN). Purified PCR products were cloned into the plasmid vector pCR™4-TOPO® using the TOPO® TA Cloning kit (Invitrogen, www.invitrogen.com) following the manufacturer's protocol. Transformants were inoculated into 10 mL of Luria-Bertani (LB) broth plus 50 mg mL⁻¹ ampicillin. Cultures were incubated overnight at 37°C in a shaking incubator.

Plasmid DNA was purified from individual clones using the Wizard Plus SV Minipreps DNA Purification System (www.promega.com) and quantified using the NanoDrop ND-1000 spectrophotometer (Nano Drop, Wilmington USA).

Clones were sent to the Research Technology Support Facility (RTSF) at Michigan State University and were sequenced using the M13F (5'-TGTAACGACGGCCAGT-3') primer. Sequences were analyzed for chimeras using Bellerophon (Huber et al., 2004) and were compared with known sequences in the GenBank database using the Basic Local Alignment Sequence Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Amplified *hao* were aligned with *hao* from the following known ammonia oxidizing bacteria: *Nitrosomonas eutropha* (CP000450), *Nitrosomonas europaea* (NC_004757) and *Nitrospira multififormis* (CP000103) using CLUSTAL W (Thompson et al., 1994) at <http://www.ch.embnet.org/software/ClustalW.html>. Phylogenetic analysis was done in MEGA4 (Tamura et al., 2007).

Table 4.1 Target genes and the PCR primers used in the study.

Process/target gene	Primer	Sequence (5'-3')	Amplicon length	Cycling condition*	Reference
Nitrification					
Bacterial <i>amoA</i>	amoA-1F	GGGGTTTCTACTGG TGGT	491	95°C for 10 min, 30 cycles of 95°C for 60 s, 50°C for 60 s, and 72°C for 60 sec 72°C for 7 min	Rotthauwe et al., 1997
	amoA-2R	CCCCTCKGSAAAGC CTTCTTC			
Archaeal <i>amoA</i>	Arch-amoA-for	CTG AYT GGG CYT GGA CAT C	256	95°C for 10 min, 30 cycle of 95°C for 20 sec, 53°C for 45 sec, and 72°C for 60 sec 72°C for 7 min	Wuchter et al., 2006
	Arch-amoA-rev	TTC TTC TTT GTT GCC CAG TA			

Table 4.1 Continued

<i>hao</i>	haoF1	TGCGTGGARTGYCA C	992	94°C for 5 min, 10 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 90 sec, 20 cycle of 94°C for 1 min, 54°C for 1 min, 72°C for 1 m in 72°C for 10 min	Schmid et al., 2008
	haoR3	AGRTARGAKYSGGC AAA			
<i>nxrA</i>	nxrAF	CAGACCGACGTGTG CGAAAG	322	94°C for 3 min 35 cycles of 94°C for 30 s, 55°C for 45 sec, 72°C for 45 sec 72°C for 5 min	Wertz et al., 2008
	nxrAR	TCCACAAGGAACGG AAGGTC			
	nxrBF1 69	TAC ATG TGG TGG AAC A	485	95°C for 4 min 35 cycles of 95°C for 40 sec, 56.2°C for 30 sec, 72°C for 60 sec 72 °C for 10 min	Maixner, 2009
	nxrBR6 38	CGG TTC TGG TCR ATC A			
Denitrification					
<i>nrf</i>	<i>nrfA</i> -2F	CAC GAC AGC AAG ACT GCC G	67	95°C for 10 min 30 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 45 sec 72°C for 7 min	Smith et al., 2007
	<i>nrfA</i> - 2R	CCG GCA CTT TCG AGC CC			
<i>nirK</i>	nirK1F	GG(A/C)ATGGT(G/T) CC(C/G)TGGCA	514	95°C for 5 min 30 cycles of 95°C 30 sec, annealing** for 40 sec, 72°C for 40 sec 72°C for 7 min; (*annealing was set initially at 45°C decreasing by 0.5 °C every cycle until 40°C then the remaining 20 cycles was at 43°C)	Braker et al., 1998
	nirK5R	GCCTCGATCAG(A/G) TT(A/G)TGG			
<i>nirS</i>	nirS1F	CCTA(C/T)TGGCCGC C(A/G)CA(A/G)T	890	95°C for 10 min 30 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 45 sec 72°C for 7 min	Smith et al., 2007
	nirS6R	CGTTGAACTT(A/G)C CGGT			
<i>nar</i>	<i>narG</i> - 1F	GAC TTC CGC ATG TCR AC	69	95°C for 10 min 30 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 45 sec 72°C for 7 min	Smith et al., 2007
	<i>narG</i> - 1R	TTY TCG TAC CAG GTG GC			
<i>nap</i>	<i>napA</i> - 1F	GTY ATG GAR GAA AAA TTC AA	55	95°C for 10 min 30 cycles of 95°C for 15 sec, 55°C for 30 sec, 72°C	Smith et al., 2007
	<i>napA</i> -	GAR CCG AAC ATG			

Table 4.1 Continued

	1R	CCR AC		for 45 sec 72°C for 7 min	
Anammox					
<i>hzo</i>	hzocl1 F1	TGYAAGACYTGYCA YTGG	470	94°C for 5 min 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 90 sec, 72°C for 10 min	Schmid et al., 2008
	hzocl1 R2	ACTCCAGATRTGCT GACC			
	hzocl2a F1	GGTTGYCACACAAG GC	289		
	hzocl2a R1	TYWACCTGGAACAT ACCC			

*Cycling conditions may be modified from references.

Results and Discussion

A molecular survey of genes involved in the nitrogen cycle was done in nitrifying reactors simulating premises plumbing. Table 4.2 shows the genes that were detected in the study. Because the system was nitrifying, genes involved in nitrification were expected to be present. No bacterial ammonia monooxygenase gene was detected in either PVC or copper suggesting the presence and activity of a different group of organisms for this first step of nitrification as previously reported (Chapter 2). Failure to detect ammonia oxidizing bacteria has been reported previously in other nitrifying systems (Hovanec and De Long., 1996; van der Wielen et al., 2009) and the activity has been attributed to other yet to be characterized organisms. While AOB were not detected, archaeal *amoA* was detected in both copper and PVC systems as previously reported (Chapter 2; Chapter 3) suggesting a role for AOA in nitrification within the system.

hao is the gene that encodes for hydroxylamine oxidoreductase which takes the product of the first step of ammonia oxidation, hydroxylamine to nitrite. In archaea, no

homologue for this gene has been found suggesting a different pathway taken by ammonia oxidizing archaea. Interestingly, *hao* was detected in the systems and a comparison of the amplified sequence with known *hao* sequences reveals a very low similarity to known ammonia oxidizing betaproteobacteria (Figure 4.3 and 4.4). Sequences obtained were not identical thus it can be hypothesized that it is carried by a diverse population of organisms. This may mean that there is an ammonia oxidizing bacteria that has a very divergent gene that escapes detection. Another possible explanation is that the gene was acquired by the archaea by horizontal gene transfer. Further analysis is needed to definitively determine the abundance and role of this gene in the system.

Using *nxrA* primers designed for *Nitrobacter*, an amplicon was obtained in the PVC sample and none for the copper. Interestingly, *nxrB* primers that are designed to amplify *nxr* from most *Nitrospira* provided amplicons from both PVC and copper. Previously, using a 16S rRNA based approach, *Nitrospira sp* were detected in both systems (Chapter 2). The combination of primers designed to amplify nitrite oxidizing bacteria suggests the probable presence of more than one nitrite oxidizing genus in the system.

Although amplicons with the expected size were not achieved using the *nir* primers, amplicons of varying sizes were obtained. Additional analyses are required to definitely determine if these are PCR artifacts resulting from non-specific priming events or indicative of the presence of *nir*-like genes in the system. No anammox genes were

detected in the system. However, because Hao can oxidize hydrazine, the possibility of anaerobic niches where anammox may occur cannot be totally discounted.

Table 4.2. Genes involved in the major transformations of nitrogen detected in the reactors.

Gene	PVC	Copper
Bacterial <i>amoA</i>	-	-
Archaeal <i>amoA</i>	+	+
<i>hao</i>	+	+
<i>nxr</i>	+	+
<i>nir</i>	-	-
<i>nrf</i>	-	-
<i>nar</i>	-	-
<i>nap</i>	-	-
<i>hzo</i>	-	-

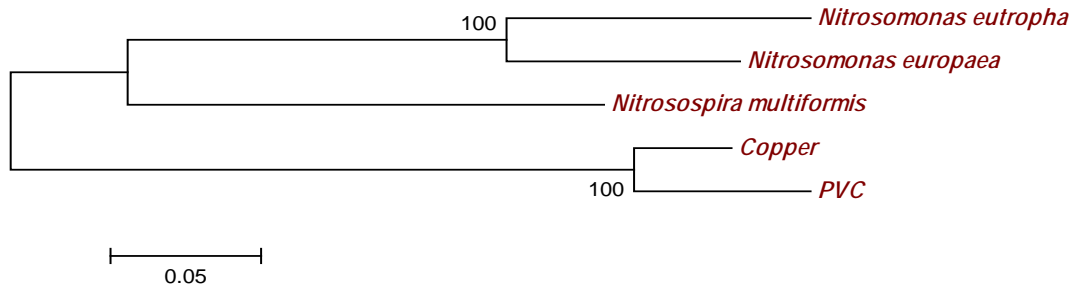


Figure 4.3. Evolutionary relationships of the amplified *hao* sequences from the copper and PVC biofilm with known ammonia oxidizing bacteria. Phylogenetic analysis was done in MEGA 4 (Tamura et al., 2007).

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N.eutropha      TGGCACATGGTGCAACCTTCTGCCACTTCACGTTGCGGCATGGCTGCCCAAACGGTGGTT
N.europaea     TGACACATGGTGCAACCTTCGGCAACTTCACGTTGCGGCATGGCTGCCCAAACAGTGGTC
Nitrosospira   TGGCACATGCTGCAGCCTTCGGCAATCTCGCGCTGCGGCATGGAGGCAAAATACCGCTACT
Copper         TGGCACATGGAGCAGCCTTCGGCAATCTCGCGCTGCGGCATACCCGCCAGATATCGGTC
PVC           TGGCACATGGAGCAGCCTTCGGCAATCTCGCGCTGCGGCATACCCGCCAGATATCGGTC
** * * * * *

N.eutropha      TCAATGTTGGCTGTGTAGTCCAGTGCCTGGGATGGACGTCGGTCCAGGCCACTGGCCATT
N.europaea     TCGATGTTGGCTGTGTAGTCCAGTGCCTGGGAGGGGCGTCCGGCAGGCCATTGTCCGTT
Nitrosospira   TCTACGTTGGCCTTCCAGTCCAGCAGTGCAGGGGCGTCCCTGCGGCCATTGCTTGTTC
Copper         TCGACGTTGGCTTCCCAATCCAGCAGTGCAGGGGACGCCCTCTCGGCCACTGGTCTTTC
PVC           TCGACGTTGGCTTCCCAATCCAGCAGTGCAGGGGACGACCTCTTGGCCACTGGTTCTTT
** * * * * *

N.eutropha      GGCCAGATCATGGTGCACGCTCGGATTCAGCTCGGCAAATTCCTCAGGTGACAGGTT
N.europaea     GGCCAGACCATGGTGTCCCGTTCGGATTCAGCTTCGGCAAATTCCTTAAGTGACAGGTT
Nitrosospira   GGCCATACCAGCGTGTCCGCTCGGATTCAGCTTCGGCAAATTCCTGACAGTGGCAGGTT
Copper         GGCCAGAGGATCGTATCAGCTCAGACTCGGCTCGGCATATTCATGAGATGGCAGTTG
PVC           GGCCAGAGGATGTGTCCGCTCGGACTCGGCTCGGCATATTCATGAGATGGCAGTTG
*****

N.eutropha      CCGCAAACGTGAGTGTAGGCATGCGTACGCTCTTGGTGTGATCTGCCTTCTTTTGGCG
N.europaea     CCGCAGGTGTCCCGCTCGGCATGCGGATGCTCTTGGTATGATCCGCTTGTCTTTTTTG
Nitrosospira   CCGCATACGTCCGCGTCCGCTCGGCATCCGCGAGATCCTTCATGTGGTCTGCCTTGTCTT
Copper         CCGCAGACATCGGCGTGGGCATCTTGGATCCACCCGGTATCCGCGGACTTTTTCGTG
PVC           CCGCAGACATCGGCTGTGGGCATCTTGGATCCACTCGGTGATCCGCGGCTTCTTCTG
*****

N.eutropha      TTGATGTCAACGTGACAGTCAATACAGCCTACTTCCCTGAGCGCTTCCCTTCTCCTAGT
N.europaea     TTGACGTCAACGTGACAGTCAATACAGCCTACTTCCCTGAGGGTTCTTTTTCCCCAGC
Nitrosospira   TTGATGTCAACGTGACAGTCAATACAGCCTACTTCCCTGAGCTTGTGCTTGGCCCCAGC
Copper         TTGATGTCCACATGGCAATCGATGACGCCACTTCCCTTAAAGTCTCTTTGGCACCAGC
PVC           TTGATGTCAACATGGCAATCGATGCAACCCACTTCCCTCAAGTTCTCTTTGACCCGAGG
*****

N.eutropha      TTGCCATGGAGCGCAGGTTGTTCTCAACTTCTTCCAGCTTGCCTTTTTGTAAAAAAGC
N.europaea     TTGCCATCGAGCGCAGGTTGTTTCAACTTCTTCCAGCTTCCCTTTCTGTAATACAGG
Nitrosospira   TTGCCATCGAGCGAAGATTCTTCTCCAGCTTCTCAAGCTTGCCTTCTTGTAGTACGTC
Copper         TTACCAGCGAGCGCAGGTTGCCTCGATTCTTCCAGCTTGGCCTTCTGTAAGAGGTG
PVC           TTACCAGCGAGCGCAGGTTGCCTCGACTTCCCTCCAGCTTGCCTTCTTATAGAACGTA
*****

N.eutropha      GGATCTTCCGGCTTGGGTTGCGTATTTGTCCAGGTTGGCGTGGGTGCTGCGTITCCAT
N.europaea     GGATCTCCGACTTGGGTTGCGAATCTTGTCAAGATTGGCGTGGGTGCTGCGCTTCCAG
Nitrosospira   GGTCTTCCAGCTTGGGTTCCGATCTTGTCCAGGTTCCGCTGCGTGTCCGCTTCCAC
Copper         TCATCTTCCGGCTGAGCTTGGGATCTGGTCCAGATTGGCATGGGTGCTTCTCTTCCAC
PVC           TCGTCTTGGAGTCAAGTTGCGAATCTTGTCAAGATTGGAGTGGGTGCTTCTCTTCCAC
** * * * * *

N.eutropha      GCTCTTACCCAAACCGGCTTTCATCAGAGTGGCATTCAACGCA
N.europaea     GCTCTTACCCATAACCGGCTTTCATCAGAGTGGCATTCCACGCA
Nitrosospira   GCACTCACCCATAACCGGCTTTCATCAGAGTGGCATTGACGCA
Copper         ATCGCGACCCAGCCGCGACTCATCGGTGTGACACTCCACGCA
PVC           ATGGCCACCCACCTGGGACTCATCGGTGTGACACTCCACGCA
*****

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Figure 4.4. Alignment using CLUSTAL W of the *hao* sequences from the reactors with the *hao* sequences of ammonia oxidizing bacteria.

Conclusion

Several genes involved in the nitrogen cycle were detected in nitrifying reactors simulating premises plumbing. The presence of a novel *hao* gene was determined but the role in a system wherein ammonia oxidizing bacteria have not been detected is yet to be elucidated. The presence of more than one population of nitrite oxidizing bacteria was also determined. This undertaking is the first to assess the genes in the system. The knowledge of the ecology of the drinking water distribution system may lead to ways by which the system can be manipulated to prevent or control nitrification.

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CHAPTER 5

OVERALL CONCLUSIONS

The work presented in this dissertation aimed to determine a link between microbial community composition and function in nitrifying simulated premises plumbing. Previously, Rahman et al (2011) investigated the application of nitrification control mechanisms on the system and found atypical responses to agents used i.e. chlorite inhibition was only noted at 20 ppm. Interestingly, responses differed between the two types of reactors, copper and PVC.

The investigation of the microbial community started with the study of the diversity of the bacterial population. The genetic fingerprinting approach PCR-DGGE was used in combination with PMA treatment (Nocker et al., 2007) to focus on the active population. The result of the analysis can be divided into three major findings: 1) the material effect, 2) the distinction of bulk from biofilm communities, and 3) temporal changes.

The influence of the type of plumbing material has been the subject of various studies as it confers one of the most complex effects on microbial growth and persistence (Berry et al., 2006; Yu et al., 2010). In this study, it was determined that unique assemblages of organisms are found in the copper and the PVC reactors. Copper that leaches from copper pipes is known to be toxic to bacteria (Trevors and Cotter, 1990). This is not absolute as demonstrated by the persistence and growth of biofilm, as well as planktonic cells in the copper reactors. However copper sensitivity can account for the

observed distinction of copper bulk from biofilm communities. This result can be interpreted as the inability of some microbes to persist in the biofilm phase on the copper surface. Exopolymers may confer protection from the toxic effects of copper and the protein: carbon ratio of the copper and PVC biofilms indicates a possible difference in exopolymer production.

Most studies on the microbial ecology have focused on the biofilm phase while the bulk phase community is overlooked. Our results show that bulk community is different from the biofilm community. Bulk samples clustered more tightly (among replicates and temporally) compared to their biofilm counterparts indicating there is lesser diversity of organisms that can persist in the bulk than the biofilm phase. However, the organisms that persist do so consistently and even without the benefits of a biofilm lifestyle. This result demonstrates the importance of studying both biofilm and bulk phases in water distribution systems.

Temporal changes were observed indicating that the communities are dynamic. New organisms are continually being introduced into the system as indicated by a shift in the profile of the BAC influent. Some of these organisms become integrated while existing members of the community are detached from the biofilm and/or flushed from the system. It should be noted that changes in the community were not accompanied by change in function, specifically nitrification. Thus, functional redundancy among species may account for the maintenance of function even with the observed changes in community composition.

Focusing on the nitrification, ammonia oxidizing bacteria were not found in the system using both a 16S rRNA-based approach as well as a targeted *amoA* approach suggesting ammonia oxidation by a different group of organisms. Recent findings have put the spotlight on ammonia oxidizing archaea as they have been found to be present in various environments and even in greater abundance than their bacterial counterparts. To date, only two mesophilic ammonia oxidizers have been isolated into pure culture but these serve as important evidence for their contribution to nitrification.

The presence and abundance of archaea was determined in reactors in relation to the bacteria to obtain an overall picture of prokaryotic diversity. Using FISH with domain-specific probes, archaea were detected along with bacteria in biofilms from both copper and PVC. An initial endpoint PCR indicated the presence of potential ammonia oxidizing archaea only in the PVC reactor but FISH showed their presence in both reactor types and prompted further investigation. Results showed that the necessary dilution step to remove PCR inhibitors also diluted the target gene to non-detection for the copper system. This was remedied by a second round of PCR using the PCR product from the first round as template. The diversity of organisms carrying the archaeal *amoA* was investigated using DGGE and the presence of at least two phylotypes was determined with both being present in the BAC influent and copper, while only one in the PVC. It can only be speculated at this point that the phylotype in the PVC reactor outcompetes the other, while in the copper system, its slight inhibition allows the other phylotype to grow and survive. Phylogenetic analysis of the amplicons reveals the affinity of the novel

archaea in the system to a known ammonia oxidizing archaeon *Nitrosotenuis uzonensis* (Hatzenpichler et al., in preparation).

In addition to their detection, the abundance of the archaea was also determined. While bacteria were found to be comparable in copper and PVC, archaea was significantly more abundant in PVC suggesting a material effect on microbial density that is domain-specific.

An enrichment using bulk fluid from the PVC reactor that oxidizes ammonia to nitrate was obtained. The enrichment was found to be photosensitive as nitrite production was inhibited in light conditions relative to cultures kept in the dark. The rate of enrichment was determined to be 0.07 ppm NO₂N/day which is much slower than in the reactors. A possible reason for this difference includes the exclusion of humics in the enrichment medium which may support growth of heterotrophs that are known to form synergistic relationships with nitrifiers. The attempt to isolate the archaeal component of the enrichment into pure culture using antibiotics that target bacteria and not archaea was not successful and similarly can be explained by the removal of heterotrophs from the system that may support the nitrifying population.

Recent discoveries involving ammonia oxidizing archaea have changed the perception of nitrification. Results of this study show the presence of potentially ammonia oxidizing archaea associated with biofilms within a nitrifying simulated premises plumbing. Further research is needed to definitively ascribe ammonia oxidation to the archaea in the system and this may ultimately have practical implications in drinking water research and management including a re-evaluation of the current models

for nitrification as well as decision making tools on choice of disinfectants, plumbing materials and control strategies to mitigate or control nitrification.

Future Directions

The results of this study provided further evidence of an effect of material effect on the microbial population composition and abundance in a simulated premise plumbing system. With the PCR-DGGE-sequencing approach, the identities of the organisms present in the system were determined. Co-migration of 16S rRNA gene fragments is possible, along with other biases of the method and might have led to non-detection of some species. A more comprehensive catalog of the organisms can be provided by metagenomic analysis using a high throughput sequencing approach. Similarly this approach can be used to provide a more thorough analysis of archaeal 16S rRNA sequences.

Because presence cannot be equated to activity, FISH coupled with microautoradiography (FISH-MAR) can be performed on the biofilms to determine the uptake of carbonate in the system, thus determining autotrophy.

The enrichment of a nitrifying consortium provides a tool to analyze the ecology of a nitrifying system. This needs to be further characterized by identifying the specific members and possibly determining their abundance in the actual reactors. In addition, the contribution of the archaeal population to nitrification can be determined by using agents that inhibit archaeal activity or growth (i.e. diphtheria toxin) and measuring nitrification inhibition.

Because the organism that carries the *hao* gene is not yet determined along with its role in nitrification, the detection and quantification of the gene in the course of the enrichment should be examined. This may provide evidence for the presence or absence of a role for it.

Since the enrichments have been grown in batch cultures, it would be interesting to grow them in continuous culture and also determine the biofilm formation capacity, noting that most heterotrophs have been selected against in this enrichment. Application of nitrification inhibitors such as the ones reported by Rahman et al (2011) can be done and the responses of the enrichment compared to those of the reactors.

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APPENDICES

APPENDIX A

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Nitrification and potential control mechanisms in simulated premises plumbing

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ABSTRACT

Indigenous drinking water organisms were used to establish nitrification in glass reactors containing copper or polyvinyl chloride (PVC) surfaces. The reactors were fed soil-derived humics as the organic carbon source and ammonium sulfate as the nitrogen source in biologically treated tap water. Water in the reactors was stagnant for 8 h and then flowed for 5 min to simulate conditions in household plumbing. Following the establishment of complete nitrification (conversion of ammonia to nitrate) in both reactor types, various inhibitors of nitrification were tested followed by a period where recovery of nitrification was observed. In one PVC reactor, copper was gradually introduced up to 1.3 ppm. To ensure that most of the copper was in the ionic form, the pH of the influent was then gradually lowered to 6.6. No significant change in nitrification was observed in the presence of copper. Chlorite was introduced into copper and PVC reactors at doses increasing from 0.2 ppm to 20 ppm. There was limited effect on the PVC system and inhibition in the copper reactor only at 20 ppm. Chloramine was tested at chlorine to ammonia ratios ranging from 0.5:1 to 5:1. Nitrification activity was impacted significantly at a 5:1 ratio and ultimately stopped, with the fastest response being in the copper system. Whenever a control mechanism was tested, there was increased release of copper from the reactors with copper coupons. In all cases, nitrification recovered when inhibitors were removed but the rates of recovery differed depending on the treatment method and coupon surface.

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1. Introduction

A topic of regulatory and public health concern in drinking water is the creation of potentially carcinogenic disinfection by-products (DBPs) when the water is chlorinated. As an alternative to chlorination, many utilities have shifted to the use of chloramines to reduce the levels of regulated DBPs to meet the Stage 2 Disinfectants/Disinfection By-Product Rule (USEPA, 2000). Although the use of chloramines as a secondary

disinfectant can reduce DBPs, there is the chance that increased levels of free ammonia in the distribution system may serve as an energy source for indigenous nitrifying organisms. Proliferation of these organisms can then result in nitrification in the distribution system. Not surprisingly, nitrification is one of the most frequent operational problems encountered by drinking water utilities that use chloramine for secondary disinfection (Skadsen, 1993; Wolfe and Lieu, 2001; Odell et al., 1996; Wilczak et al., 1996; Seidel et al., 2005).

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Nitrification is a microbial process by which reduced nitrogen compounds (primarily ammonia) are sequentially oxidized to nitrite and nitrate. This process can have a detrimental impact on water quality. Due to nitrification, chloramine residual, pH, alkalinity and dissolved oxygen of water decrease. As nitrification can cause a decrease in pH, some utilities may be susceptible to elevated levels of soluble metal contaminants such as lead (Zhang et al., 2009), leading to Lead and Copper Rule (LCR) violations. Nitrification can also cause biological instability through production of soluble microbial products which may support the growth of heterotrophic bacteria in low nutrient environments (Rittmann et al., 1994).

Studies on nitrification in drinking water have mostly been done in distribution mains or at the treatment plant level and studies on premises plumbing are lacking. Premises plumbing not only has higher surface to volume ratios but also has about 10 times more length than water mains (NRC, 2006). In addition, it stands to reason that favorable conditions for nitrification such as low or no disinfectant, long water age and warmer temperatures exist in premises plumbing. This gap in knowledge led to investigations on possible control for nitrification in premises plumbing. The studies took place in laboratory reactors designed to simulate the surface area to volume ratios, flow conditions, and water quality that may be encountered in these systems. Ammonia concentrations chosen represented a worst case scenario where all the chloramine added at the regulatory limit (4 ppm) had decayed to release ammonia. Two commonly utilized premises plumbing materials, copper and polyvinyl chloride (PVC), were used. Copper is the most widely used metal for household plumbing systems and more than 90% of domestic plumbing material in the US is made of copper (Oskarsson and Norrgren, 1998) while PVC pipes are also a very common plumbing material (NSF, 2008).

Three separate strategies based on realistic approaches for drinking water systems were evaluated. First, the inhibitory effect of copper was examined by (1) comparing nitrification in PVC vs copper reactors and (2) by introducing known amounts of copper (gradually increasing concentration) into a nitrifying reactor with PVC coupons. Previous work has shown that nitrification in pure cultures is either enhanced or inhibited by copper, depending on its concentration. Loveless and Painter (1968) found that 0.005–0.03 ppm of Cu^{+2} stimulated the growth of the ammonia oxidizer *Nitrosomonas* while Skinner and Walker (1961) observed enhanced growth at higher concentrations of 0.1–0.5 ppm copper. However, these higher concentrations were found to be inhibitory by Loveless and Painter (1968). Zhang and Edwards (2005) observed slight inhibition of nitrification for pure cultures in the presence of 5 ppb copper while 25 ppb copper had a slightly stimulatory effect. In the same study, a much higher concentration of 500 ppb copper significantly inhibited nitrification. Zhang and Edwards (2010) also reported inhibition of nitrification at copper levels greater than 100 $\mu\text{g/L}$. In cases where there is free ammonia, copper–ammonia complexes such as $[\text{Cu}(\text{NH}_3)_2]^{+1}$, (Sato et al., 1988) and cupric tetraamine $[\text{Cu}(\text{NH}_3)_4]^{+2}$ (Lee et al., 1997) may also be responsible for inhibition of nitrifiers. It is also important to know the form in which the copper exists because Cu^{+2} ions are believed to be responsible for the inhibition of nitrifying bacteria (Braam and Klapwijk,

1981; Hu et al., 2003). Cupric ions in the vicinity of the cell membrane may cause damage by depolarization and impairment of receptors or transporter molecules (Alt et al., 1990), and may bind proteins and block the function of proteins in the exopolymeric substance (EPS) of the bacteria (Geesey and Jang, 1989). Ion speciation can be controlled by the pH (Braam and Klapwijk, 1981; Edwards et al., 1996), and this was considered as part of the experimental design.

A second set of experiments was done to determine the impact of chlorite on nitrification. The use of chlorite as a control mechanism for nitrification has been proposed for full scale distribution systems and storage tanks. Hynes and Knowles (1983) showed that chlorite interfered with the first step of nitrification, specifically in the oxidation of ammonia to nitrite by *Nitrosomonas europaea*. Several studies in both laboratory and full scale drinking water distribution systems have been conducted to investigate the effect of chlorite on nitrification. McGuire et al. (1999) found that low levels (0.2 ppm) of chlorite caused a significant reduction in the culturability of ammonia oxidizing bacteria (AOB). In the same study, the experience of the Gulf Coast (Texas) Water Authority (GCWA) which uses chlorine dioxide as the primary disinfectant and chloramine as the secondary disinfectant was reported. Chlorite was detected in their distribution system in the range of 0.25–0.35 ppm and although the conditions were conducive to nitrification, it did not occur suggesting the inhibitory effect of chlorite on nitrification. In another study conducted in plug flow reactors in Tucson AZ, continuous feed of chlorite at concentrations as low as 0.1 ppm was found to prevent nitrification (McGuire et al., 1999). Chlorite was also used to prevent nitrification in parts of the Glendale, CA distribution system (McGuire et al., 2009). Interestingly, chlorite has been found to be ineffective in controlling nitrification in other studies. McGuire et al. (1999) reported a nitrification episode in the Corpus Christi Texas distribution system that continued even after dosing with chlorite. Similarly, Karim and LeChevallier (2006) reported the recurrence of nitrification in a pilot system where initially the use of 0.5 ppm chlorite controlled nitrification. In another study the presence of chlorite in water reservoirs prevented the onset of nitrification, but once nitrification started, introducing chlorite was not effective and a 0.2 ppm dose of chlorite in a nitrifying reservoir inhibited nitrification for only two weeks (McGuire et al., 2006). To investigate the impact of chlorite on nitrification in the simulated premises plumbing system, doses were incrementally increased from 0.2 to 20 ppm.

A third potential control mechanism was maintaining a chloramine disinfectant residual within the plumbing system. Chloramine, although considered as a weaker disinfectant than chlorine for suspended cells, is thought to be more effective for disinfecting biofilms (LeChevallier et al., 1988; Wahman et al., 2009), and since most nitrifying bacteria are present as biofilms rather than planktonic cells in both natural and engineered systems (Schramm et al., 1996; Okabe et al., 2005; Kindaichi et al., 2006), the use of chloramine was also examined. In an actual water distribution system, it may be possible to either distribute water with a stable residual or recreate chloramine through booster chlorination. To investigate this experimentally, chlorine was applied to attain different chlorine to ammonia ratios.

All of these experiments took place in reactors that had been undergoing complete nitrification (conversion of ammonia to nitrate) for one year. Importantly, the reactors had undefined mixed population biofilms originating from Bozeman tap water and were not inoculated with specific nitrifying organisms. The operating conditions were carefully chosen to represent conditions in premises plumbing while allowing for meaningful sampling strategies and control of variables. During each test, effluent copper concentrations were measured to assess the influence of that strategy on copper release. As such, the results provide insights on how indigenous nitrifying biofilm communities respond to potential control strategies and how these strategies influence effluent water quality.

2. Materials and methods

2.1. Reactors

Domestic plumbing systems were simulated using a modified CDC reactor (Goeres et al., 2005). Modifications included adding parallel coupons to solid rods, adding a base plate, and changing the stir blades to either polyvinyl chloride (PVC) or copper (Fig. 1). The surface area of the coupons, base plate and stir blades was calculated to create the same surface to volume ratio as that of a six foot long $\frac{3}{4}$ " diameter domestic plumbing pipe. The PVC or copper was washed with 0.1 N NaOH three times prior to use. Volume of the reactors is 120 ml.



Fig. 1 – Modified CDC reactor showing the paired copper coupons (1.5 × 1.7 cm inside, 1.5 × 1.3 cm outside on the rods), base plate (bottom of the reactor) and the blade in the center.

2.2. Operational scheme

To simulate periods of stagnation in home plumbing the reactors were flushed with peristaltic pumps with shear created by the stirplate for 5 min followed by 8 h stagnation periods. The feed pumps and stirplates were controlled by timers that were offset by 1 min with the stirplate starting before the pumps. At the end of 5 min the stirplates stopped, followed by the pumps. The stirplates were set to create a rotational speed of the blade of 300 rpm, which was approximately equivalent to a velocity of 3 ft/s in the bulk water. The cycle was repeated three times per day.

Two sets (four reactors each) of modified CDC reactors equipped with two different types of coupons (PVC and copper) were used in this investigation. All reactors had been in operation for more than one year and showed signs of stable, complete nitrification as measured by conversion of ammonia to nitrate.

2.3. Stock/feed solution preparation

All reactors were fed with a combination of mineral amended reverse osmosis (RO) water, biologically treated Bozeman tap water, and a humic substances organic feed. The RO water was amended to create an alkalinity of 35 mg/L as CaCO₃ and a stable pH of 8.15. Constituents of the RO water + mineral feed were MgSO₄ (39.6 mg/L), NaHCO₃ (59.6 mg/L), CaSO₄·2H₂O (25 mg/L), Al₂(SO₄)₃·18H₂O (0.62 mg/L), CaCl₂·2H₂O (20.80 mg/L), and Na₂SiO₃·9H₂O (26 mg/L). Ammonium sulfate was added to provide a final concentration in the reactor of 0.71 mg/L as N. Biologically treated water provided as a separate, parallel influent. It was created by flowing Bozeman tap water (surface water source, no background ammonia, chlorinated) through a granular activated carbon column followed by flow through a biologically active carbon column to provide a continuous inoculum of indigenous organisms (10⁴ CFU/ml of heterotrophic plate count (HPC)) that was the only source of microorganisms to the reactors. This water also contained sufficient background phosphate for microbial growth at the organic carbon level used. Organic carbon was supplied to the reactors in a third, separate influent in the form of soil-derived humic substances. 50 grams of Elliot silt loam soil (International Humic Substances Society) was added to 500 ml of 0.1 N NaOH and mixed for 48 h. This solution was centrifuged at 10,000 × g for 20 min. The supernatant was collected in carbon free glassware (baked at 390 °C for 5 h) and stored at 4 °C in the dark. Total organic carbon content of the humics was measured using a Dohrman DC-80[®] and subsequently diluted to the appropriate concentration using the RO water feeding the reactors to provide a concentration in the reactors of 4 mg/L as dissolved organic carbon. The RO water/biologically treated Bozeman tap water/organic carbon feed ratio was 50:5:1.

Other amendments and modifications of the influent feed are described below for each set of specific experiments.

2.4. Sampling

Water was collected from the reactors at the end of the 8 h stagnation period three times weekly. Samples were analyzed

for ammonia, nitrate, and nitrite. Weekly samples were analyzed for heterotrophic plate counts, ammonia oxidizing bacteria, and nitrite oxidizing bacteria.

Free $\text{NH}_3\text{-N}$ was measured using a HACH 2000 spectrophotometer using the salicylate method (HACH method 10023) at 655 nm immediately after the samples were collected. Nitrite was analyzed using a HACH 2000 spectrophotometer and the diazotization method. Reaction of nitrite with sulfanilic acid and forms an intermediate diazonium salt that couples with chromotropic acid to produce a pink colored complex measured at 507 nm. Nitrate in filtered samples (0.2 μm pore size polyethersulfone) was measured using a Dionex[®] ion chromatography system with a CD20 conductivity detector and GP40 gradient pump unit. An AS4A column and DS3 detection stabilizer was also used in this method. The Dionex[®] ion chromatography system was calibrated using five sodium nitrate standards (1, 0.5, 0.2, 0.1, 0 ppm of $\text{NO}_3\text{-N}$). To minimize experimental error, after every seven measurements a standard solution of nitrate was measured to check the accuracy of the measurement. If the obtained measurement of the standard was outside 90–110% of the standard value then the calibration was repeated and sample was measured again (Standard Methods, 1995).

Heterotrophic plate counts were done according to Standard Methods (1995) 9215A using R2A agar plates. Plates were incubated at 20 °C for 7 days, and then the number of colonies in the plates was counted using a Quebec colony counter. For the chloramine experiments, the disinfectant was neutralized with sodium thiosulfate prior to dilution and plating.

Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) populations were enumerated using the most probable number (MPN) technique (Rowe et al., 1977) using Costar[®] Clear-Bottom 96 well microtiter plates. The mineral medium used for AOB contained per liter: $(\text{NH}_4)_2\text{SO}_4$, 330 mg; KH_2PO_4 , 100 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 40 mg; CaCl_2 , 15 mg and 1 ml of a trace-element solution. The trace-element solution contained per liter: Na_2EDTA , 4292 mg; $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 1988 mg; $\text{MnCl}_2 \cdot \text{H}_2\text{O}$, 99 mg; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 24 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 24 mg; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 17 mg; ZnCl_2 , 68 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 24 mg and H_3BO_3 , 62 mg. Bromothymol blue (5ml/L of 0.04% solution in water) was added as a pH indicator. The pH was adjusted to 8 using 1 M NaOH before autoclaving at 110 °C for 15 min. The NOB medium had the same composition except that it did not contain $(\text{NH}_4)_2\text{SO}_4$ and bromothymol blue, and was supplemented with 34.5 mg/L NaNO_2 . The pH was adjusted to 6.5 with 1 M NaOH before autoclaving at 110 °C for 15 min. After inoculation the microtiter plates were sealed with polyester tape to prevent evaporation and incubated for nine weeks at 20 °C in the dark. After the incubation period AOB and NOB presence was determined by detecting nitrite and nitrate in the medium by adding 40 μl of 0.2% diphenylamine in H_2SO_4 in the well. The absorbance of the blue color was measured at 630 nm with a microplate reader (EL808 ultra microplate reader BioTek instruments[®]). The blue color indicated nitrite or nitrate had formed and the well was scored as positive. Griess Ilosvay reagent (Alexander and Clark, 1965) was used to detect nitrite. A red color was produced within 5 min and absorbance measured after 5 min at 540 nm with the microplate reader. If nitrite in the well was detected it was scored as

negative for NOB. The difference between the two measurements then allowed for differentiation between AOB and NOB. The MPNs were calculated according to Rowe et al. (1977).

Biofilm samples were collected at the end of each experiment's test period prior to the recovery phase. One coupon was removed from the reactor and placed in a glass tray (baked at 390 °C for 5 h) containing the autoclaved RO water. The coupon was then scraped using an autoclaved rubber policeman inside a laminar flow hood. The biomass with water was poured in a sterilized 50 ml Falcon[®] tube, which was then homogenized with a homogenizer (Bio-homogenizer[®] Model M133/12810, ESGE[®]) for 30 s. From the homogenized biomass, samples were taken for MPN and HPC analysis.

2.5. Statistical analysis

Paired t-test analysis was done using Microsoft Excel on the data to see if there are significant differences between two treatments. The level of significance for all tests was $\alpha = 0.05$.

2.6. Experimental approach

2.6.1. Effect of copper ion on nitrification

To test the effect of copper on nitrification, copper in the form of CuSO_4 was added to the influent of one nitrifying PVC reactor. Initially 15 ppb copper was continually dosed and incrementally raised to 1.3 ppm, which is the action limit for copper according to the Lead and Copper Rule. Each concentration of copper was maintained for two weeks. Another PVC reactor was used as the control where no copper was added. Because nitrification is presumed to be more affected by free copper (Cu^{+2}) (Braam and Klapwijk, 1981), and the amount of Cu^{+2} in solution is a function of pH, the pH of the influent with 1.3 ppm of copper was then reduced gradually from 8.15 to 6.6 by 0.3 units every two weeks. Another PVC reactor with the influent at the same pH but with no copper was used as a control.

When copper was added to the PVC reactor at lower concentrations (less than 400 ppm) an ICPMS (inductively coupled plasma mass spectrometer) was used to measure the copper. At higher concentrations a HACH 2000 spectrophotometer (method 8506,560 nm wavelength) was used. Both total and dissolved copper in the effluent water were measured. Dissolved copper is operationally defined as the portion of the copper which passes through a 0.45 μm pore size syringe filter. In the presence of colloidal species that can pass through the filter, the method represents an upper bound to truly soluble copper. When the pH was adjusted, a copper ion selective electrode (Cu-ISE), (Orion cupric electrode, model, 94–29, Boston, MA) was used to measure the free copper (Cu^{+2}) in the water. The electrode was calibrated using standard cupric ion solutions according to manufacturer's direction before measuring the sample. Cupric ion at 1.3 ppm total copper and pH 6.6 was measured and was found to be in the 0.90 ± 0.10 ppm range.

2.6.2. Effect of chlorite on nitrification

Laboratory grade sodium chlorite was added to the influent of one PVC and one copper reactor. Initially 0.2 ppm of chlorite was added and gradually increased to 2.0 ppm and then

a shock load of 20 ppm of chlorite was dosed. Each concentration of chlorite was tested for two weeks. Chlorite was measured by a modification (McGuire et al., 1999) of the US Environmental Protection Agency (USEPA, 1993) method 300 with the Dionex® ion chromatography system described above equipped with an AS9 column and 100 µl sampling loop. Chlorite was then discontinued and the recovery of nitrification in both the PVC and copper system was observed. Copper concentrations in the copper reactor effluent were also monitored.

2.6.3. Effect of chloramine on nitrification

To represent different scenarios that can occur in a distribution system due to chloramine decay, different amounts of chlorine were added in the ammonia-containing RO water influent of two nitrifying copper and PVC reactors. The added sodium hypochlorite formed chloramine with the ammonia in the influent at different chlorine to ammonia ratios. Initially a 0.5:1 ratio was applied and gradually raised to 5:1. Both free and total chlorine were measured using a Lamotte DC1100® colorimeter with the DPD colorimetric method (Standard Methods, 1995, method 4500-Cl). At the beginning of the measurement phosphate buffer was added to the sample to maintain a pH between 6.2 and 6.5. Dissolved and total copper in the copper reactor effluents were also measured.

At the end of chloramine exposure, chlorine was discontinued and recovery of nitrification in PVC and copper reactors was compared.

3. Results

All experiments were done in reactors where stable, complete nitrification (conversion of ammonia to nitrate) had been occurring for one year. Before initiation of the experiments, there was no difference in ammonia conversion between PVC and copper, and there was good reproducibility in effluent water quality between pairs of reactors with the same coupon materials (data not shown). All testing consisted of a phase where the reactors were exposed to a stepwise change in water quality (addition of copper/change of pH, addition of chlorite, or increase in chlorine to ammonia ratio) and compared to a control reactor with stable influent water quality followed by a recovery period where complete nitrification was again established. As such, the data demonstrated the efficacy of the treatment method in addition to the robustness of the nitrification process.

3.1. Effect of copper on nitrification

To eliminate the influence of the copper substratum on the results, these experiments were conducted with copper ion added to a PVC reactor. Copper doses were incrementally increased from 15 to 1300 ppb. At the lower copper doses there was little difference in NH_3 utilization measured by percent disappearance of influent to effluent concentrations in the effluent. At higher copper doses (i.e., 600–1300 ppb) intermittent small decreases in NH_3 utilization were observed. Overall, there was no statistically significant difference in the effluent ammonia concentrations of the copper reactor

compared to the control until 600 ppb was reached. After 600 ppb added copper, the difference was significant ($p < 0.05$) but actual measurements differed by a maximum of only 0.05 mg/L $\text{NH}_3\text{-N}$ ($n = 6$ for each copper dose). A similar trend was seen with nitrate measurements; there were very small differences with a maximum variation of 0.04 mg/L $\text{NO}_3\text{-N}$ between the control and copper dosed systems. After the highest copper dose was reached, and to ensure all copper was as Cu^{+2} , the pH of the reactor was gradually lowered to 6.6. Another PVC reactor without copper where the pH was also adjusted was used as a control. There were statistically significant differences in effluent ammonia and nitrate levels at all pH values (7.8–6.6 at 0.3 increments) but the actual amounts were no more than 0.04 mg/L $\text{NH}_3\text{-N}$ or $\text{NO}_3\text{-N}$. In all cases, there was no more than 0.26 mg/L $\text{NH}_3\text{-N}$ in the control or copper exposed reactor effluents (range 0.16–0.26) and $\text{NO}_3\text{-N}$ ranged from 0.65 to 0.43 mg/L. Only trace amounts of nitrite were measured (data not shown).

There were no significant differences in AOB or NOB MPN counts in the reactor effluents from the copper vs control reactors. Values ranged from 7 to 35 MPN/ml for AOB and from 4 to 41 MPN/ml for NOB. These results are reported because utilities will typically only measure bulk water nitrifier numbers since biofilm interrogation is not feasible. Similarly, there were no differences in the HPC values, with the range from 4×10^4 to 6×10^5 per ml. Increasing copper doses were not statistically correlated with MPN or HPC values.

3.2. Effect of chlorite on nitrification

Because chlorite has been used in full scale distribution systems to control nitrification with varying effect, this chemical was added in increasing doses (0.2–20 ppm) to nitrifying PVC and copper reactors. Reactors that did not receive chlorite were maintained as controls. As seen in Fig. 2, low range chlorite (i.e., 0.2–2 ppm) did not affect nitrification in the PVC reactor. When the dose was increased to 20 ppm the PVC reactor was slightly affected as it temporarily had lower $\text{NH}_3\text{-N}$ utilization, but even in the presence of chlorite, complete ammonia loss rebounded. The temporary decreases in $\text{NH}_3\text{-N}$ utilization may have been the result of detaching biomass, adaptation to the chlorite, or other unknown factors.

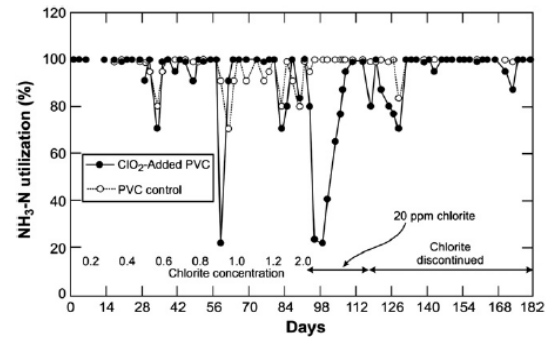


Fig. 2 – $\text{NH}_3\text{-N}$ utilization (%) in bulk water for chlorite dosed and control PVC reactors.

The copper reactor was relatively unaffected by lower chlorite doses, but at 20 ppm chlorite, $\text{NH}_3\text{-N}$ utilization dropped to zero (Fig. 3). After chlorite was discontinued it took almost two months to re-establish complete nitrification.

The bulk water $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ as percentages of the added $\text{NH}_3\text{-N}$ were calculated. For the both reactors, chlorite at the low range (0.2–2.0 ppm) did not significantly affect the $\text{NO}_2\text{-N}$ percentage. At 20 ppm chlorite, $\text{NO}_2\text{-N}$ percentage increased noticeably to about 1–3% of the added ammonia in both systems, but the magnitude of this change was only in the ppb range. In the case of $\text{NO}_3\text{-N}$, the percent of ammonia converted decreased from near 100%–60% in the PVC reactor the day that the chlorite was increased to 20 ppm and rebounded to the original level of near 100% within 10 days even when this level of chlorite was maintained. In the case of the copper reactor, the percent conversion dropped to less than 5% after a week of exposure to 20 ppm chlorite and did not rebound for almost two months.

Effluent total and dissolved copper concentrations in chlorite added and control copper were also measured. The 95% confidence interval for total and dissolved copper for the chlorite dosed reactor (0.8 ± 0.04 and 0.69 ± 0.03 ppm) was higher than that for control reactor (0.71 ± 0.02 and 0.63 ± 0.02 ppm). A paired t-test for both effluent total and dissolved copper was averaged for chlorite concentrations from 0.1 to 2 ppm and for 20 ppm. In all cases, there was significantly more total and dissolved copper in reactors that had received chlorite.

The MPN values for AOB and NOB were comparable to those found in the previous copper experiment. The only difference was that at 20 ppm chlorite, no NOB were found in the effluent of both the PVC and copper reactors. HPC values were unaffected and similar to those in the previous copper experiment.

3.3. Effect of chloramine on nitrification

The effect of chloramine on nitrification was investigated by gradually increasing the amount of free chlorine fed to the influent of nitrifying PVC and copper reactors. Initially chlorine was added at 0.5:1 chlorine to ammonia ratio and gradually raised to a 5:1 ratio, with a total/combined chloramine

dose of 3.55 mg/L. NH_3 utilization in the chloramine dosed PVC, copper and control reactors are shown in Fig. 4. Bulk $\text{NH}_3\text{-N}$ utilization decreased significantly only at the 5:1 chlorine to ammonia ratio, with occasional decreases at the lower ratios. Note that the 5:1 ratio was maintained for two months and there was a long period of acclimation where the ammonia utilization gradually declined. Even at the 5:1 ratio after two months of exposure, the copper reactor continued to utilize around 20% of the ammonia. When chloramine was discontinued, the copper reactor regained full nitrification after three weeks. In contrast, the PVC reactor ceased ammonia utilization at the higher ratio and it required approximately six weeks to recover its nitrifying ability.

The percent conversion of ammonia in the influent to $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ in the reactors after 8 h of stagnation was calculated for all chlorine to ammonia ratios. Only in the case of the 5:1 ratio in the PVC reactor did the nitrite level initially increase from the detection limit and peaked at two weeks to approximately 1.4% of the added nitrogen. This level then again decreased to no detectable nitrite. There was no change in nitrite in the copper reactors as a result of increased chlorine to ammonia ratios; it remained at the limit of detection suggesting complete conversion to nitrate. For the copper reactor, the percent of ammonia converted to nitrate dropped one week after attaining the 5:1 ratio to an average of 18% conversion and it took two weeks to reach an average of 12% in the PVC reactor. When chlorine addition was terminated, there was a rebound of total conversion of ammonia to nitrate approximately three weeks later for the PVC reactor. This time was considerably longer (seven weeks) for the copper reactor, and corresponds to the complete loss of ammonia (Fig. 4).

Total and dissolved copper concentrations in the effluent of the monochloramine-dosed and control copper reactors were measured at each chlorine to ammonia ratio. The 95% confidence intervals for total and dissolved copper for the monochloramine-dosed reactor (0.83 ± 0.02 and 0.73 ± 0.02) are higher than that for control reactor (0.75 ± 0.02 and 0.63 ± 0.01). A paired t-test showed that the p values for total and dissolved copper were significantly different. It appears that the presence of monochloramine significantly increased the copper concentration in the water.

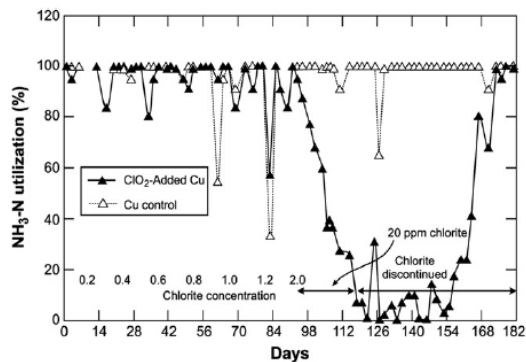


Fig. 3 – $\text{NH}_3\text{-N}$ utilization (%) in bulk water for chlorite dosed and control copper reactors.

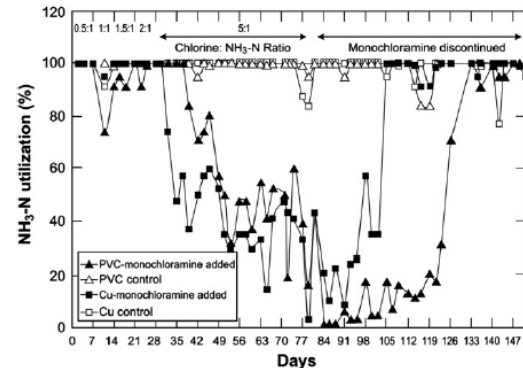


Fig. 4 – $\text{NH}_3\text{-N}$ utilization (%) in bulk water for chloramine dosed (PVC, copper) and control (PVC, copper) reactors.

The average MPN value for AOB and NOB in the reactor water are shown in Table 1. In general, there were fewer AOB and NOB in reactors that received chlorine. No NOB were detected in the presence of chloramine at the 5:1 ratio in either reactor which is consistent with the increased level of nitrite that was measured. HPC effluent counts were unaffected and comparable to those of the control reactors that did not receive chlorine (data not shown).

3.4. Effect of copper, chlorite and chloramine on biofilm cell numbers

AOB/NOB and HPC numbers in the biofilm were determined at the end of each experiment and before the recovery phase (Table 2). AOB/NOB abundance is typically about 3 logs lower than the HPC with the exception of the highest chlorine to ammonia (5:1) ratio in the copper reactor, where the AOB were present at numbers greater than that of the heterotrophs. No NOB were detected in the copper reactor biofilms at the termination of the chlorite and chloramine experiments. A lack of detection of NOB in the biofilm and the bulk water is consistent with the increased levels of nitrite measured in these reactors.

4. Discussion

In this project three different strategies for controlling nitrification in household plumbing were studied. The inhibitory effect of copper, chlorite, and a chloramine residual on nitrification were tested. The impact of each control measure on the release of total and ionic copper from copper coupons into solution was also evaluated. Throughout the experiments in the control reactors, there was total loss of ammonia, no detectable nitrite, and near complete conversion of ammonia to nitrate. This suggests that nitrite was likely formed but converted to nitrate within the 8 h stagnation period of the experimental system and that complete nitrification was occurring. Complete nitrification is contrary to what has often

Table 1 – Average ($n = 2$) MPN per ml for ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) at different Cl_2 to $\text{NH}_3\text{-N}$ ratios. ND = none detected.

Chlorine to $\text{NH}_3\text{-N}$ ratio		NH_2Cl added PVC reactor	Control PVC reactor	NH_2Cl added copper reactor	Control copper reactor
0.5:1	AOB	31	37.7	10.7	38.1
1.0:1.0		27.6	31.5	10.4	14.5
1.5:1		9.7	52.2	6.9	27.6
2:01		7.2	9.5	8.7	17
5:01		12.7	31.4	13.2	21.2
0.5:1	NOB	48.8	59.1	106.7	30.3
1.0:1.0		9.7	23.3	23.5	51.5
1.5:1		21	351.9	28.5	170.9
2:01		8.4	793.6	10.2	61.5
5:01		ND	310.1	ND	48.3

Table 2 – Cell numbers for autotrophic and heterotrophic populations in the biofilm at the end of each experiment. AOB – ammonia oxidizing bacteria, NOB – nitrite oxidizing bacteria, HPC – heterotrophic plate counts. ND = none detected.

	AOB (MPN/cm ²)	NOB (MPN/cm ²)	HPC (CFU/cm ²)
Copper added PVC	5.7×10^3	4.4×10^3	1.6×10^6
pH Control PVC	2.8×10^3	3.2×10^3	7.7×10^5
Chlorite added PVC	3.5×10^2	7.7×10^1	1.5×10^6
NH_2Cl added PVC	9.2×10^1	7.9×10^2	1.2×10^5
PVC-control	2.4×10^3	2.8×10^3	1.2×10^6
Chlorite added copper	1.3×10^1	ND	1.8×10^5
NH_2Cl added copper	1.4×10^2	ND	9.8×10^1
Copper-control	1.1×10^3	8.0×10^2	1.8×10^5

been observed in nitrifying water distribution systems where there is incomplete nitrification, nitrite accumulation, and high nitrite-N concentrations (Wolfe et al., 1988).

4.1. Added copper

Copper introduced to the nitrifying PVC reactor did not have a significant effect on ammonia utilization, nor on nitrate concentration in the reactor. The numbers of HPC, AOB and NOB were also unaffected by copper. This observation contradicts the results from other researchers (Skinner and Walker, 1961; Loveless and Painter, 1968; Martin and Richard, 1982; Zhang and Edwards, 2005, 2010), who reported that low (5 ppb–0.56 ppm) copper concentrations inhibited nitrification. It is possible that inhibition may have occurred if concentrations had been increased to higher levels as those used by Tomlinson et al. (1966) and Meiklejohn (1950). The discrepancy in copper sensitivity between the results of this work and that of others may be because the previous work was done with pure cultures or activated sludge, and the copper tolerance for those biological systems may be much different from the biofilm grown in the reactors used in this project. Another possible explanation is that organic matter contains functional groups (Sarathy and Allen, 2005) that bind metals to form less bioavailable complexes (Loveless and Painter, 1968; Dodge and Theis, 1979; Greclius et al., 1982) that are therefore less inhibitory (Kim et al., 2006). The humics used in this research may have acted in this capacity although the copper in the reactor water was detected in the ionic form.

Added copper also had no impact on the HPC numbers in the reactors. This is in contrast to the findings of others (Thurman and Gerba, 1989; Artz and Killham, 2002; Kim et al., 2002; Teitzel and Parsek, 2003; Lehtola et al., 2004) who reported that copper has a toxic effect on heterotrophs in drinking water. Again this discrepancy may be due to the difference in experimental conditions and bacterial populations. Another potential explanation is that the reactors used in this study contained intact, natural biofilms that had been present for over a year while other studies used suspended organisms and/or fresh copper surfaces.

Copper toxicity depends on the concentration of cupric ion (Cu^{+2}) (Braam and Klapwijk, 1981), and this ionic form

becomes more abundant at a lower pH (Edwards et al., 1996). Even when the pH of the reactor was lowered to 6.6 to ensure that the added copper was in the cupric ion form, there was no effect on nitrification or the numbers of AOB, NOB or HPC in the reactor effluent or in the biofilm.

4.2. Chlorite

Chlorite was chosen as another potential control mechanism because previous studies by McGuire et al. (1999) showed that chlorite ion (0.2–1.0 ppm) in distribution systems can inhibit nitrification. Results in this project contradict their reports. The PVC reactor was initially impacted only at the unrealistic dose of 20 ppm chlorite but then regained the ability to nitrify in the presence of this concentration. At this high concentration there was an impact on nitrification in the copper system that persisted after the cessation of chlorite addition. McGuire et al. (1999) mentions that chlorite did not inhibit nitrification in one system, which according to the author may be due to the presence of higher ammonia (1.4 mg/L). Karim and LeChevallier (2006) also reported that chlorite was unable to hinder nitrification. All these studies were done with low doses of chlorite (0.2–1.0 ppm) and their flow pattern, water quality and bacterial population/biofilm characteristics may be significantly different from this project. Another possible explanation is that chlorite inhibits the activity of *Nitrosomonas europaea* and *Nitrobacter winogradski* (Hynes and Knowles, 1983), but may be inactive toward other groups of AOB and NOB that may be predominant in the reactors.

The planktonic and biofilm heterotrophic populations were unaffected by chlorite exposure. The trend in heterotrophic numbers supports previous work by Gagnon et al. (2005) where chlorite at 0.1–0.25 ppm was ineffective in inactivating heterotrophic bacteria. Similar to HPC, AOB values did not show any effect due to chlorite exposure. The planktonic NOB population remained unchanged for all concentrations of chlorite except at 20 ppm, when they could not be detected. NOB were also not detected in the copper reactor's biofilm at the end of exposure period to the high chlorite concentration. Hynes and Knowles (1983) reported that pure cultures of AOB (*N. europaea*) and NOB (*N. winogradski*) were inhibited by chlorite, and that the AOB are 50 times more sensitive to chlorite inhibition than NOB. In our experiments it appeared that the NOB were more sensitive than the AOB. Again this may be due to the differences in experimental setup, pure cultures vs environmental biofilm, water quality and other factors. It is also possible that the methods used in this work did not enumerate all of the potential nitrifying organisms in the reactors.

A potential impact of chlorite on copper plumbing could be an increased release of copper into the water. As chlorite (ClO_2^-) could be transformed to chlorine dioxide (ClO_2) in an acidic environment (Gates, 1989) that could be created by oxidation of ammonia during nitrification by biofilm on the surfaces, copper corrosion could increase due to the oxidative nature of chlorine dioxide. Therefore, there could be an unintended result of elevated copper release when chlorite is applied, and this was seen in these experiments. For this reason, and because chlorite is a regulated compound, the

utilities should carefully evaluate chlorite before implementing it as a nitrification control strategy.

4.3. Chloramine

According to a survey by Seidel et al. (2005), optimizing the chlorine to ammonia ratio is the most common nitrification control technique. The chlorine to ammonia-N weight ratio used to form monochloramine typically varies from 3:1 to 5:1 (Wilczak, 2006). Several studies in full scale chloraminated systems have determined that a minimum 2–3 mg Cl_2/L chloramine residual should be maintained to prevent nitrification (Wolfe et al., 1990; Lieu et al., 1993; Kirmeyer et al., 1995; Odell et al., 1996; Harrington et al., 2002). A combination of chloramines does and optimizing the chlorine to ammonia ratio has been shown to be the easiest and most cost-effective way to control/prevent nitrification (Lieu et al., 1993).

The results of this work can be compared to several studies where the chlorine to ammonia ratio has been measured in pilot and full scale systems and related to nitrification. McGuire et al. (2004) showed that nitrification occurred in a pilot system where chloramine was applied at a 3:1 ratio. Similarly, for two covered finished water reservoirs in southern California which were initially chloraminated at a 3:1 ratio, nitrification was significantly reduced after raising the ratio to 5:1 (Wolfe et al., 1988). A Florida utility rarely experienced nitrification when the combined chlorine residual was above 1 mg/L at a chlorine to ammonia ratio of 5:1 (Liu et al., 2005). A study done by Karim and LeChevallier (2006) showed that monochloramine applied at a 3:1 ratio was not able to control nitrification, but it was effective at a 5:1 ratio.

Based on the above information, the impact of an incremental increase in the chlorine to ammonia ratio on nitrification was tested. The intent was to test situations in (1) a system where chloramine residual was regained and/or (2) households downstream of booster chlorination that was used to recreate chloramines from free ammonia (Wolfe and Lieu, 2001; Wilczak, 2006). At the starting ratio of 0.5:1, about 0.35 mg/L of total chlorine was present. This was chosen because Holt et al. (1995) showed reduction of nitrification at total chlorine concentrations of more than 0.3 mg/L. However, at an 8 h stagnation time, this low ratio did not have an impact on nitrification and organisms responsible for nitrification could be found in the bulk water and biofilm. Very minor transient impacts were seen with increasing ratios of 1:1, 1.5:1, and 2:1. There are several possible explanations for these results. Nitrifying bacteria form protective layers (slime layer or capsules) mainly composed of polysaccharides (Prosser, 1986) as a defensive mechanism against unfavorable environmental conditions such as low pH. These capsules protect organisms so that they are more resistant to disinfectants (Stewart and Olson, 1996). Cunliffe (1991) detected nitrifiers in 64% of the samples collected in South Australia and of them 20.7% contained more than 5.0 mg Cl_2/L of monochloramine. The author hypothesized that as the nitrifiers grow in aggregates or in biofilm attached to the surface, they remain unaffected by disinfectant and the nitrifiers detected in samples containing high chloramine residual may have been detached shortly before or during the sampling

period. Higher AOB have been detected in biofilm than water in the Metropolitan Water District of Southern California distribution system (Stewart and Lieu, 1997). Regan (2001) postulated on the protective mechanism of biofilms. Monochloramine is mass transport limited in biofilm, so bacteria inside the biofilm are not exposed to the disinfectant. Another protective mechanism may be the relative ratio of growth vs disinfection. Harrington et al. (2003) stated that if AOB growth rate driven by ammonia concentration exceeds the AOB inactivation rate by monochloramine, then theoretically AOB can grow in the presence of monochloramine. Fleming et al. (2005) proposed nitrification potential curves based on the relative concentration of chlorine (biocide) and free ammonia (food). According to them the threshold chlorine value is 1.6, above which nitrification would be prevented, without any influence from free ammonia concentration. At chlorine concentrations below 1.6, the nitrification potential depends on the ratio of chlorine and free ammonia.

Only when the 5:1 Cl_2 to $\text{NH}_3\text{-N}$ ratio was reached was there a consistent reduction of nitrification during the 8 h of stagnation, and the affect of this ratio agrees with results from Karim and LeChevallier (2006) and Lieu et al. (1993). Likewise, in work by Harrington et al. (2002), nitrification did not occur when the total chlorine concentrations were more than 2.2 mg/L and the biocide to food ratio was 1.9 mg Cl_2 /mg of N or more. The most pronounced effect was on the PVC reactor, which is in contrast to the chlorite effect which was most obvious on the copper reactor. Interestingly, the HPC numbers in the biofilm on the copper surfaces were lower than those of the AOB, suggesting that the disinfectant had greater activity on the HPC than the nitrifiers. No planktonic NOB were detected in monochloramine exposed reactors at the 5:1 ratio and they were also not detected in the biofilm of the copper reactor. A possible explanation is that the NOB were more vulnerable to disinfection than the AOB (Wolfe and Lieu, 2001).

These results also support observations that once nitrification starts in full scale systems, higher levels of chloramine may not be an effective control method. Skadsen (1993) reported that a chloramine dose of 8 mg Cl_2 /L was not effective in controlling nitrification in the Ann Arbor, Michigan distribution system. This may be due to the fact that nitrite can degrade chloramine residuals before it can inactivate the nitrifying bacteria (Wolfe et al., 1988; Kirmeyer et al., 1995; Odell et al., 1996). Also, maintaining monochloramine at a high ratio close to 5:1 in full scale systems is not always easy, and is sometimes associated with dichloramine formation, and taste/odor problems and higher DBP formation (Skadsen and Cohen, 2006).

This project also provides some insight on effect of chloramine on copper corrosion. It has been found that chloramine increased copper corrosion (Ingleson et al., 1949). Enhanced copper solubility during periods of chloramination with excess ammonia present was observed for Champaign IL tap water (AwwaRF, 1990). Ammonia has a strong complexation constant for cupric ion (Schock et al., 1995). On the other hand, according to MacQuarrie et al. (1997) and Rahman et al. (2007), application of monochloramine results in a decrease in copper pipe corrosion. Current results found higher total and dissolved copper concentrations in the reactors where the disinfectant was added.

4.4. Copper vs PVC surfaces

Copper and PVC were chosen because of their use in plumbing systems and also because of the potential antimicrobial effect of copper vs PVC. One could also hypothesize that copper materials may be more prone to nitrification in chloraminated systems because monochloramine can rapidly decay through reactions with a copper plumbing system (Edwards and Nguyen, 2005), therefore providing more free ammonia for nitrification. Therefore, pipe material could significantly influence the nitrification process.

In the conditioned control reactors with no added chlorite or chlorine, there was no difference in ammonia utilization between the two surfaces, and the HPC, AOB and NOB numbers were similar. The lack of efficacy of copper against these organisms was supported by the experiments where copper was added to the PVC reactor and no decrease in nitrification was seen. In the presence of chlorite or combined chlorine, there were some differences between the two systems. In the case of chlorite, nitrification in the copper system was affected to a greater extent with a loss of nitrification at the highest concentration (20 ppm) and an extended recovery time after chlorite addition ceased. This could be because of the production of chlorine dioxide (Gates, 1989) on the metal surface. However, in the presence of combined chlorine, the PVC system was more greatly impacted; nitrification ceased and it required nearly six weeks for recovery. In the copper reactor, there was never a complete loss of nitrification, and complete nitrification was again attained only three weeks after chlorine addition stopped. This suggests that the mechanism of chloramine decay on the copper surface, as reported by Edwards and Nguyen (2005), could be at play.

4.5. Relationship between HPC and nitrification

Some research has shown that there is a correlation between HPC values and nitrification in water systems with HPC increasing when nitrification occurs. Wolfe et al. (1990) reported that HPC and AOB population were highly correlated in distribution system water, and that this may be explained due to the dependence of HPC on AOB for carbon fixation. However, other researchers reported that the relationship is very site specific (Donnelly and Giani, 2005), likely because HPC can grow in response to other available organics and their numbers are also affected by residual disinfectant. Throughout these experiments there was no consistent relationship between HPC and AOB/NOB MPN values. This may be due to the presence of humic substances in the reactors.

5. Conclusions

- Copper surfaces or copper added to reactors at concentrations up to 1.3 ppm within a pH range of 6.6–8.15 did not inhibit nitrification.
- Chlorite was effective at inhibiting nitrification only at an unrealistic dose (20 ppm), on copper surfaces. There was limited, transient control on reactors with PVC surfaces at 20 ppm chlorite.

- Chloramine at a Cl_2 to $\text{NH}_3\text{-N}$ ratio of 5:1 managed nitrification in the copper reactor and was able to control it in the PVC system.
- The addition of chlorite and chloramines may increase copper corrosion.
- No correlation between HPC and AOB/NOB was found.
- Time to recovery after cessation of addition of chlorite and chloramine varied but did occur, suggesting that nitrification in premises plumbing is a robust process.

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APPENDIX B

SUPPLEMENTARY FIGURES

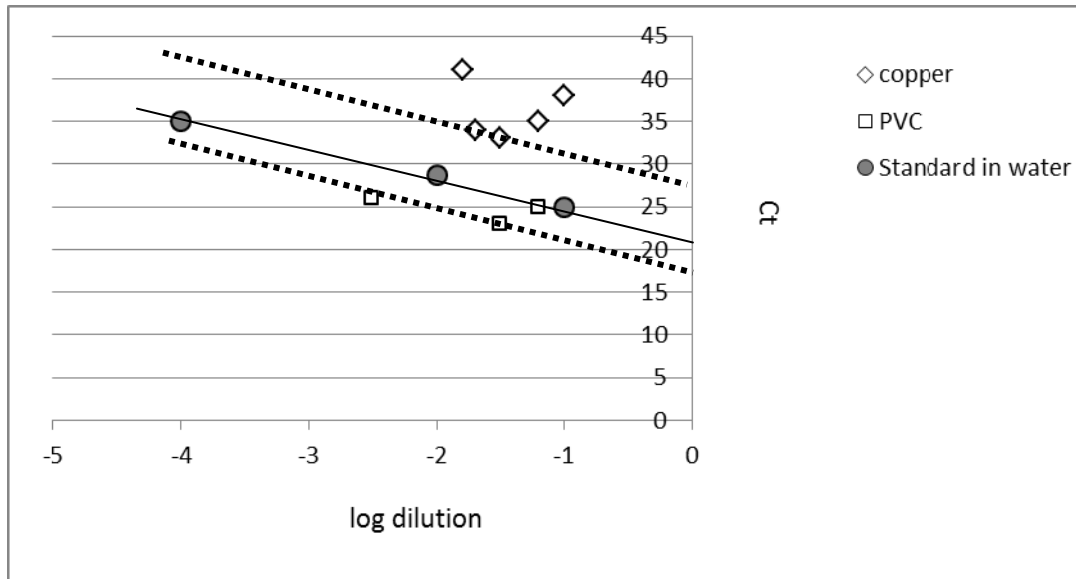


Figure S1. Ct values for various dilutions of copper and PVC samples. Nonlinearity at lower dilutions suggests presence of PCR inhibitors in the samples.

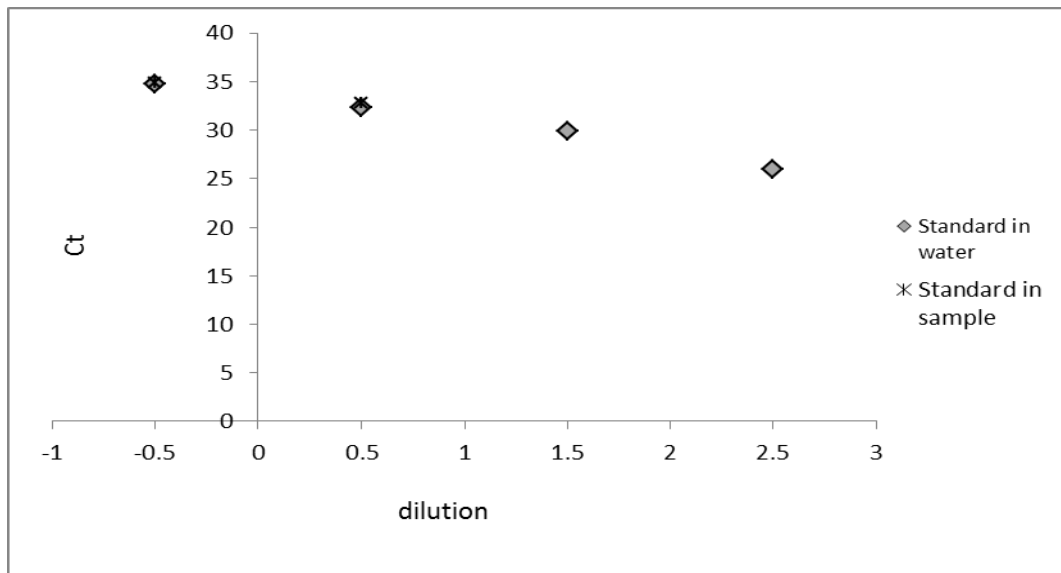


Figure S2. Example of a standard mixed with a 1/10 diluted sample. Standard with sample has similar value as standard indicating no inhibition conferred by sample.

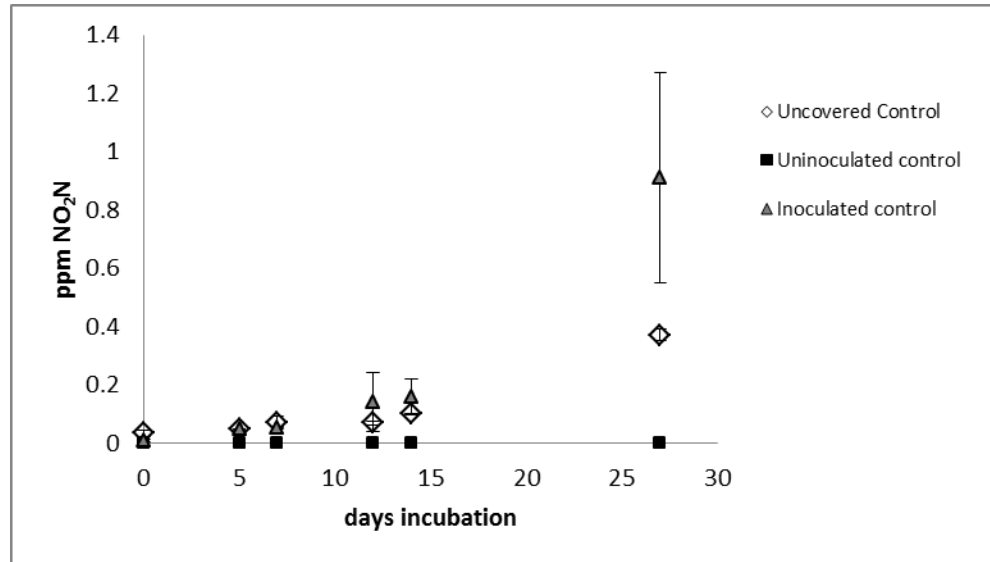


Figure S3. Enrichments incubated in dark and light conditions showing a photoinhibition of nitrite production in the enrichments exposed to light.

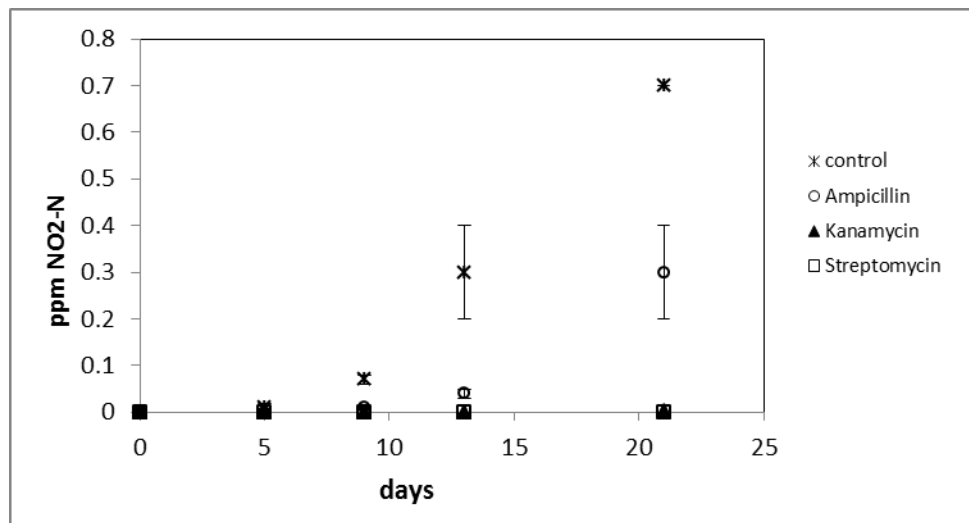


Figure S4. Enrichments with various antibiotics showing inhibition of nitrite production compared to the control.

APPENDIX C

REACTOR FEED AND HUMICS PREPARATION

Influent Water (mg/ L) pH 8.15

MgSO ₄	39.6
NaHCO ₃	56.9
CaSO ₄ 2H ₂ O	25
Al ₂ (SO ₄) 18H ₂ O	0.62
CaCl ₂ 2H ₂ O	20.80
Na ₂ SiO ₃ 9H ₂ O	26
(NH ₄) ₂ SO ₄	3.75

Preparation of Humics solution (Rahman, 2008)

50 gm of Elliot silt loam soil (International Humic Substances Society) was added to 500 mL of 0.1 N NaOH and mixed for 48 hours. The solution was centrifuged at 10,000 X g for 20 minutes and the supernatant was collected in carbon-free glassware (combusted at 390° C for five hours) and stored at 4° C in the dark. Total organic carbon content of the humics was measured using a Dohrman DC-80® and diluted to the appropriate concentration using the RO water feeding the reactors.

APPENDIX D

MODIFIED SYNTHETIC CRENARCHAEOTA MEDIUM

(MODIFIED FROM KÖNNEKE ET AL., 2005)

Modified synthetic Crenarchaeota medium (modified from Könneke et al., 2005)

Basal Medium (per L)

NaCl	1 g
MgCl ₂ .6H ₂ O	5 g
MgSO ₄ .7H ₂ O	5 g
CaCl ₂	1.5g
KBr	0.1 g
Non-chelated trace element mixture*	1 mL
Vitamin solution**	1 mL
KH ₂ PO ₄ (4g/L)	10 mL
Selenite tungstate solution***	1 mL
Bicarbonate solution (1M)	1 mL
NH ₄ Cl (1M)	1 mL
Al ₂ (SO ₄) 18H ₂ O	0.62 mg
Na ₂ SiO ₃ .9H ₂ O	26 mg

*Non-chelated trace element mixture (Widdel and Bak, 1992)

Distilled water	987 mL
HCl (25%)	12.5 mL
FeSO ₄ .7H ₂ O	2100 mg
H ₃ BO ₃	30 mg
MnCl ₂ .4H ₂ O	100 mg
CoCl ₂ .6H ₂ O	190 mg
NiCl ₂ .6H ₂ O	24 mg
CuCl ₂ .2H ₂ O	2 mg
ZnSO ₄ .7H ₂ O	144 mg
Na ₂ MoO ₄ .2H ₂ O	36 mg

Autoclaved in bottles

**Vitamin Solution (Widdel and Bak, 1992)

Sodium phosphate buffer (10mM; pH 7.1)	100mL
4-Aminobenzoic acid	4 mg
D(+)-Biotin	1 mg
Nicotinic acid	10 mg
Calcium D(+)-panthothenate	5 mg
Pyridoxine dihydrochloride	15 mg

The solution is filter sterilized and kept at 4°C in the dark

***Selenite-tungstate solution (Widdel and Bak, 1992)

Distilled water	1000 mL
NaOH	0.4 g
Na ₂ SeO ₃ .5H ₂ O	6 mg
Na ₂ WO ₄ .2H ₂ O	8 mg

Autoclaved as described previously

APPENDIX E

REAGENTS USED FOR NO₂ AND NH₄ ASSAY

Griess Ilosvay Reagent (Alexander and Clark, 1965)

Solution 1 0.6 g sulfanic acid in 70 mL hot distilled water + 20 mL concentrated HCl adjust final volume to 100 mL with distilled water

Solution 2 0.6 g alpha-naphthylamine and 1 mL concentrated HCl adjust volume to 100 mL with distilled water

Solution 3 16.4 g $\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$ and add water to 100 mL

Store solutions separately in dark bottles in the refrigerator. Mix three solutions in equal proportion prior to test.

Ammonia Phenate method reagents (Eaton et al., 2005)

Reagents:

- a) Phenol Solution: Mix 11.1 mL liquefied phenol (>89%) with 95% v/v ethyl alcohol to a final volume of 100 mL. Prepare weekly
- b) Sodium nitroprusside, 0.5% w/v: Dissolve 0.5 g sodium nitroprusside in 100 mL deionized water. Store in amber bottle for up to 1 month.
- c) Alkaline citrate: Dissolve 200 g trisodium citrate and 10 g sodium hydroxide in deionized water. Dilute to 1000 mL.
- d) Sodium hypochlorite, commercial solution ~5%
- e) Oxidizing solution: Mix 100 mL alkaline citrate solution with 25 mL sodium hypochlorite. Prepare fresh daily.
- f) Standard ammonium solution

Procedure (Volumes modified from Eaton et al. (2005) in 96 well microplate)

To 250 μL sample, add with thorough mixing after each addition, 10 μL phenol, 10 μL sodium nitroprusside solution and 10 μL oxidizing solution. Cover samples with plastic wrap and let color develop at room temperature in subdued light for at least one hr. Measure absorbance at 640 nm.

APPENDIX F

REFERENCES FOR APPENDICES

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