Characterization of the metabolic properties and ecological functions of *Thaumarchaeota* in terrestrial environments

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ABSTRACT

Characterization of the metabolic properties and ecological functions of *Thaumarchaeota* in terrestrial environments

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Anthropogenic perturbation of global nitrogen cycles, from fossil fuel combustion to excessive N-fertilizer use, has led to a number of environmental problems including eutrophication, acidification, loss of biodiversity, and increased emission of greenhouse gases. The cycling of reactive nitrogen (Nr) is governed by a variety of microbially mediated processes, including nitrification, denitrification, ammonification, dissimilatory nitrate reduction to ammonium (DNRA) and anaerobic ammonia oxidation (anammox). For over a century, aerobic ammonia oxidation, the first and rate-limiting step in nitrification, was thought to be carried out only by ammonia oxidizing bacteria (AOB). Recent studies, however, suggest that ammonia oxidizing archaea (AOA), assigned to the newly designated phylum *Thaumarchaeota*, are actively involved in global nitrogen and carbon cycles. While *Thaumarchaeota* are abundant and ubiquitous across diverse ecosystems and appear to have substantive roles in nitrification in marine environments, their contributions to nitrification in terrestrial environments and their related metabolic properties are less well understood. This dissertation examines metabolic properties, phylogenetic diversity, and ecological functions of *Thaumarchaeota* in terrestrial

environments using both cultivation and cultivation-independent molecular techniques. In particular, two terrestrial systems were investigated: freshwater sediments and the plant rhizosphere.

In studies on Columbia River freshwater sediments, I employed a combination of analytical and molecular approaches to characterize the biogeochemical gradients and populations of ammonia oxidizers at a fine vertical scale, for the purpose of determining the environmental factors shaping the distribution of AOA and AOB (**Chapter 3**). Depth profiles of ammonia monooxygenase subunit A (*amoA*) gene abundance and expression in AOA and β-AOB, and archaeal *amoA* diversity were analyzed. AOA generally had both higher abundance of *amoA* genes and gene transcripts than β-AOB, regardless of season and depth, suggesting potentially important roles of AOA in nitrification. Composition changes in putative AOA populations across steep O_2 and E_h gradients were observed, and higher AOA diversity corresponded to steep E_h gradients. A combination of environmental factors including oxygen, nitrite and nitrate concentrations, and E_h showed significant correlations with archaeal *amoA* abundance. Furthermore, to determine potential nitrification activity by AOA and AOB in freshwater sediments, nitrification-coupled growth assays were performed in sediment slurry incubations with the addition of an ammonia oxidation inhibitor allylthiourea (ATU) and a variety of organic substrates (**Chapter 4**). Results showed that β-AOB were the dominant ammonia oxidizers in sediment incubations with either organic or inorganic amendments, despite the fact that AOA were more abundant than β-AOB in natural sediments. These results suggested that some *Thaumarchaeota* might not be autotrophic ammonia oxidizers, and

may have alternative ecological strategies, such as heterotrophic or mixotrophic metabolisms, to maintain their high abundance in freshwater sediments.

Using a plant-root enrichment culture system, we characterized the diversity and metabolic potential of mesophilic soil *Thaumarchaeota* selected with organic and inorganic amendments (**Chapter 5**). Comparative analysis of 16S rRNA and *amoA* genes indicated that specific archaeal clades were selected under different conditions, whereas AOB were not detected in this enrichment. Three *amoA*-containing clades were identified, while a fourth clade identified by 16S rRNA gene analysis alone, designated the "root clade," yielded no corresponding *amoA* gene with the primers used. Analysis of archaeal community composition by PCR-single stranded conformation polymorphism (PCR-SSCP) under different culture conditions revealed that the root clade was present only in media with organic amendment, while *amoA*–containing clades were present in media with either organic or inorganic amendment. We hypothesized that the root clade archaea were heterotrophs, assimilating organic carbon in root extract as substrates. Furthermore, results from gene abundance and expression analyses, together with potential nitrification activity assays suggested differential contributions by the clades to nitrification in our system. Taken together, our results indicate diverse metabolic lifestyles in soil *Thaumarchaeota*.

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INTRODUCTION

1.1 Background

1.1.1 The global nitrogen cycle

The nitrogen cycle is one of the most important biogeochemical cycles on earth. Nitrogen is an essential element to all living organisms. It is a component of biological building blocks such as proteins and nucleic acids, and also a crucial component of primary nutrients. Although about 78% of the atmosphere is nitrogen gas, most of it is unavailable for biological use. Only reactive nitrogen (Nr), produced by lightning and atmospheric deposition, industrial synthesis and biological nitrogen fixation, is available to organisms (Galloway et al., 2004; Gruber and Galloway, 2008). Therefore, nitrogen is often a limiting nutrient, especially in the open ocean and some terrestrial environments (Galloway et al., 2004; Gruber and Galloway, 2008). The nitrogen cycle is regulated by a variety of oxidation and reduction reactions mediated by specific microorganisms and their functional enzymes, including nitrogen fixation, nitrification and denitrification, anaerobic ammonia oxidation (Anammox), ammonification and dissimilatory nitrate reduction to ammonium (DNRA) (Figure 1.1). Nitrification and nitrifying microorganisms play important roles in controlling the availability of nitrogen for primary production, and thus influence the global carbon cycle (Yool et al., 2007; Yool, 2011). Denitrification and Anammox are the major pathways to convert Nr to reduced forms.

Over the past century, the nitrogen balance has been substantially disturbed by increasing anthropogenic Nr input into the environment. The excessive use of nitrogen

fertilizers in agriculture often results in eutrophication of water bodies, which is signified by toxic algal blooms. Eutrophication has severe environmental consequences, including changing food web structure in aquatic systems, increasing anoxia and hypoxia in water columns, and deteriorating drinking water sources (Camargo and Alonso, 2006). Another important issue caused by human activity is the production of N_2O, a major greenhouse gas contributing to global warming and ozone depletion, from N-fertilizers use to increasing fossil fuel combustion. Over the past several decades, the scientific community has sought to address the questions of how the global nitrogen cycle is impacted by human activity, and how microorganisms mediate the nitrogen cycle in response to anthropogenic perturbation. Along the way, many exciting discoveries have been made to help shape a more complete picture of global nitrogen cycle. For example, the process of anammox (Mulder et al., 1995; Strous and Jetten, 2004), and aerobic ammonia oxidation within the domain *Archaea* (Venter et al., 2004; Treusch et al., 2005; Könneke et al., 2005) have recently been discovered, and dramatically changed our view of the global nitrogen cycle. The work presented in this thesis was focused on ammonia oxidizing archaea (AOA), or *Thaumarchaeota*. The following sections summarize recent findings in the research on these novel archaea.

1.1.2 *Thaumarchaeota* - a newly proposed phylum in the *Archaea*

Archaea, one of the three domains of life, is generally less well studied compared to the *Bacteria* and *Eukaryota* (Woese, 1987;1998; Woese et al., 1990). The domain *Archaea* was originally thought to be composed of two distinct phyla, the *Euryarchaeota*, and *Crenarchaeota*. In the past, *Crenarchaeota* were believed to live only in extreme environments, and to occupy narrow ecological niches, until recent

discoveries of nonthermophilic members. Our limited knowledge of these microorganisms is mainly because of the great difficulty in cultivating most mesophilic members by traditional approaches. Recent advances in culture-independent molecular techniques provided opportunities to study these unculturable microorganisms directly from environmental samples, which dramatically broadened our knowledge of their diversity and physiology.

Mesophilic *Crenarchaeota*, whose 16S rRNA genes are deeply divergent from thermophiles, were first discovered in 1992 separately by DeLong (DeLong, 1992) and Fuhrman *et al* (Fuhrman et al., 1992). Mesophilic *Crenarchaeota* were later found to be ubiquitous and abundant in almost every environment (Hallam et al., 2006a; Leininger et al., 2006; Francis et al., 2005), comprising 20–30% of the marine prokaryotes in the open ocean below euphotic zones (>150 m) (Karner et al., 2001; Herndl et al., 2005) and 1–5% of the total microbial community in many soils (Ochsenreiter et al., 2003; Buckley et al., 1998). In addition to being numerically abundant, mesophilic *Crenarchaeota* have also been shown to be functionally important. Since the first discovery of the ammonia monooxygenase *(amoA)* gene in the archaeal genome (Treusch et al., 2005; Schleper et al., 2005; Venter et al., 2004), the key gene required in ammonia oxidation, increasing evidence has suggested that mesophilic *Crenarchaeot*a potentially play important roles in the nitrogen and carbon cycles.

In 2008, genomic analysis of "*Candidatus* Cenarchaeum symbiosum", a member of ammonia oxidizing archaea, led to the proposal of a novel phylum within the *Archaea* domain, *Thaumarchaeota* (Brochier-Armanet et al., 2008), in place of the previous designation of "mesophilic *Crenarchaeota*." This new designation was supported by

subsequent genomic analysis (Brochier-Armanet 2011a; a2011b; Pester et al., 2012). The designation of *Thaumarchaeota* has also been supported by their distinct phylogenetic position, characteristic marker genes, and unique metabolisms and physiology (Brochier-Armanet et al., 2008; Brochier-Armanet et al., 2011a;2011b; Hallam et al., 2006b; Walker et al., 2010). Comparative analysis of *Thaumarchaeota* and *Crenarchaeota* genomes showed that more than 250 proteins, including replication and DNA-binding proteins, are encoded in the *Thaumarchaeota* genome, but not in the *Crenarchaeota* genome (Hallam et al., 2006a; Walker et al., 2010; Spang et al., 2010; Pester et al., 2011; Blainey et al., 2011; Spang et al., 2012). Furthermore, *Thaumarchaeota* possess a phylum-specific membrane lipid called thaumarchaeol, the characteristic core glycerol tetraether (GDGT) membrane lipid containing a cyclohexane group (Damsté et al., 2012; Spang et al., 2010; Pitcher et al., 2011a), that is absent in the membranes of hyperthermophilic *Crenarchaeota*. In addition, *Thaumarchaeota* also use a novel cell division system as well. Both *FtsZ* and *Cdv* cell division systems are present in thaumarchaeal genomes, including *Nitrosopumilus maritimus*, "*Ca.* C. symbiosum", "*Ca.* Nitrosoarchaeum limnia" and "*Ca.* Nitrososphaera gargensis", while thermophilic *Crenarchaeota* lack the *FtsZ* genes (Hallam, et al., 2006a; Walker et al., 2010; Blainey et al., 2011; Spang et al., 2012).

To date, only two *Thaumarchaeota* isolates have been obtained in pure culture for further physiological and metabolic characterization: (i) the group 1.1a representative *N. maritimus*, and (ii) the group 1.1b representative *Nitrososphaera viennensis* (Könneke et al., 2005; Tourna et al., 2011). Thus far, a few complete or nearly complete genomes of *Thaumarchaeota* strains have been reported (Table 1.1), most of which belong to group

1.1a including *N. maritimus* SCM1, "*Ca.* C. symbiosum" A, "*Ca.* N. limnia" SFB1 and BD20, "*Ca.* Nitrosoarchaeum Koreensis" MY1 and AR1, "*Ca.* Nitrosopumilus salaria" BD31 and "*Ca.* Nitrosopumilu sediminis" AR2 (Walker et al., 2010; Hallam et al., 2006a; Blainey et al., 2011; Kim et al., 2011; Mosier et al., 2012a; 2012b; Park et al., 2012a; 2012b). The only complete genome within group 1.1 b is "*Ca. N. gargensis*" (Spang et al., 2012).

All of the sequenced genomes contain the ammonia monooxygenase (AMO) encoding genes *amoABC*, suggesting that most *Thaumarchaeota* have the genetic machinery for ammonia oxidation. However, the mechanism of ammonia oxidation in *Thaumarchaeota* has not been fully characterized. In ammonia oxidizing bacteria, ammonia is oxidized first to the intermediate hydroxylamine by AMO, then to nitrite by hydroxylamine oxidoreductase (HAO), but no HAO homologue has been found in published thaumarchaeal genomes (Hallam et al., 2006a; Walker et al., 2010; Blainey et al., 2011; Spang et al., 2012). Therefore, *Thaumarchaeota* might use an alternative mechanism or different enzyme complex in place of HAO for ammonia oxidation. A recent study reported that *N. maritimus* was able to use hydroxylamine as an intermediate for ammonia oxidation, suggesting that marine *Thaumarchaeota* possibly catalyze hydroxylamine oxidation using a novel enzyme complex (Vajrala et al., 2013).

In terms of central carbon metabolism, most *Thaumarchaeota* are believed to be autotrophs, because all of the key genes involved in the 3-hydroxypropionate/4-hydroxybutyrate carbon fixation pathway have been identified in the published genomes (Hallam et al., 2006b; Berg et al., 2010; Walker et al., 2010; Blainey et al., 2011; Kim et al., 2011; Mosier et al., 2012a; 2012b; Spang et al., 2012). Along with the genomic

evidence, autotrophic carbon fixation by *Thaumarchaeota* has also been observed in pure and enrichment cultures, and has been shown by metabolic studies using stable isotopic probing (SIP) and CARD-FISH/microautoradiography (FISH-MAR) techniques (Könneke et al., 2005; Hatzenpichler et al., 2008; Zhang et al., 2010; Pratscher et al., 2011).

In contrast, there is also evidence suggesting that not all of the *Thaumarchaeota* are autotrophs; in fact, some members within this phylum might be able to assimilate organic substrates. This idea has been supported by both genomic and metabolic investigations. For example, genes involved in coding the nearly complete oxidative TCA cycle, as well as a number of transporters for organic compounds, such as amino acids, were identified in several thaumarchaeal genomes (Walker et al., 2010; Blainey et al., 2011; Spang et al., 2012), indicating the genomic potential of *Thaumarchaeota* to assimilate small organic compounds. In addition to genomic potential, the metabolic ability to incorporate amino acids and other organic carbon compounds by some *Thaumarchaeota* has been suggested by several studies of the open oceans, using microautoradiography, or by measuring the natural distribution of radiocarbon in archaeal lipids (Herndl et al., 2005; Ouverney and Fuhrman, 2000; Ingalls et al., 2006; Varela et al., 2011). Recently, a study of nitrifying organisms in wastewater treatment plants indicated that some *Thaumarchaeota* might not be autotrophs, because the maximum energy generated from autotrophic ammonia oxidation in the refinery plant could not support the high abundance of *Thaumarchaeota* found there (Muβmann et al., 2011). Furthermore, heterotrophy or mixotrophy by some *Thaumarchaeota* has also been implied by the study of the soil isolate *Nitrososphaera viennensis* (Tourna et al., 2011), which required the addition of pyruvate to achieve a

high growth rate and was able to use urea as the sole energy source. Similar findings were shown in our study of a root-colonizing *Thaumarchaeota* enrichment culture (Xu et al., 2012) and are presented in Chapter 5 of this thesis.

The most common substrate for *Thaumarchaeota* is inorganic ammonium, while organic substrates such as urea or nitrogen derived from remineralization have also been suggested (Tourna et al., 2011; Alonso-Sáez et al., 2012; Levičnik-Höfferle et al., 2012; Lu and Jia, 2013). Genes responsible for urea hydrolysis and transport, and more recently for cyanate transport were identified in some *thaumarchaeal* genomes (Walker et al., 2010; Blainey et al., 2011; Spang et al., 2012; Mosier et al., 2012a; Park et al., 2012b), suggesting that *Thaumarchaeota* might be able to use organic nitrogen to fuel their energy requirements. The growth of *Thaumarchaeota* on urea as sole energy source has also been observed in the soil isolate *N. viennensis* (Tourna et al., 2011). Recent studies suggested that urea utilization may be an alternative strategy for *Thaumarchaeota* under energy-stress conditions such as the dark polar oceans (Alonso-Sáez et al., 2012) and acid soils (Levičnik-Höfferle et al., 2012; Lu and Jia, 2013).

Since the first isolation of *Thaumarchaeota* in pure culture, a variety of thaumarchaeal cultures from diverse environments have also been obtained and investigated. These studies showed that *Thaumarchaeota* potentially have diverse metabolisms and physiology. For example, the description of giant archaea within the *Thaumarchaeota* phylum from sulfidic marine mats (Muller et al., 2010) suggested that they are physiologically very different from other known *Thaumarchaeota*. The world of enigmatic *Thaumarchaeota* is starting to be revealed.

1.1.3 Ammonia oxidation by *Thaumarchaeota*

Nitrification, the aerobic oxidation of ammonia to nitrate via nitrite, plays a central part in the nitrogen cycle. This two-step process is mediated sequentially by ammonia-oxidizing and nitrite-oxidizing microorganisms (Prosser, 1989; Kowalchuk and Stephen, 2001). Ammonia oxidation is the first and rate-limiting step in nitrification. For over a century, this process was thought to be carried out by only a few genera of bacteria from the β- and γ-Proteobacteria lineages (Purkhold et al., 2000; Kowalchuk and Stephen, 2001). Shortly after the discovery of *amoA*-like genes in archaeal genome fragments in ocean (Venter et al., 2004) and soils (Treusch et al., 2005; Schleper et al., 2005), the first AOA isolate, *Nitrosopumilus maritimus,* was obtained in pure culture from tropical marine sediments at the Seattle Aquarium, demonstrating that AOA could indeed perform autotrophic ammonia oxidation and fix CO_2 (Könneke et al., 2005). Subsequently, numerous studies demonstrated ammonia oxidation potential by archaea in AOA enrichment cultures (la Torre et al., 2008; Hatzenpichler et al., 2008; Tourna et al., 2011) and by in situ nitrification activity assays in various environments (Zhang et al., 2011; Lam et al., 2007), suggesting a potentially significant involvement of archaea in nitrification.

Ammonia monooxyganse subunit A *(amoA)* gene has been widely used as a molecular marker in environmental microbiology for detecting the presence of ammonia oxidizing microorganisms. Primers targeting archaeal *amoA* sequences have been frequently used in quantitative PCR (qPCR) to examine the abundance and distribution of AOA in diverse environments (Francis et al., 2005). One major disadvantage of qPCR-based approaches is the requirement for known sequences and the possibility of missing

unknown genes. Nevertheless, archaeal *amoA* genes appear to be ubiquitous and widely distributed in almost every environment on earth, including open oceans (Francis et al., 2005; Hallam 2006a; Mincer et al., 2007; Lam et al., 2007), estuaries and rivers (Beman et al., 2008; Mosier and Francis, 2008; Santoro et al., 2008; Cao et al., 2011; S. Liu et al., 2013), lakes (Herrmann et al., 2009; Pouliot et al., 2009; Auguet et al., 2011; Auguet and Casamayor, 2013; Vissers et al., 2013b), Arctic and Antarctic waters (Agogué et al., 2008; Kalanetra et al., 2009; Alonso-Sáez et al., 2012), different soils (Leininger et al., 2006; He et al., 2007; Nicol et al., 2008; Di et al., 2010; Gubry-Rangin et al., 2010; Zhang et al., 2010; Pratscher et al., 2011; Pester et al., 2012), plant rhizospheres (Sliwinski and Goodman, 2004; Herrmann et al., 2008; Chen et al., 2008; Xu et al., 2012), freshwater sediments (Ye et al., 2009; French et al., 2012; Wu et al., 2013; Dang et al., 2013), hot spring mats and sediments (la Torre et al., 2008; Hatzenpichler et al., 2008; Reigstad et al., 2008; Spear et al., 2007), coral reefs (Beman et al., 2007; Siboni et al., 2008; Z. Liu et al., 2011; Siboni et al., 2012), and wastewater treatment plants (Park et al., 2006; T. Zhang et al., 2009; Muβmann et al., 2011). Furthermore, AOA frequently outnumber AOB in many aquatic (Hallam et al., 2006a; Mincer et al., 2007; Beman et al., 2008), and terrestrial habitats (Leininger et al., 2006; Zhang et al., 2012; Pratscher et al., 2011; Herrmann et al., 2012), indicating that they potentially play important roles in mediating the global nitrogen cycle.

While AOA are extremely abundant and frequently dominant in diverse habitats, their *in situ* activity and their relative contribution to nitrification in different environments has not always been examined in tandem with gene quantification. In the marine water column, AOA appeared to be the dominant ammonia oxidizers, suggested by a number of

studies (Wuchter et al., 2006; Beman et al., 2008, Santoro et al., 2009). Archaeal cell counts and *amoA* gene copies were positively correlated with the peaks of nitrite production and nitrification rate in the open oceans, whereas AOB were detected with low abundance or undetected, and were not correlated with ammonia oxidation activity (Beman et al., 2008, Santoro et al., 2009).

The picture in terrestrial environments, however, is not clear as contradictory findings have been reported. For example, in an agricultural soil (Jia and Conrad, 2009) and nitrogen-rich grass soils (Di et al., 2009), the AOB abundance was correlated with nitrification activity and CO_2 fixation, while the growth of AOA was independent of nitrification activity despite their high abundance. It is possible that the relatively high concentration of ammonium in those soils inhibited the growth of AOA that are more prevalent in oligotrophic environments. In other studies, AOA growth was positively correlated with soil nitrification, especially in acid soils (Offre et al., 2009; Gubry-Rangin et al., 2010; Zhang et al., 2010). Interesting to note is that, in most of these studies, the active AOA populations performing autotrophic ammonia oxidation were found to be belong to group 1.1a that is less abundant in soils, instead of the group 1.1b that are more prevalent there. It has been suggested that some *Thaumarchaeota*, especially members of group 1.1b, might be heterotrophic nitrifiers, oxidizing ammonia at a slower rate and assimilating organic matter, but their contribution to nitrification remains largely unknown (Muβmann et al., 2011; Xu et al., 2012).

Despite all the uncertainties in determining their relative contribution to nitrification, different ratios of AOA and AOB across diverse environments do raise interesting questions: what are the key environmental factors shaping the ecological niches of AOA

and AOB? Thus far, many site-related environmental factors have been investigated, including ammonium level (Hatzenpichler et al., 2008; Martens-Habbena et al., 2009; Di et al., 2010; Verhamme et al., 2011; Lehtovirta-Morley et al., 2011; French et al., 2012), oxygen concentrations (Martens-Habbena et al., 2009; Lam et al., 2009; Park et al., 2010; Pitcher et al., 2011b; French et al., 2012), carbon and nitrogen sources (Di et al., 2009; 2010; Levičnik-Höfferle et al., 2012; Pester et al., 2012), salinity (Mosier and Francis, 2008; Santoro et al., 2008), temperature (Tourna et al., 2008, French et al., 2012, Wu et al., 2013), pH (Nicol et al., 2008; Gubry-Rangin et al., 2010; French et al., 2012; Lu et al., 2012), light (Merbt et al., 2012; French et al., 2012), energy stress (Valentine, 2007), and sulfide level (Erguder et al., 2009). Of these, substrate type and concentration, and pH were found to be very influential (Martens-Habbena et al., 2009; Erguder et al., 2009; Gubry-Rangin et al., 2011; Verhamme et al., 2011; Hatzenpichler, 2012; Prosser and Nicol, 2012). AOA appear to be predominant in nutrient-limited environments such as oligotrophic ocean and soils. This is likely due to their extremely high substrate affinity and low substrate threshold ($\leq$ 10 nM for *N. maritimus*) (Martens-Habbena et al., 2009), which is more than 100-fold lower than the minimum concentration required for the growth of cultivated AOB (Prosser, 2007; Bollmann et al., 2002). A preference by AOA for low ammonium concentrations has also been observed in enrichment culture studies (Hatzenpichler et al., 2008; French et al., 2012), and supported by environmental studies (Di et al., 2010; Verhamme et al., 2011). The pH is believed to indirectly affect the structure of the nitrifying community by controlling the speciation and concentration of substrate. The ratio of AOA to AOB was significantly increased in soils when pH was under 5, which was explained by low availability of substrate ammonia under low pH due

to protonation (Nicol et al., 2008; Gubry-Rangin et al., 2011). Therefore, when the ammonia concentration is low, AOA likely outcompete AOB due to their high substrate affinity, or their ability to utilize alternative substrates in low-energy environments. Additional environmental factors, for example, the variety and concentration of organic materials, could potentially be important and await further investigation.

1.2 Objectives of this thesis

The major goals of my Ph.D. research were: 1) to assess the potential contribution of AOA and β-AOB to nitrification in Columbia River freshwater sediments; 2) to investigate the biogeochemical factors shaping the distribution of AOA and β-AOB in freshwater sediments; and 3) to examine the metabolic properties of terrestrial *Thaumarchaeota*, with respect to their ability to perform autotrophic ammonia oxidation. I have used a combination of three different approaches to investigate above questions: 1) precise analytical methods to measure biogeochemical variables; 2) molecular techniques to quantify genes and gene transcripts, examine community composition, and analyze phylogenetic diversity; and 3) traditional cultivation approaches and molecular methodology to monitor ammonia oxidation activity coupled with the growth of AOA and β-AOB. γ-AOB were not studied in this work, because this group only contains a few marine strains and is usually undetectable in other environments.

1.2 Significance

The work in this thesis expands our current understanding of the phylogenic diversity and metabolic properties of *Thaumarchaeota* in terrestrial environments. Freshwater rivers are among the most impacted ecosystems by anthropogenic input of Nr, however, the microbial assemblages involved in nitrification in freshwater systems are poorly

studied. This study on Columbia River sediments provides insights into the dynamics of ammonia oxidizing communities along geochemical gradients and across different seasons, and the potential contribution of ammonia oxidizing archaea and bacteria to nitrification in freshwater sediments. In addition, the work from plant-root enrichments provides insights into the phylogenic diversity and metabolic potential of diverse *Thaumarchaeota*. The results obtained complement our current knowledge of nitrifying microorganisms in freshwater sediments and the rhizosphere, and build a more comprehensive picture of the global nitrogen cycle.

1.4 Structure of this thesis

The organization of this thesis is described in this section. Common methods employed in multiple chapters are presented in detail in Chapter 2. Chapters 3 through 5 present three individual projects, including introduction, material and methods, results, discussions, tables and figures sections. Chapter 3 describes the biogeochemical and biomolecular profiles of ammonia oxidizing archaea and bacteria in Columbia River freshwater sediments using a combination of precise analytical methods and quantitative molecular approaches, and examines the environmental factors shaping the niche differentiation of AOA and β-AOB. Chapter 4 presents the autotrophic ammonia oxidation potential by AOA and β-AOB in freshwater sediment slurry incubations with amendments of organic substrates and an ammonia oxidation inhibitor. Chapter 5 describes the selective growth of different *Thaumarchaeota* clades with organic and inorganic amendments in a root-colonizing enrichment culture. Chapter 6 presents the summary of results, implications and future directions. Chapter 7 lists all of the references cited.

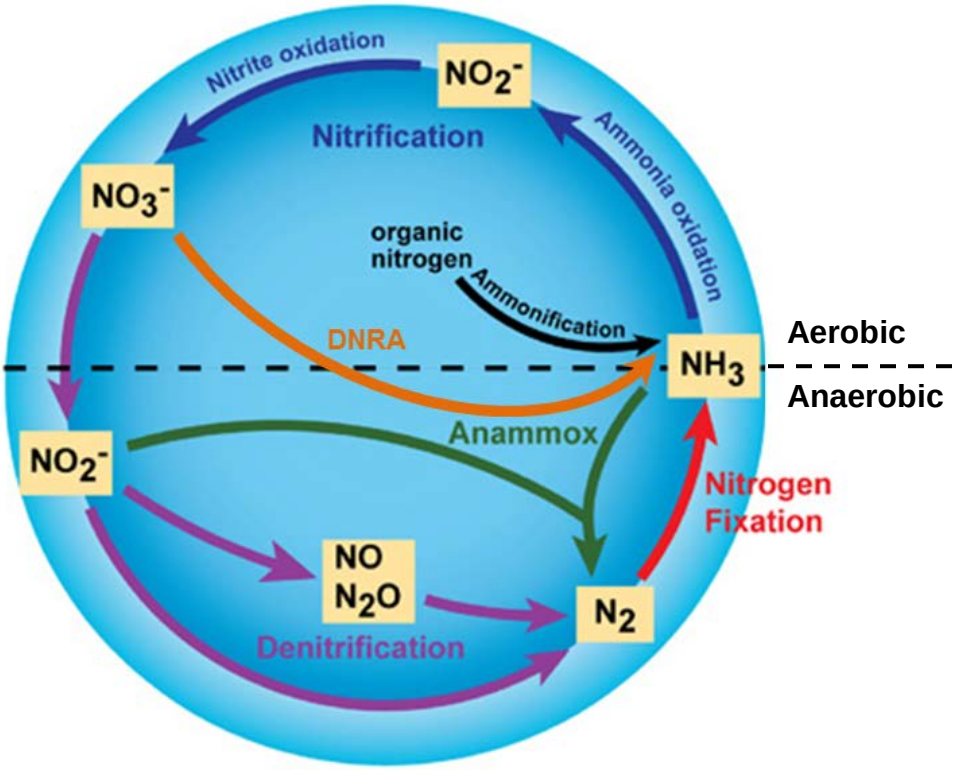

Figure 1.1 A cartoon of the nitrogen cycle (modified from Bernhard et al., 2008). The dashed line separates aerobic (above) from anaerobic (below) processes.

Table 1.1 Description of *amoA* containing *Thaumarchaeota.*

***Thaumarchaeota* species**	*Nitrosopumilus maritimus* SCM1	*Ca.* Nitrosopumilus sediminis AR2	*Ca.* Nitrosopumilus salaria BD31	*Ca.* Cenarchaeum symbiosum A	*Ca.* Nitrosoarchaeum koreensis MY1	*Ca.* Nitrosoarchaeum limnia SFB1	*Ca.* Nitrosotalea devanaterra	*Nitrososphaera viennensis* EN76	*Ca.* Nitrosophaera gargensis	*Ca.* Nitrosocaldus yellowstonii
Availability	pure culture	enrichment	enrichment	natural enrichment (marine sponge)	enrichment	enrichment	enrichment	pure culture	enrichment	enrichment
Affiliation	Group 1.1a	Group 1.1a	Group 1.1a	Group 1.1a	Group 1.1a	Group 1.1a	Group 1.1a-associated	Group 1.1b	Group 1.1b	ThAOA
Original habitat	salt water aquarium	marine sediment within Arctic circle	estuary sediment	marine sponge *Axinella mexicana*	soil from the rhizosphere of *Carogana sinica*	low-salinity estuary sediment	acidic soil	garden soil	hot spring microbial mats	hot spring
T_{opt}	28°C	25 °C	N/A[a]	10 °C	25 °C	N/A[a]	25 °C	35 °C	46 °C	72°C
pH_{opt}	7.4-7.6	8.1	N/A[a]	N/A[a]	6.5-7.0	N/A[a]	4.0-5.0	7.5	N/A[a]	N/A[a]
Growth rate	0.78/ day (max)	0.65/ day (max)	N/A[a]	N/A[a]	N/A[a]	0.2/day (max)	0.37/day (max)	0.043-0.53[c]	N/A[a]	0.8/day (max)
Inhibition by ammonium	2 mM	N/A[a]	N/A[a]	N/A[a]	20 mM	N/A[a]	N/A[a]	10-15 mM	3.08 mM	N/A[a]
Genome size	1.65 Mb	1.69 Mb	>1.57 Mb[b]	2.05 Mb	>1.61 Mb[b]	>1.77 Mb[b]	N/A[a]	N/A[a]	2.83 Mb	N/A[a]
GC content	34%	34%	34%	58%	33%	32%	N/A[a]	N/A[a]	48%	N/A[a]
References	Konneke et al., 2005 Marten-Habbena et al., 2009 Walker et al., 2010	Park et al., 2010 & 2012	Mosier et al., 2012	DeLong and Preston 1996 Hallam et al., 2007	Kim et al., 2011 Jung et al., 2011	Blainey et al., 2011 Moiser et al.,2012	Lehtovirta et al., 2011	Tourna et al., 2011	Hatzenpichler et al., 2008	de la Torre et al., 2008

[a]: not applicable
[b]: unclosed draft genome
[c]: with the addition of pyruvate

CHAPTER 2
GENERAL METHODS

2.1 Overview

This chapter describes in detail all of the general methods used in my experiments, including DNA and RNA extraction, purification and quantification, construction of plasmids for quantitative PCR (qPCR), media preparation and chemical analysis. The general methods listed in this chapter will not be described in detail in other chapters, while chapter 3-5 have additional methods specific to questions addressed in each chapter .

2.2 Nucleic acid extraction, purification and quantification

Total DNA was extracted from sediments or cell pellets using the Fast-DNA spin kit for soil (MP Biomedicals, Solon, OH) according to the manufacturer's protocol. Up to 0.5 gram sediment samples or cell pellets were added to the lysing matrix tubes that contains 1.4 ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead, and were bead beat with 978 µl Sodium Phosphate Buffer and 122 µl MT buffer. After cell lysis, cell debris was pelleted by centrifugation, and the supernatant was collected into a new 2 mL tube. Following the protein precipitation step, the supernatant was well mixed with resuspended Binding Matrix in a 15 mL tube, and transferred onto a SPIN filter for washing. Finally, DNA was eluted in 100 µL of nuclease-free water, and stored at -20 °C for future use.

Total RNA was extracted using a modified phenol-chloroform method with CTAB extraction buffer (Smith et al., 2010; Griffiths et al., 2000). Cell pellets or 0.5 gram sediment were dissolved in 600 µL CTAB extraction buffer, transferred into 2 mL tube

containing 0.1 and 1 mm silica beads, and mixed with 700 μL phenol:choloroform, 50 μL of 10% SDS and 50 μL Sodium Lauryl Sarcosine. After bead beating, the tube was incubated for 10 minute at room temperature with frequent shaking, and then centrifuged at 13500 rpm for 5 minutes. The aqueous phase was collected into a new 2 mL microcentrifuge tube, followed by a second extraction. After pooling the aqueous phase from two extractions, nucleic acid was re-extracted with 800 μL phenol:chloroform, and then residual phenol:chloroform was removed with 800 μL chloroform. The aqueous phase was collected into a new 2 mL microcentrifuge tube, and incubated with 0.1 volume of 3 M sodium acetate at pH 5.2 and 0.7 volume of isopropanol on ice for 1-2 hours, followed by centrifugation at 12000 rpm for 30 minutes at 4 °C. The precipitated RNA pellet was washed with 500 μL ethanol twice, air dried, and then digested with *DNAse* to remove genomic DNA using Ambion's TURBO DNA-free^{TM} kit (Ambion, Austin, TX) according to the manufacturer's protocol. In a 50 μL reaction, the RNA sample was incubated with 2 μL TURBO *DNase* and 5 μL TURBO *DNase* buffer at 37 °C for 30 minutes. After digestion, *DNase* was inactivated by adding 10 μL resuspended TURBO *DNase* Inactivation Reagent. Digested RNA was further purified with Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

DNA and RNA concentrations were quantified with PicoGreen and RiboGreen dye (Invitrogen Corp., Carlsbad, CA), respectively, on a Nanodrop fluorometer (Thermo Scientific, Wilmington, DE).

2.3 Construction of plasmids for qPCR

The standards used for quantitative PCR were constructed from the Columbia River freshwater sediment clones. Archaeal and bacterial *amoA* gene fragments were PCR-amplified with sediment total DNA using Arch-amoAF/Arch-amoAR (Francis et al., 2005) and amoAF1/amoAR2 (Rotthauwe et al., 1997), respectively. The PCR products were confirmed by gel electrophoresis for the correct size and a single band. PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI), and cloned into *E. coli* with the pCR2.1 vector system using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. White colonies were screened for the presence of inserts by colony-based PCR amplification. Plasmids containing archaeal or bacterial *amoA* genes were extracted from clones, and purified using the Qiagen plasmid midi kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Purified plasmids were linearized by digestion with the restriction enzyme *XbaI*, and confirmed the correct size by gel electrophoresis. The plasmids were sequenced at the OHSU DNA service center to verify. Concentrations of linearized plasmids were measured using PicoGreen dye (Invitrogen Corp., Carlsbad) on a NanoDrop fluorometer (Thermo Scientific, Wilmington, DE). A serial dilution of plasmids, spanning 6 orders of magnitude (10^1-10^6), was used as standard curve for the quantification of gene copy numbers. Standard curves were linear with R^2>0.99.

2.4 Chemical analysis

Ammonium concentrations were determined used a fluorometric method (Kérouel and Aminot, 1997; Holmes et al., 1999) or a colorimetric Ammonia Assay Kit (Sigma-Aldrich, St. Louis, MO). For the fluorometric method, working reagent (WR) was

freshly prepared in a 1 liter brown polyethylene and consisted of 40 mg/L sodium sulfite, 40 g/L sodium tetraborate and 50 ml/L orthophthaldialdehyde (OPA) in ethanol. The reaction was conducted in a ratio of 4:1 sample to WR in a 15 mL centrifuge tube (with 4 mL of sample or standards), and incubated in the dark for 3-4 hours. Each time, a serial dilution of NH_4Cl solution was prepared (0.5-500 μM) and used to generate a standard curve for the quantification of ammonium concentrations. The fluorescence was measured using the excitation wavelength of 350 nm and the emission wavelength of 420 nm. The detection limit for the fluorometric method was 10 nM (Holmes et al., 1999). For the colorimetric ammonia assay, ammonia (100 μL) was reacted with a-ketoglutaric acid and NADPH (1 mL) in the presence of 10 μL L-glutamate dehydrogenase (GDH) to form L-glutamate and NADP (Mondzac et al., 1965). The decrease of the absorbance at 340 nm due to NADPH oxidation was proportional to the ammonia concentration, and measured with a spectrophotomer (Van Anken and Schiphorst, 1974). The detection range was 0.2-15 μg/L (Van Anken and Schiphorst, 1974).

Nitrite and nitrate concentrations were determined spectrophotometrically using either the Nitrate/Nitrate Colorimetric Assay Kit or the Nitrite/Nitrate Fluorometric Assay Kit (Cayman Chemical, Ann Arbor, MI) in a two-step process. Nitrate was first reduced to nitrite with nitrate reductase and cofactor NADPH. The total nitrite and nitrate was reacted with Griess Reagents to form a purple product that was determined colorimetrically at the wavelength at 540 nm (Green et al., 1982; Nims et al., 1995). Alternatively, the total nitrite and nitrate was reacted with 2,3 diaminonaphthalene (DAN reagent) to form a fluorescent product that could be measured on a plate-reader with the excitation and emission wavelength of 375 and 417 nm, respectively (Misko et al., 1993;

Miles et al., 1995). The detection limit of nitrite using colorimetric and fluorometric methods was 2 μM and 30 nM, respectively (Nims et al., 1995; Misko et al., 1993).

2.5 Media for growth and activity assays

Media described here were used for enriching *Thaumarchaeota* and for quantifying the ammonia oxidation potential by AOA and AOB.

Site water medium used in sediment slurry incubations (Figure 2.1) was freshly collected Columbia River water (pH 7.6-7.9) filtered through a 0.2 micron filter with only the addition of the nitrogen source, typically 250 μM ammonium chloride, and was bubbled with air through a 0.2 micron sterile Millex Vent Filter Unit (Millipore Corp., Billerica, MA) by an aquarium pump.

Synthetic freshwater *Crenarchaeota* medium contained 50-500 μM NH_4Cl, 1-2 mM $NaHCO_3$, 10 mM HEPES (pH 7.0), 1 g/L NaCl, 0.4 g/L $MgCl_2 \cdot 6H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.5 g/L KCl, 0.02 μM selenite-tungstate solution, vitamin solution and trace element solution (la Torre et al., 2008). Vitamin solution (1 liter) contained 4 mg 4-aminobenzoic acid, 1 mg biotin, 10 mg nicotinic acid, 5 mg calcium pantothenate, 15 mg pyridoxine dihydrochloride and 100 mL 10 mM sodium phosphate suffer (pH 7.1) (Widdel et al., 1992). Trace element solution (1 liter) contained 7.5 mM $FeSO_4 \bullet 7H_2O$, 0.5 mM H_3BO_3, 0.5 mM $MnCl_2 \bullet 4H_2O$, 0.8 mM $CoCl_2 \bullet 6H_2O$, 0.1 mM $NiCl_2 \bullet 6H_2O$, 0.01 mM $CuCl_2 \bullet 2H_2O$, 0.5 mM $ZnSO_4 \bullet 7H_2O$, 0.15 mM $Na_2MoO_4 \bullet 2H_2O$ and 100 mM HCl (Widdel et al., 1992).

The medium used for semi-recyclostat (Figure 2.2) contained normal basal salts, vitamin solution, 250 mM sodium bisulfite, 100 μg/mL streptomycin and carbencillin with the addition of 5g/L (wet wt) tomato root extract when appropriate (Simon et al.,

2005). Harvest tomato roots were frozen in liquid nitrogen, ground into a fine powder in a mortar, and stored at -80°C. To prepare root extract, powdered root material was measured into sterile Milli-Q H_2O and vortexed for 5 min. Large particles were removed by coarse filtration, and root extract was sterilized by filtering through a 0.6 and 0.22-µm filter sequentially. Basal salts (20X in 0.5 liter) contained 7.84 g KH_2PO_4, 14.75 $Na_2HPO_4 \bullet 7H_2O$, 1.58 g $(NH_4)_2SO_4$, 1.65g KCl and 110 mL Daniel's Mineral Elixir (in 500 mL, pH=7 with Na_2CO_3) that includes 0.2103g KH_2PO_4, 0.1097g K_2HPO_4, 0.0193 g $MgCl_2 \bullet 6H_2O$, 0.0135 g $CaCl_2 \bullet 2H_2O$, 0.3489 g NH_4Cl, 0.3 g NaCl and 0.08 g Na_2CO_3 and 5 mL Trace elements. Trace element solution (100X in 100 mL,) contained 149.9 mg nitrilotriacetic acid, 48.6 mg $FeCl_2 \bullet 6H_2O$, 0.5 mg $CoCl_2 \bullet 6H_2O$, 0.5 mg $Na_2MoO_4 \bullet 2H_2O$ and 10 mg $NiCl_2 \bullet 6H_2O$. Vitamin solution (100X in 1 liter) included 20 mg thiamine HCl, 20 mg Ca-D-panthotherate, 20 mg nicotinamide, 20 mg riboflavin, 20 mg pyridoxime HCl, 1 mg p-aminobenzoic acid, 0.5 mg biotin, 0.2 mg vitamin B_{12} and 0.125 mg folic acid. The medium was regularly sparged with mixed gas of N_2, O_2 and CO_2.

Figure 2.1 A photo showing the set up of sediment slurry incubations.

Figure 2.2 A photo showing the semi-recyclostat used to enrich *Thaumarchaeota*.

CHAPTER 3
GEOCHEMICAL AND MOLECULAR CHARACTERIZATION OF AMMONIA OXIDIZING ARCHAEA AND BACTERIA IN COLUMBIA RIVER FRESHWATER SEDIMENTS

3.1 Introduction

The nitrogen cycle, controlling nitrogen availability for key microbial processes such as primary production and decomposition, is one of the most important biogeochemical cycles on earth (Zehr and Ward, 2002). Anthropogenic inputs of Nr from excessive use of nitrogen fertilizers to fossil fuel combustion have substantially disturbed the global nitrogen balance and led to a number of environmental problems, including eutrophication of aquatic systems, deterioration of water quality, and increased emission of N_2O as greenhouse gas (Galloway et al., 2003; 2004; 2008; Gruber and Galloway, 2008). Estuaries and freshwater rivers are among the most impacted ecosystems, due to the large inputs of Nr from agricultural activities (Rabalais, 2002; Galloway et al., 2004). The Columbia River (CR) is the second largest river by flow in the United States, with a drainage basin of 660,480 km^2, and also the largest freshwater source in the Pacific Northwest region (Simenstad, 1990). The Columbia River is also characterized by strong tidal cycles and seasonal fluctuations in nutrients (Sullivan et al., 2001; Gilbert et al., 2013).

Nitrification, the microbial oxidation of ammonia to nitrite and then to nitrate, plays a central role in the global nitrogen cycle. It is also a crucial process in mitigating nitrogen-related environmental problems, Nr derived from organic matter and the removal of Nr via denitrification (Galloway et al., 2004; Gruber and Galloway, 2008). Ammonia

oxidation is the first, and often the rate-limiting step in nitrification (Kowalchuk and Stephen, 2001). For over a century, this process was thought to be carried out by only two groups of ammonia oxidizing bacteria (AOB) belonging to the sub-phyla β and γ-Proteobacteria (Purkhold et al., 2000; Kowalchuk and Stephen, 2001). However, the discovery of genes encoding the putative archaeal ammonia monooxygenase (AMO) in marine and soil clones has altered our bacteria-centered view of nitrification (Venter et al., 2004; Treusch et al., 2005; Hallam et al., 2006a). Since the first isolate of the ammonia oxidizing archaea (AOA), *Nitrosopumilus maritimus*, was obtained in pure culture from marine sediments (Könneke et al., 2005), numerous studies have demonstrated archaeal ammonia oxidation in pure (Tourna et al., 2011) and enrichment cultures obtained from diverse environments, including the oceans and estuaries (Santoro and Casciotti, 2011; Park et al., 2010), different soils (Lehtovirta-Morley et al., 2011; Jung et al., 2011; Kim et al., 2012), hot spring sediment and microbial mats (la Torre et al., 2008; Hatzenpichler, 2012), plant roots (Xu et al., 2012), and low-salinity and freshwater sediments (Mosier et al., 2012; Blainey et al., 2011; French et al., 2012). Based on the phylogenetic and metabolic uniqueness of AOA, a new and deeply-branched phylum of archaea, *Thaumarchaeota*, was proposed (Brochier-Armanet et al., 2008), and this new term is supported by a number of subsequent studies (Spang et al., 2010; Brochier-Armanet et al., 2011a; 2011b; Gupta and Shami, 2011; Pester et al., 2011; 2012; Pelve et al., 2011).

The archaeal ammonia monooxyganse subunit A (*amoA*) gene has been widely used as a molecular marker in determining AOA abundance and distribution (Francis et al., 2005). The archaeal *amoA* genes appear to be ubiquitous in almost every environment on earth (Francis et al., 2005; Treusch et al., 2005; Leininger et al., 2006; Park et al., 2006;

Hallam et al., 2006a; Beman et al., 2007; Mincer et al., 2007; Mosier and Francis, 2008; Torre et al., 2008; Herrmann et al., 2008; Pouliot et al., 2009; Xu et al., 2012; Muβmann et al., 2011). It is often the archaea rather than the bacteria that appear to be dominant, based on enumeration of their respective *amoA* genes, in a variety of aquatic (Hallam et al., 2006a; Mincer et al., 2007; Beman et al., 2008) and terrestrial (Leininger et al., 2006; Pratscher et al., 2011; Zhang et al., 2012) environments, suggesting that AOA potentially play significant roles in the global nitrogen cycle. Furthermore, *Thaumarchaeota* appear to be metabolically diverse, based on the analysis of sequences of metabolic genes and physiological characterization of *Thaumarchaeota* in pure and enrichment cultures (la Torre et al., 2008; Martens-Habbena et al., 2009; Walker et al., 2010; Brochier-Armanet et al., 2011a; 2011b; Tourna et al., 2011; Blainey et al., 2011; Lehtovirta-Morley, et al., 2011; Nunoura et al., 2011; Xu et al., 2012; Spang et al., 2012).

While archaeal *amoA* genes are extremely abundant and frequently outnumber bacterial *amoA* genes in diverse habitats, *in situ* nitrification activity attributed to archaea has rarely been quantified, particularly in freshwater systems. In addition, the linkage between AOA abundance and environmental factors has not been fully elucidated. Thus far, a number of environmental factors potentially shaping niche diversification of AOA and AOB have been investigated, including substrate concentrations, salinity, pH, temperature, C:N ratio, light and sulfide levels (Könneke et al., 2005; Martens-Habbena et al., 2009; Di et al., 2010; Francis et al., 2005; Santoro et al., 2008; He et al., 2007; Nicol et al., 2008; Erguder et al., 2009; French et al., 2012). Among all the environmental factors examined, the key determinant is the substrate concentration. Metabolic investigations of *Nitrosopumilus*-like AOA revealed their extremely high substrate

affinity and low substrate threshold (≤ 10 nM ammonium) (Martens-Habbena et al., 2009; Park et al., 2010). As a result, AOA are often found to be numerically dominant in oligotrophic aquatic and terrestrial habitats (Hatzenpichler et al., 2008; Lehtovirta-Morley et al., 2011; Tourna et al., 2011, Wuchter et al., 2003; Pearson et al., 2004; Kuypers et al., 2001), in contrast to AOB, which are frequently abundant in the environments with high nutrient availability such as fertilized soils (Jia and Conrad, 2009; Di et al., 2010).

In this study, I attempted to investigate environmental factors shaping niche diversification of AOA and AOB in the Columbia River intertidal freshwater sediments, by employing a combination of precise analytical methods and molecular approaches. The CR freshwater sediments were collected at low tide in July 2010, April and August 2012 at Cathlamet Island. Biogeochemcial properties of the freshwater sediments were characterized at a very fine vertical scale. The *amoA* genes and gene transcripts were quantified for both AOA and β-AOB by quantitative PCR (qPCR), and the shifts in AOA community composition were examined by PCR-Single Stranded Conformation Polymorphism (PCR-SSCP). In addition, a clone library of archaeal *amoA* genes were constructed, sequences were generated and a representative phylogenetic tree was analyzed. The correlation between the environmental parameters (O_2, E_h, ammonia, nitrite and nitrate) and changes in *amoA* gene abundance, expression and diversity was further analyzed using Canonical Correspondence analysis (CCA) and Pearson's correlation. Finally, nitrification activity coupled growth assays were performed in sediment slurry incubations to examine potential nitrification activity by AOA and β-AOB in the CR sediments.

3.2 Materials and methods

3.2.1 Site description and sampling procedures

During 2009-2012, freshwater sediment cores were collected at Cathlamet Island (46.172 ° N, 123.355 °W) (Figure 3.1) in the Columbia River throughout different seasons. The bulk geochemical properties of the sediments were measured by Agri-Check (Umatilla, OR) (Table 3.1). Triplicate core samples were collected for fine-scale geochemical characterization, and duplicate or triplicate sediment cores were used for molecular analysis. The temperatures of the water column, surface and deeper sediments (5 cm below the sediment surface) were recorded on site.

3.1.2 Sediment core collection, handling and processing

Intact sediment cores were collected at low tide using modified 60 mL sterile syringes (Fisher Scientific, Pittsburgh, PA). All sediment cores were sealed by sterile rubber stoppers, placed on ice and transferred to the laboratory (OHSU science and engineering campus, Beaverton, 97006) promptly. Each sediment core was fractionated into 2 mm subsections and homogenized in a sterile container. The sediment fractions were stored at −80 °C and processed within one week.

3.1.3 Geochemical measurements using microelectrodes

Oxygen and E_h profiles of the sediment cores were generated at 2 mm intervals vertically throughout each intact core on site using a Unisense Oxygen Microsensor PA 2000 (Uni- sense, Aarhus, Denmark) and an Oxidation-Reduction Potential Microelectrode (Microelectrodes Inc., Bedford, NH), respectively, and were used to guide subsequent sediment fractionation. The detection limit of the oxygen probe was 0.3 µM (Revsbech, 1989; Gundersen et al., 1998). The sensitivity of E_h probe was 0.1 mV.

3.1.4 Geochemical measurement of pore water

Sediment pore water was obtained from the supernatant of individual fractions by centrifugation, and filtration through 0.2-µM pore size filters (Fisher scientific, Pittsburgh, PA). Dissolved nitrogen species in pore water were analyzed for different depths using different analytical methods. Ammonium concentrations were determined using an OPA-based fluorometric method (Holmes et al.,1999). Nitrite and nitrate concentration was measured using a Nitrite/Nitrate fluorometric Assay Kit (Cayman chemical, Ann Arbor, MI), with the excitation and emission wavelengths of 375 and 417 nm, respectively (Misko et al., 1993; Miles et al., 1995). The detection limits for ammonium and nitrite plus nitrate were 10 nM and 0.2 µM, respectively (Holmes et al., 1999, Miles et al., 1995).

3.1.5 Nucleic acid extraction, purification and quantification

Total DNA was extracted from 0.5 gram sediment fractions using the Fast-DNA spin kit (MP Biomedicals, Solon, OH). Total RNA was extracted using a modified phenol-chloroform method with CTAB extraction buffer (Griffiths et al., 2000; Smith et al., 2010). Extracted RNA was treated with *DNase* to remove genomic DNA using the Ambion's TURBO DNA-free^{TM} kit (Ambion, Austin, TX), and then purified with the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacture's protocol. DNA and RNA concentrations were quantified with PicoGreen and RiboGreen dyes (Invitrogen Corp., Carlsbad, CA), respectively, on a Nanodrop fluorometer (Thermo Scientific, Wilmington, DE). DNA purity was examined using a dilution series of template by qPCR to ensure there was no template inhibition, and to determine the linear range of amplification. PCR controls indicated that there was no DNA contamination in RNA preps.

3.1.6 Quantitative PCR

Archaeal and β-bacterial *amoA* genes were quantified from total sediment DNA using the archaeal *amoA* primer set 353F/487R designed from freshwater sequences by Dr. Holly Simon (Table 3.6), and amoA1F/amoA2R primer set (Rotthauwe et al., 1997), respectively. Total DNA was PCR amplified in a 25-µl reaction mixture containing 12.5 µl SYBR-Green Supermix (Bio-Rad Laboratories, Hercules, Ca), 11 µl of PCR water, 0.25 µl of each primer (200 nM final concentration), and 1 µl of template DNA. PCR was conducted in a MyiQ real-time qPCR detection system (Bio-Rad laboratories, Inc, Hercules, CA) and amplified using the following PCR cycling conditions: 1 min denaturation at 94°C, followed by 40 cycles consisting of 30 s of denaturation at 94°C, 45 s of annealing at 54°C or 56°C for archaea and bacteria, respectively, and 45 s of elongation at 72°C, with a final extension step at 72 °C for 4 min. A melting curve protocol began after amplification and consisted of 1 min at 95 °C, followed by 1 min at 55 °C and 80 10-s steps with a 0.5 °C increase in temperature at each step. PCR threshold value and the amplification efficiency of individual wells (82.4% to 98.6%) were calculated using the LinReg PCR program (Ramakers et al., 2003). Inhibition was tested for by serial dilution of templates, and was not observed. Archaeal and bacterial *amoA* gene standards were generated with plasmids extracted from CR sediment clones (described in the Chapter 2). The linear amplification ranges were from 10^1 to 10^6 template copies with R^2 values > 0.9 for all assays.

3.1.7 qRT-PCR

Total purified RNA was converted to cDNA with oligo dT primers using the SuperScript III First-Strand Synthesis Supermix for qRT-PCR, according to the

manufacturer's protocol (Invitrogen Corp., Carlsbad, CA). cDNA was amplified using the quantitative PCR protocols described above.

3.1.7 PCR, *amoA* gene cloning, and sequencing.

Total DNA extracted from the top 10 cm of sediment was pooled and amplified by PCR using archaeal *amoA* gene-specific primers. PCR was performed in 50 µl reactions with 200 nM each of the primers Arch-amoAF and Arch-amoAR (Francis et al., 2005) using the following PCR protocol: 95°C for 5 min; followed by 30 cycles consisting of 94°C for 45 s, 54°C for 60 s, 72°C for 60 s; and 72°C for 15 min. PCR products were confirmed for expected size on agarose gels, purified by Promega Wizard SV gel and PCR purification kit (Promega Corp., Madison, WI), and cloned with the TOPO TA vector system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. White colonies were screened for the presence of inserts by colony-based PCR amplification using Arch-amoAF and Arch-amoAR (Francis et al., 2005). Ninety-six-well plates inoculated with positive clones were incubated in LB medium (with 40 µg/mL kanamycin) overnight at 37 °C prior to sequencing. Sequences were generated by bidirectional sequencing using the vector primers M13-FWD and M13-REV on an ABI 3730XL sequencing platform at Beckman Coulter Genomics (Beckman Coulter Inc., Brea, CA).

3.1.8 Phylogenetic analysis

DNA sequences were screened for high quality (low baseline noise and high signal) and full length (>600 bp) before being analyzed. Archaeal *amoA* gene sequences were assembled and edited using CLC Genomics Workbench (Version 4.0, CLC bio, Aarhus, Denmark). Sequences were searched against the GeneBank database using basic local

alignment search tool (BLAST) algorithm (Altschul et al., 1990) for closest matching sequences. The sequences were aligned using the MUSCLE algorithm (Edgar, 2004) in MEGA 5.05 (Tamura et al., 2011). Phylogenetic trees were constructed in the MEGA 5.05 program with neighbor joining, maximum likelihood, and maximum parsimony methods. Bootstrap analysis was performed with 1000 iterations to estimate the reliability of phylogenetic trees. Trees constructed with different methods showed similar topology. Only the phylogenetic tree constructed using the neighbor joining method is shown.

Diversity indicators including Chao1 richness estimator (Chao et al., 2005) and Shannon-Weaver index (Shannon, 2001) were calculated based on 97% sequence identity in the FastGroupII program (http://fastgroup.sdsu.edu/) (Yu et al., 2006).

3.1.9 PCR-SSCP

PCR–single-strand conformation polymorphism (PCR-SSCP) analysis was conducted by using the fluorescently labeled archaeal *amoA* primers FAM-353F/487R. Total extracted DNA was PCR amplified in a 50-µl reaction mixture containing 25 µl of IQ Supermix (Bio-Rad), 22 µl of PCR water, 0.5 µl of each primer (200 nM final concentration), and 2 µl of template DNA. The reaction mixtures were subjected to touchdown PCR as follows: 1 min at 94°C, followed by 20 touchdown cycles consisting of 30 s of denaturation at 94°C, 1.5 min of annealing at 64°C, and 1.5 min of elongation at 72°C, followed by a melting curve protocol described above. During touchdown, the annealing temperature was reduced every two cycles by 0.5°C, until the final annealing temperature of 54°C was reached. This was followed by 15 cycles of regular PCR. After amplification, the PCR products were purified using a Promega Wizard SV Gel and PCR purification kit (Promega Corp., Madison, WI) according to the manufacturers' protocols.

After purification, each PCR product was combined with 15 µl of SSCP stop solution (Lonza, Basel, Switzerland), denatured at 95°C for 3 min, and immediately transferred onto ice. The products were electrophoresed on 1× MDE (Lonza Group, Ltd., Basel, Switzerland) polyacrylamide gels at 300 V for 22 h at 17°C. The gels were scanned on a Typhoon variable mode imager (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and images were analyzed using GelCompar software II (Applied Maths, Austin, TX). Peak height cutoffs ranged from 5 to 20 relative fluorescence units, depending on the signal intensity.

3.2.10 Statistical analysis

Canonical correspondence analysis (CCA) was performed to investigate the relationships between AOA community compositions on and environmental variables using XLSTAT statistical software (Addinsoft, Paris, France). Environmental variables analyzed were E_h, ΔE_h, O_2, and ammonium, nitrite and nitrate concentrations. In the CCA maps, the length of the arrow corresponds to how strong the environmental factor is correlated to molecular variance. In addition, correlations between molecular abundance and environmental factors were analyzed using Pearson Correlation (v1.0.6) in Statistics Software (v1.1.23-r7) (Wessa, 2012).

3.2.11 Ammonia oxidation potential

Surface sediments were collected, homogenized, and inoculated (20 grams) into 200 mL filtered site water with the addition of 250 µM ammonium chloride. Sediment slurries were incubated in 500 mL flasks in the dark, at 22 °C. Aerobic conditions were maintained by continuously bubbling air through a 0.2 micron sterile Millex Vent Filter Unit (Millipore Corp., Billerica, MA) by an aquarium pump. Potential nitrification

activity was assessed by monitoring changes in the concentrations of ammonium, nitrite and nitrate over time, and in the abundance of *amoA* genes for both AOA and β-AOB. Ammonium concentrations were determined by measuring the color absorbance at 340 nm (Mondzac et al., 1965; Van Anken and Schiphorst, 1974) using a Sigma-Aldrich Ammonia Assay Kit (St. Louis, MO). Nitrite and nitrate concentrations were determined by measuring the color absorbance at 540 nm with the Nitrite/Nitrate Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI). The detection limits for nitrite, nitrate and ammonium were 2 μM, 2.5 μM and 10 μM, respectively. Growth of AOA and β-AOB was determined by quantifying *amoA* gene copy in the beginning (Day 0) and at the end of (Day 7) of each experiment using qPCR.

3.3 Results

3.3.1 Seasonal fluctuations in the biogeochemistry of Columbia River freshwater sediments

Sediment samples were collected at the freshwater end (46.172°N, 123.355°W) of the Columbia River estuary, which is affected by both strong tides and seasonal river discharges (Sigleo and Frick, 2007; Chawla et al., 2008). In spring, freshwater sediments were influenced by high water discharge from heavy winter precipitation and early spring snowmelt in the Pacific Northwest; while in summer, these sediments became more stratified.

Bulk chemical analysis showed that the sediment texture was silt loam, containing 39.3 % sand, 54.3% silt and 6.4% clay. The pH in the bulk sediment was near neutral, ranging between 6.4 and 7.2 (Table 3.1), and was around neutral to slightly basic in the site water (up to pH 7.9, data not shown). Nitrate concentration in the bulk sediment was

1─3 mg/kg, and the ammonium concentration was 10.6─31.5 mg/kg (Table 3.1). The concentrations of ammonium, TOC, and dissolved Mn and Fe were higher in summer (2010 and 2012) than those in spring (2012) (Table 3.1), suggesting seasonal fluctuations in nutrient availability.

Oxygen profiles exhibited steep gradients in the surface sediments. Oxygen concentrations rapidly decreased from fully saturated to below the detection limit (0.3 μM) within the first centimeter of all sediments (Figure 3.3A). Oxygen depletion occurred at shallower depths in summer (2-6 mm) than in spring (8 mm) (Figure 3.3A), indicating potentially higher oxygen consumption rate in summer sediments. E_h profiles had more variation across different seasons (Figure 3.3A). In summer sediment cores (July 2010 & Aug 2012), E_h values were more positive (oxidizing) at the top and became increasingly negative (reducing) with depth. In spring (April 2012) sediment cores, E_h was still relatively positive at the top and remained mostly unchanged throughout the cores. The absence of steep E_h gradients in spring sediment was potentially caused by more mixing occurring in spring due to high water discharge (Peterson et al., 1984; Sigleo and Frick, 2007). In contrast, the redox gradient was less disturbed in summer due to low flow and more stratification.

Depth profiles of dissolved nitrogen species in pore water were measured for sediments collected in April and August 2012 (Figures 3.3B, 3.3C). In spring sediment pore water, the total concentration of nitrate and nitrite decreased with depth near the surface, but gradually increased at deeper depths (up to 25 μM, Figure 3.3C). The minimum nitrate and nitrite concentration (~3 μM) was found around 12 mm below the surface. The ammonium concentration, on the other hand, remained relatively low (< 13

μM) for the first 20 mm below the surface with a slight uptrend, and then spiked between 25 and 30 mm (up to 73 μM, Figure 3.3B). In contrast, in summer, the concentration of nitrate and nitrite was very low near the surface (~2 μM, Figure 3.3C), while the ammonium concentration was relatively high (~50 μM, Figure 3.3B). As the depth increased, both nitrate (and nitrite) and ammonium increased, up to 28 and 93 μM, respectively. It is also interesting to note that there appears to be a threshold depth for nitrate (and nitrite) and ammonium, respectively, beyond which no significant difference between spring and summer profiles was observed (5 mm for nitrate and nitrite, and 26 mm for ammonium) (Figure 3.3B, 3.3C).

3.3.2 Effects of short-term frozen storage on the quantification of *amoA* gene transcripts

The processed sediment samples required short-term storage prior to RNA extraction and purification, and gene expression analysis. Sample preservation is especially critical to ensure the quality and integrity of RNA. RNA*Later* is a common stabilization reagent used to preserve RNA, but it didn't have the expected effect on soils and sediments samples (Rissanen et al., 2010). In contrast, whole-core freezing and storage of sediments did not affect the integrity of biomolecules such as DNA and RNA (Brow et al., 2010). To examine whether short-term frozen storage of sediment samples without adding stabilization reagent would affect RNA yield or abundance of measured gene transcripts, three different approaches were examined: 1. RNA was extracted immediately without freezing or storing of sediments (unfrozen control); 2. Sediments were flash frozen in liquid nitrogen and stored at -80 °C for one week followed by extraction of RNA (frozen 1-week); and 3. Sediments were preserved in RNA*Later,* flash frozen and stored at -

80 °C for one week prior to extraction of RNA (frozen 1-week in RNA*Later*). For each approach, RNA was extracted from triplicate subsamples of homogenized sediment. The total RNA yield was quantified using RiboGreen dye, and the abundance of archaeal and bacterial *amoA* transcript was quantified by qPCR (Figure 3.2). In the absence of RNA*later*, there was no significant difference in total RNA yield between the unfrozen controls and 1-week frozen storage. In contrast, with RNA*later* preservation, total RNA yields were significantly reduced after frozen storage samples (Figure 3.2A). Similar results were observed for archaeal and bacterial *amoA* transcripts using qRT-PCR (Figure 3.2B). With RNA*later* preservation and frozen storage, *amoA* transcripts were one-to-two orders of magnitude less abundant, while transcript abundance was similar between unfrozen controls and samples frozen for one week without RNA*later*. The poor performance of RNA*Later* for environmental samples containing humic acids such as soil and sediments has been reported (Rissanen et al., 2010). This is likely due to the high ammonium sulfate in RNA*Later* solution, which will co-precipitate humic and nucleic acids, resulting in significant reductions of nucleic acid yields during storage and extraction. Overall, the results suggested that short-term frozen storage at -80 °C without preservatives did not affect total RNA yield or *amoA* transcript abundance in our sediment samples. Therefore, this was the approach I subsequently used when collecting samples for RNA analysis.

3.3.3 Sediment depth profiles of *amoA* gene abundance and expression

Quantitative PCR was used to determine the abundance and vertical distribution of both bacterial and archaeal *amoA* genes in CR freshwater sediments collected across different seasons (Figure 3.4A, B). qPCR analysis showed that the abundance of archaeal

amoA genes (5.6×10^5 to 5.8×10^6) was as much as 2 orders of magnitude higher than bacterial *amoA* genes (1.5×10^4 to 1.8×10^6) in all samples, regardless of depth or season. Both archaeal and bacterial *amoA* gene abundance remained relatively stable. Interesting to note that the AOA:β-AOB *amoA* gene abundance ratio (normalized to abundance ratio of the surface sediment) also showed seasonal patterns (Figure 3.4C): Little change was observed with depth in spring sediments (April 2012), but the ratio increased with depth in summer sediment samples (July 2010 & August 2012). Higher ratios were observed at the depths of 10 mm and greater, suggesting possible seasonal impacts on the AOA:β-AOB abundance ratio.

Furthermore, *amoA* transcript abundance was quantified using qRT-PCR. Expression of *amoA* genes was detected in both AOA and β-AOB throughout the sediment cores (Figure 3.5). Similar to the abundance data, archaeal *amoA* transcripts were more abundant (1.1×10^3 to 1.0×10^5) (Figure 3.5A) than bacterial *amoA* transcripts (1.8×10^2 to 1.2×10^4) at most depths (Figure 3.5B). The *amoA* gene expression in both AOA and β-AOB was generally greater in surface sediments, and gradually decreased with depth. There was a second peak of *amoA* transcript abundance for both AOA and β-AOB observed at around 26~36 mm (Figure 3.5), which corresponded to the decrease of ammonium concentration and increase of nitrite and nitrate concentration (Figure 3.3B, C). This concurrency might indicate potential nitrification activity by these ammonia oxidizing assemblages at these depths of CR freshwater sediments. In addition, higher AOA: β-AOB gene expression ratios (up to 16) were observed at surface sediments, and the ratio decreased in deeper sediments regardless of seasons (Figure 3.5C).

3.3.4 Correlation between molecular abundance and environmental parameters

Pearson's correlation analysis was performed to examine the relationships among environmental variable and *amoA* gene and transcript abundance in AOA and β-AOB (Table 3.4). In summer sediments, O_2, E_h and the sum of nitrate and nitrite all showed significant correlations (p <0.01) with molecular data (Table 3.4). The *amoA* gene abundance in both AOA and β-AOB was positively correlated to nitrite and nitrate concentration, and inversely correlated to positive E_h. Gene expression in AOA, but not β-AOB, was inversely correlated to the sum of nitrite and nitrate. Furthermore, O_2 was positively correlated only with archaeal *amoA* gene expression. In particular, archaeal *amoA* gene abundance was positively correlated to nitrate levels, while archaeal *amoA* gene expression was positively correlated to oxygen saturation. Additionally, no correlation was observed between NH_4^+ concentrations and molecular data in the sediments examined.

3.3.5 Archaeal *amoA* gene phylogeny

Archaeal *amoA* genes were sequenced in clones recovered from summer sediments, and designated as "CRCISA" for Columbia River Cathlamet Island sediment *amoA*. Phylogenetic trees were constructed with archaeal *amoA* gene sequences from CR sediments and other environments, and a representative tree is shown in Figure 3.6. Eighty-eight percent of *amoA* sequences (74) were grouped into two major clades - referred to herein as Clade 1.1A1 and Clade 1.1A2, which clustered within marine group 1.1a (DeLong and Pace, 2001). Ten additional sequences placed within group 1.1b, also referred to as the "soil" group (DeLong and Pace, 2001). All sequences comparisons were performed over 590 nucleotides (nt).

Clade 1.1A1 contained 60 sequences sharing ≥ 94% identity (with one exception: CRCISA-E03) (Table 3.3). Sequences in Clade 1.1A1 were 97─99% identical to sequences recovered from freshwater sediment in other sediments including Chaohu lakes (JQ698552), Dojiang and Pearl River from China (JQ312228, JX305896), diverse soil (FJ839431, JQ404240), and aquatic (JN375935, HQ401437) environments. Clade 1.1A1 *amoA* gene sequences were also 94─96% similar to the "*Ca. Nitrosoarchaeum limnia*" SFB1 sequence (Blainey et al, 2011), but only 81%─90% similar to the *N. maritimus amoA* sequence.

The 14 sequences in Clade 1.1A2, were 93-99% similar to *amoA* sequences recovered from freshwater systems including lake (JX488442), river (HM443615), wetland (JQ941737) and other terrestrial environments such as grassland soil (JQ403884), an oligotrophic cave (JQ319440), and a groundwater treatment plant(s) (EU852677, JF271980). Clade 1.1A2 *amoA* gene sequences were also 93-98% similar to an enrichment culture clone AOA-DW (JQ669394) recovered from freshwater sediment (French et al., 2012). Sequences in Clade 1.1A2 were ≥92.1% similar to one another, and 79.2-82.5% and 70.7-74.8% similar to sequences in Clade 1.1A1 and Clade 1.1B, respectively.

Sequences in Clade 1.1B, showing high identity to sequences from different soils and sediments, were ≥75.9% similar to one another, and 70.7-75.8% similar to sequences from Clade 1.1A1 and Clade 1.1A2. While this clade only represented small portion (12%) of *amoA* gene library, it was highly diverse. Seven operational taxonomic units (OTUs) were present in 10 sequences based on a 3% cutoff value, while only 8 OTUs

were identified in the 60 sequences in Clade 1.1A1 (Table 3.4). Similar results were indicated by the diversity indices calculated using the Chao1 richness estimator and Shannon-Weaver index (Table 3.4).

3.3.6 Archaeal *amoA* diversity profiles across geochemical gradients

AOA community composition was examined using PCR-SSCP with the archaeal *amoA* primer set 353F/487R (Table 3.6). The AOA community was always dominated by a few different phylogenetic types (phylotypes) (Figure 3.7: red arrows) regardless of season or depth, but the relative abundance of these dominant clades changed with depth. Vertical changes in AOA richness were not observed in spring sediment (Figure 3.7 B). But in summer sediments, particularly the July 2010 samples, AOA diversity increased significantly in 10-20 mm sediment fractions, compared to the diversity in surface and bottom sediment fractions (Figure 3.7A). This higher archaeal *amoA* gene richness corresponded to a very steep redox gradient, which was, unfortunately, not observed in other sediment cores.

Canonical correspondence analysis (CCA) was used to examine correlations between the archaeal *amoA* community composition and environmental variables. Figure 3.8 shows representative CCA maps, correlating the AOA diversity to a set of environmental variables. Three environmental variables were included in CCA analysis: oxygen, E_h and ΔE_h ; dissolved nitrogen species were additionally considered in August 2012 (Figure 3.8 B). CCA plots (Figure 3.8) described 97.66% and 92.31% of the total variance in July 2010 and August 2012 sediments, respectively, whereas 75.99% (July 2010) and 65.29% (August 2012) of variance was explained by axis F1, and 27.12% (July 2010) and 19% (August 2012) of variance was explained by axis F2. Results indicated that oxygen and

ΔE_h had the highest correlations with archaeal *amoA* community composition (Figure 3.8). In particular, AOA communities in the surface sediment fractions were associated with high level of oxygen, and always grouped together separately from those in deeper sediment fractions (Figure 3.8). Additionally, AOA communities with higher richness were positively correlated with ΔE_h.

3.3.7 Potential nitrification activity coupled with growth

Freshwater sediment slurries were incubated in the laboratory with the addition of ammonium to measure potential nitrification activity. After 7-day incubation in aerobic filtered site water, the ammonium was converted to nitrite plus nitrate in a near stoichiometric fashion (Figure 3.9). Nitrite by itself was undetectable in the incubations, suggesting rapid nitrite oxidation by nitrite oxidizing bacteria coexisting with ammonia oxidizers in these sediments The fold-increase of the β-AOB *amoA* gene over the 7-day incubation was greater than that of AOA *amoA* (5 vs. 1.9). Furthermore, the ratio of AOA:β-AOB *amoA* abundance decreased from 1.3 to 0.5 (Table 3.5), suggesting that the incubation conditions used might favor the growth of β-AOB.

3.4 Discussion

3.4.1 Seasonal variability in biogeochemistry and ammonia oxidizing assemblages in Columbia River freshwater sediments

The seasonal variability of ammonia oxidizing assemblages in freshwater systems has been reported in several recent studies (Auguet et al., 2011; Vissers et al., 2013a; 2013b). These systems are characterized by a wide range of hydrodynamic and biogeochemical conditions, indicating the prevalence of seasonal changes in ammonia oxidizers across different types of environments. Noteworthy is that the underlying environmental factors

that result in the variability, however, can be quite different, and are usually site specific. For example, temperature and conductivity were suggested as the main drivers influencing AOA and AOB distribution in an oligotrophic lake (Vissers et al., 2013a), while ammonium concentration was indicated as a major factor in an wetland ecosystem (Sims et al., 2012).

In the case of the Columbia River sediment examined in this study, strong seasonal patterns were observed for a number of environmental variables. E_h showed relatively steep redox gradients in the summer cores but little change in the spring cores (Figure 3.3A). This is mostly likely due to the unique climate in the Pacific Northwest (Simenstad, 1990), causing more mixing at sediment-water interface during the spring but more stratification in the Columbia River through the dry summer. Oxygen depletion occurred at shallower depths in summer than in spring, likely because of greater stratification and higher oxygen consumption caused by heterotrophic activities in summer. With respect to ammonium, higher concentrations were observed in the summer cores (Figure 3.3B), which was likely due to higher remineralization rate in summer (Gilbert et al., 2013; Moeller, 2011). High ammonium concentration (up to 400 μM) were also reported in other studies of freshwater lake sediments during summer stratification (Nowlin et al., 2005; French et al., 2012). With respect to nitrate and nitrite, contrasting depth profiles were observed between the spring and summer cores, however, only in the surface sediments (< 15 mm) (Figure 3.3C). Lower nitrate concentrations were observed in the surface sediments in summer (Figure 3.3C). This result is consistent with other studies showing that the measured nitrate concentrations were lowest in later summer in the Columbia River, likely due to the higher consumption rate by primary producers

(Sullivan et al., 2001; Gilbert et al., 2013). In addition, this work was mainly focused on the surface sediments, which were less likely to be affected by groundwater. A more comprehensive evaluation of nitrification activity in CR sediment would require investigation in deeper sediments, where more complex hydrogeological and biogeochemical conditions are expected due to interactions between river water and groundwater, creating hyporheic zones (Hinkle et al., 2001; Santoro et al., 2008).

Although strong seasonal fluctuations were observed in CR sediment biogeochemistry, the populations of both AOA and AOB appeared to be relatively stable with AOA consistently having higher *amo*A abundance than AOB. It is possible that we did not have enough samples representing different seasons to capture the temporal patterns of ammonia oxidizing assemblages. Nevertheless, a higher AOA to β-AOB ratio of *amoA* gene abundance was only observed in deeper sediments in summer, , and the higher ratio was mainly caused by a decrease in β-AOB abundance, potentially suggesting unfavorable growth conditions for β-AOB over AOA in deeper stratified sediments. Seasonal differences were not observed for *amoA* transcript abundance, although higher *amoA* gene expression was observed for AOA than for β-AOB regardless of depth. In other freshwater systems, higher archaeal *amoA* gene and/or transcript abundance determined by qPCR has also been reported (Santoro et al., 2008; Herrmann et al., 2008; Liu et al., 2011; Auguet et al., 2012; Hugoni et al., 2013), although nitrification activity by AOA and AOB was not examined in those studies.

3.4.2 Environmental factors relevant to ammonia oxidizers in freshwater sediments

Factors shaping the ecological niches of AOA and AOB in freshwater systems have been recently investigated in a few environmental studies (Auguet et al., 2011; 2012;

Auguet and Casamayor, 2013; S. Liu et al., 2013) and laboratory enrichments (French et al., 2012; Wu et al., 2013). In CR freshwater sediments I examined, multiple environmental parameters were correlated with molecular characteristic of ammonium oxidizing assemblages. Understanding the significance of these correlations is challenging because of (*i*) the complexity of the environments that involve many interacting but uncharacterized physical and biogeochemical processes and (*ii*) the limited number of parameters that were measured. Nevertheless, my correlation analysis showed several interesting aspects that could be important for nitrification in CR sediments.

Several studies have suggested that O_2 is an important factor shaping niches of AOA and AOB (Francis et al., 2005; Santoro et al., 2008; Beman et al., 2008; Lam et al., 2009; French et al., 2012), possibly due to the extremely high substrate affinity of AOA to oxygen (Martens-Habbena et al., 2009; Park et al., 2010; French et al., 2012). Based on Pearson Correlation analysis, strong covariance was found between O_2 and AOA *amoA* transcript abundance in summer sediments. The *amoA* transcripts were most abundant near the surface where O_2 concentrations were relatively high, and steadily decreased as O_2 was depleted with depth. Between 20 and 35 mm depths, however, the *amoA* transcript abundance of both AOA and AOB increased (Figure 3.5). Assuming that *amoA* transcript abundance is a suitable indicator for nitrification activity, the transcript profiles observed indicate two sediment fractions with higher nitrification activity: One was at the surface sediment, which was O_2 dependent, and the other was at deeper depths, which was most likely O_2 independent. However, based on gene expression data, AOA appears to positively correlate with high oxygen concentration, potentially indicating that O_2

might not play an significant role in shaping the niches of AOA and AOB in these sediments.

Ammonium concentrations have been reported also as an important factor for nitrification in oligotrophic freshwater systems, mainly by limiting AOB abundance under low-ammonium conditions (Auguet et al., 2011; 2012; Auguet and Casamayor, 2013; S. Liu et al., 2013; French et al., 2012; Wu et al., 2013). However, we did not observe any correlations between NH_4 and molecular data, which is likely because the ammonium concentration in our system was relatively high and not a limiting factor. In addition, a positive correlation was shown between nitrate concentration, the product of nitrification, and both archaeal and β-bacterial *amoA* gene abundance, suggesting both groups may be involved in nitrification in CR freshwater sediments. Similar correlations between nitrate concentrations and AOA abundance have also been reported in other freshwater studies (Auguet et al., 2011; S. Liu et al., 2013; Vissers et al., 2013b).

In addition to the parameters discussed above, pH has recently been shown to be an important environmental factor for nitrification in freshwater systems (F. Liu et al., 2011; French et al., 2012; S. Liu et al., 2013). AOA were found to be more abundant than AOB at lower pH, likely because of low ammonia availability due to protonation (Nicol and Schleper, 2006; Gubry-Rangin et al., 2011). Moreover, a sediment incubation study showed an interesting correlation between temperature and AOA abundance and diversity, suggesting that incorporation of labeled bicarbonate by archaea only occurred at warmer temperature (37ºC), in contrast to little incorporation at lower temperature (4-25ºC) (Wu et al., 2013). Another potentially important but poorly studied environmental factor - organic carbon - has recently been shown to positively affect the AOA/AOB diversity

ratio in freshwater systems (S. Liu et al., 2013). Organic carbon is particularly interesting because it might be an alternative substrate for some *Thaumarchaeota*. The ability to assimilate organic substrates by AOA has been suggested by a number of studies, including genomic investigations (Hallam et al., 2006b, Blainey et al., 2011, Spang 2012 et al.), culture studies (Tourna et al., 2011; Xu et al., 2012), environmental isotope labeling studies, and the natural distribution of radiocarbon (^{14}C) in archaeal lipids (Ouverney & Fuhrman 2000; Ingalls et al., 2006, Mussmann et al., 2011). Whether it is a major driver of niche differentiation in AOA and AOB requires more investigations.

3.4.3 Diverse community of AOA in Columbia River freshwater sediments

Analysis of archaeal *amoA* phylogeny revealed a relatively diverse AOA community in CR freshwater sediments. Sequences were clustered into three major clades: two large clades associated with group 1.1a, and one small clade clustered with group 1.1b. The most abundant AOA clades in CR freshwater sediments, associated with group 1.1a, showed high sequence similarity with *amoA* sequences recovered from other freshwater sediments and water sources (Figure 3.6). The dominance of group 1.1a-like archaea in freshwater sediments has been reported in other studies as well (Herfort et al., 2009; Auguet et al., 2011; Hugoni et al., 2013). Recently, group 1.1a-affiliated AOA strains, enriched from freshwater sediments, demonstrated autotrophic ammonia oxidation in sediment incubations (French et al., 2012), indicating a potentially important role of these group 1.1a archaea in nitrification in freshwater systems. It is interesting to note that the AOA prevalent in freshwater systems do not appear to be *Nitrosopumilus*-like archaea that are abundant in ocean waters (Auguet et al., 2011; 2012); but instead grouped together with sequences recovered from the same ecosystems representing the

"freshwater *amoA* ecotype," (Auguet et al., 2011; Cao et al., 2013). Despite their high abundance in freshwater systems, the physiological characterization of freshwater *Thaumarchaeota* is largely lacking, and only a few enrichment strains were obtained (Blainey et al.,2011; Mosier et al., 2012c; Santoro et al., 2011; French et al., 2012).

In addition to the dominant 1.1a clades, it is also worth noting that clade 1.1B, despite its low abundance, possessed high diversity in CR freshwater sediments. The Chao1 richness estimator suggested that we did not sequence all the diversity within this clade (Table 3.3). Furthermore, the ecological functions and metabolic pathways of these diverse 1.1b *Thaumarchaeota* archaea are still largely unknown. Based on prior studies, AOA in this group have not been directly linked to nitrification activity in freshwater sediments, although ammonia oxidation by group 1.1b *Thaumarchaeota* was observed in soils (Kim et al., 2012; Tourna et al., 2011). Whether all the members in group 1.1.b *Thaumarchaeota* are able to perform ammonia oxidation remains to be seen. Studies have suggested that some group 1.1b *Thaumarchaeota* might use alternatives to autotrophic ammonia oxidation, e.g., heterotrophic and mixotrophic metabolisms (Muβmann et al., 2011; Tourna et al., 2011; Xu et al., 2012). Further studies using group-specific primers and stable isotopic labeling will be necessary to address the above questions.

Furthermore, changes in AOA diversity along biogeochemical gradients were analyzed in CR freshwater sediments. Both PCR-SSCP results and CCA plots of data from summer sediment samples suggested that the AOA community composition was strongly correlated with ΔE_h. Specifically, higher AOA diversity was associated with steep E_h gradients, indicating a likely geochemically-dependent pattern of *amoA* phylotypes. Steep E_h gradients, characterized by drastic vertical change in redox potential,

might allow microorganisms occupying different ecological niches to compete for resources. This hypothesis has been suggested by another study of stratified sediments (Jones, 1979). It is tempting to speculate that in relatively stable environments with only a single substrate available, AOA communities are dominated by a few group 1.1a archaea, while in environments in which rapid changes in energy or nutrient sources are common, higher archaeal diversity is present. In such a case, competition for resources may occur not only between *Thaumarchaeota* and AOB, but also among different AOA phylotypes. Niche differentiation among different AOB phylotypes has been reported (Bollmann et al., 2002). Similar theory could also be applied for *Thaumarchaeota*, as physiological characterizations and genomic studies on strains in pure and enrichment cultures have already demonstrated potentially high metabolic diversity (Hallam et al., 2006b; Hatzenpichler et al., 2008; la Torre et al., 2008; Martens-Habbena et al., 2009; Blainey et al., 2011; Tourna et al., 2011; Lehtovirta-Morley et al., 2011; French et al., 2012; Spang et al., 2012).

3.4.4 Significance of archaeal nitrification in Columbia River freshwater sediments

In the sediments examined, AOA showed greater *amoA* gene abundance and higher gene expression than β-AOB regardless of depth and season. However, analyzing genes and transcripts alone will not reveal nitrification activity. Ammonia oxidation assays coupled with growth measurements were performed to examine the ammonia oxidation potential activity by AOA and β-AOB. Sediment incubation studies suggested that both AOA and β-AOB may be involved in sediment nitrification, but β-AOB responded faster to exogenous ammonium amendment. Similar results have also been reported in recent incubation studies of freshwater sediments (French et al., 2012; S. Liu et al., 2013),

indicating that β-AOB grew better under most incubation conditions in the laboratory. Using bacteria-biased ammonia oxidation inhibitors such as allylthiourea (ATU) will help further differentiate the relative contributions of AOA and AOB to nitrification in freshwater sediments (Santoro and Casciotti, 2011).

Furthermore, evidence suggested that some archaeal ammonia oxidizers prefer nitrogen from remineralization rather than inorganic nitrogen. This may be because the rate of release keeps nitrogen concentrations relatively low. A recent study of acidic soil showed that AOA only responded to organic nitrogen amendments, and not to inorganic nitrogen additions (Levičnik-Höfferle et al., 2012). Urea has been considered as a possible important nitrogen source in acid soils (Nicol et al., 2008; Gubry-Rangin et al., 2011; Lehtovirta-Morley et al., 2011). Recently, a study reported that some polar *Thaumarchaeota* were able to use urea to fuel nitrification in Arctic waters (Alonso-Sáez et al., 2012). Whether utilizing organic nitrogen offers an ecological advantage to AOA over AOB remains to be examined.

To conclude, molecular characterization of ammonia oxidizing assemblages showed a highly abundant, diverse and potentially active AOA community in CR freshwater sediments. Various geochemical gradients were shown to have effects on the vertical distribution of AOA abundance and diversity in freshwater sediments. Although their *in situ* activity remains to be further explored, AOA, together with AOB, play important roles in regulating nitrification process in Columbia River sediments.

3.5 Tables and figures

Table 3.1 Bulk chemical analysis of sediments (top 5 cm) collected at low tide from the Columbia River through different seasons[a].

Sampling Site location/Time	Bulk Chemical Analysis*						
	pH	**NO_3**	**NH_4**	**Mn**	**Fe**	**TOC%**	**TKN%**
Cathlamet Island-July 2010	6.4	1.0	16.3	58	100	2.47	NA
Cathlamet Island-April 2012	7.2	1.3	10.5	11	88	1.73	0.142
Cathlamet Island-August 2012	6.8	3.0	31.5	55	113	2.15	0.128

[a] All the nutrient and metal concentrations were measured in mg/kg except TOC and TKN.

Table 3.2 Percent sequence identity, over 635 nt, of CRCISA clones.

Clade name	Group 1.1A1	Clone E03 (Group 1.1A1)	Group 1.1A2	Group 1.1B1
Group 1.1A1	≥ 94.0%	86.0-89.5%	79.2-82.5%	71.7-75.8%
Group 1.1A2		77.3-79.7%	≥ 92.1%	70.7-74.8%
Group 1.1B1		81.5-84.8%		≥ 75.9%

Table 3.3 Diversity indicators of archaeal *amoA* sequences recovered from CR freshwater sediments.

Clade	**Sequence No.**	**OTU (97%)**	**Shannon index**	**Chao1 index**
Group 1.1A1	60	7	1.32	9.0
Group 1.1A2	14	4	1.23	4.0
Group 1.1B	10	8	1.83	19.5
Total	84	19	2.20	29.0

Table 3.4 Pearson correlations for variables in CR freshwater sediments in August[a].

		amoA abundance		*amoA* expression		Geochemical variables			
		AOA	β–AOB	AOA	β–AOB	O_2	E_h	NH_4	NO_2+ NO_3
amoA abundance	AOA	1.000							
	β–AOB	0.729	1.000						
amoA expression	AOA	-0.424	-0.424	1.000					
	β–AOB	-0.483	-0.483	**0.827***	1.000				
Geochemical variables	O_2	-0.195	-0.030	**0.742***	0.360	1.000			
	E_h	**-0.708***	**-0.450***	**0.775***	**0.728***	0.475	1.000		
	NH_4	-0.411	-0.281	-0.109	0.127	-0.278	0.063	1.000	
	NO_2 + NO_3	**0.678***	**0.675***	**-0.590***	-0.430	-0.388	**-0.635***	-0.239	1.000

[a] Bold with superscript ***** indicates that the correlation is statistically significant (p<0.01).

Table 3.5 Quantification of archaeal and bacterial *amoA* gene abundance in the beginning (Day 0) and at the end (Day 7) of incubation[a].

	Day 0	Day 7	Fold change
AOA	1.4×10^{6} (3.4×10^{4})	2.7×10^{6} (3.1×10^{5})	1.9
β-AOB	1.1×10^{6} (3.5×10^{5})	5.5×10^{6} (1.9×10^{5})	5.0
AOA: β-AOB	1.3	0.5	

[a] Means and standard deviations (in parenthesis) of *amoA* gene copies per gram sediment are shown; values were estimated from duplicate incubations (tested in replicate PCRs).
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Table 3.6 PCR primer sequences and annealing temperatures used in this study.

Target gene	Primer	Sequences (5'–3')	Annealing temperature	Citation
Archaeal *amoA* gene	Arch-amoAF	STAATGGTCTGGCTTAGACG	53 °C	Francis *et al.* (2005)
	Arch-amoAR	GCGGCCATCCATCTGTATGT		
	353F	AGCACTCCTTGATACTGTTTGG	54 °C	This study
	487R	TGTATGGTGGYAATGTTGGTC		
Bacterial *amoA* gene	amoAF1	GGTGCGTGGTTCTCCTTAG	56 °C	Rotthauwe *et al.* (1997)
	amoAR2	GTGTTTGTTTCTCTTTGTTGCC		

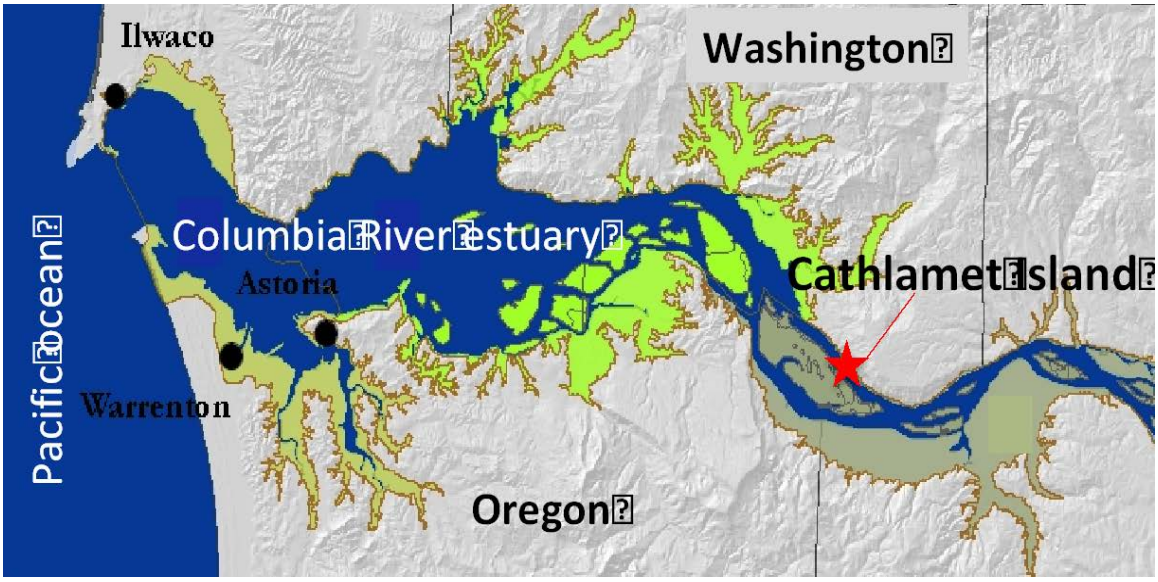
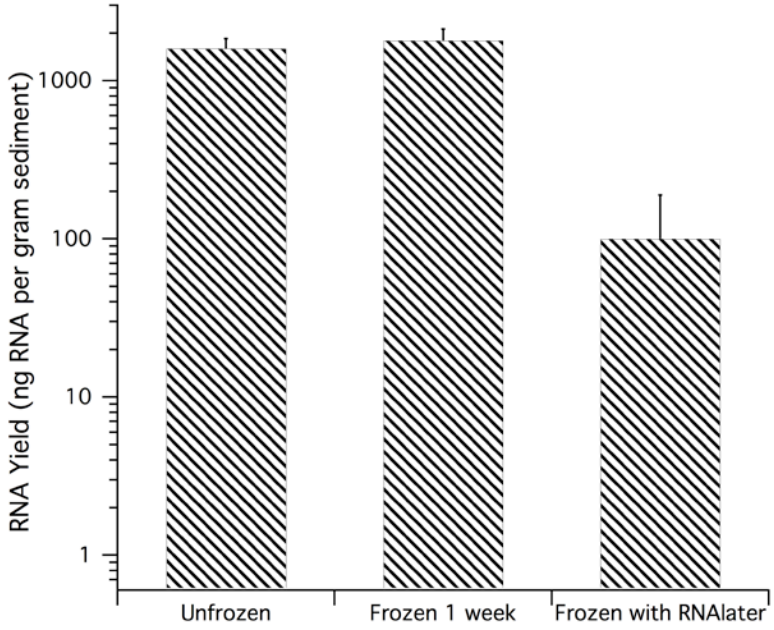
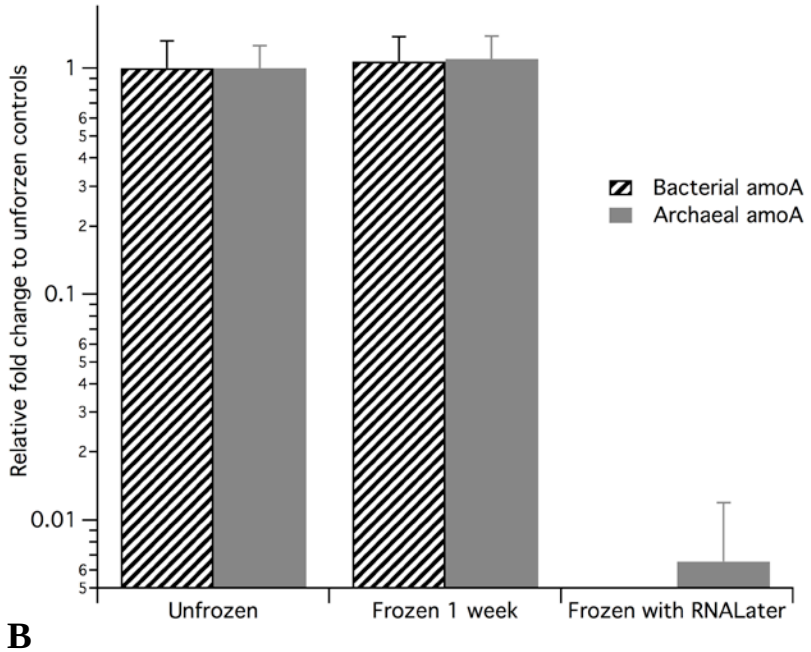


Figure 3.1 Map of the lower Columbia River showing the sampling location at Cathlamet Island (46.172 ºN, 123.355 ºW), where freshwater sediment and site water samples were collected.



A



B

Figure 3.2 Comparative analysis of total RNA yield (A), and the relative fold change of β-bacterial and archaeal *amoA* transcripts (B) using three different approaches to sample preservation prior to RNA extraction and purification. Error bars represent standard deviations from triplicate RNA extractions.

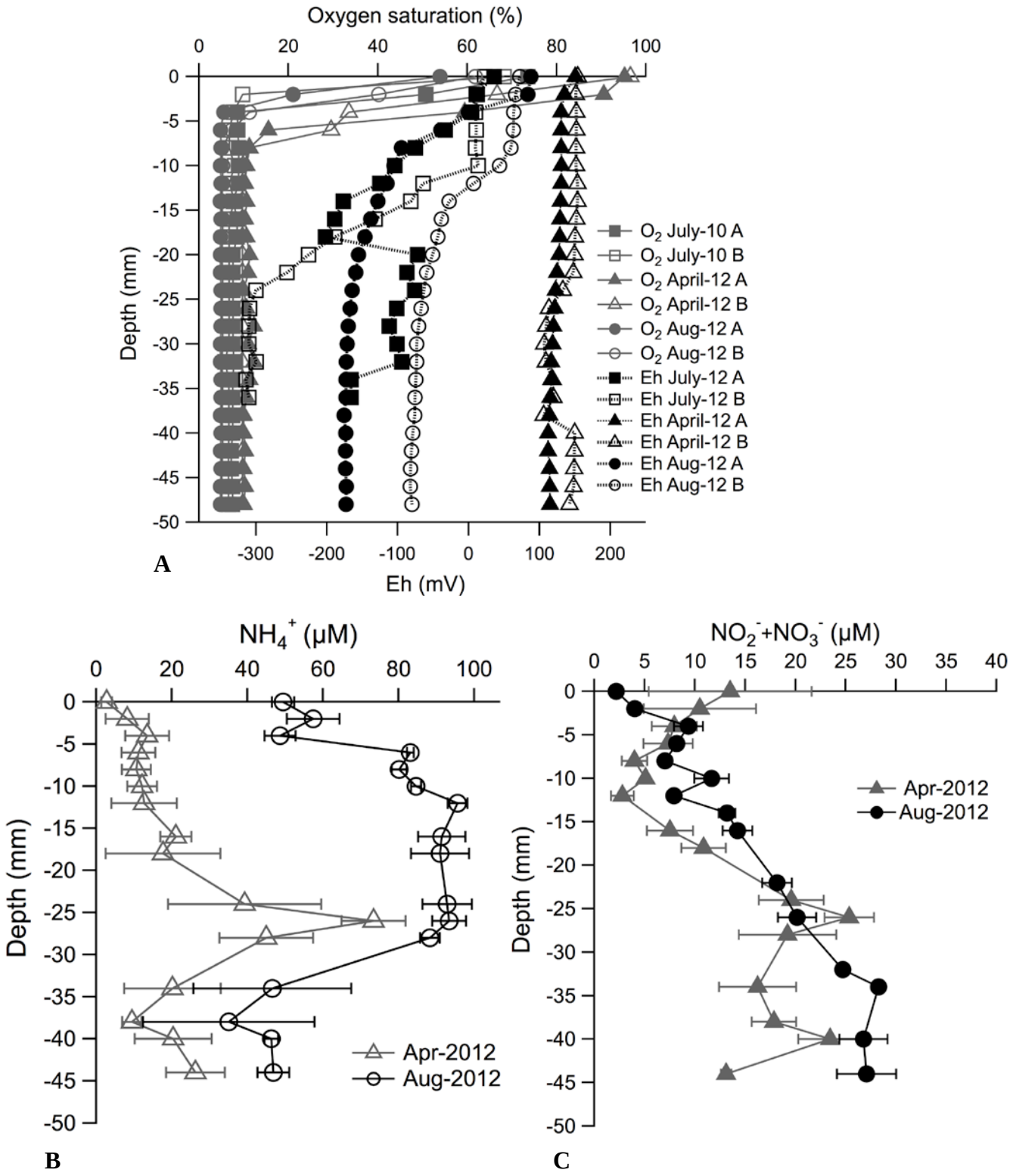
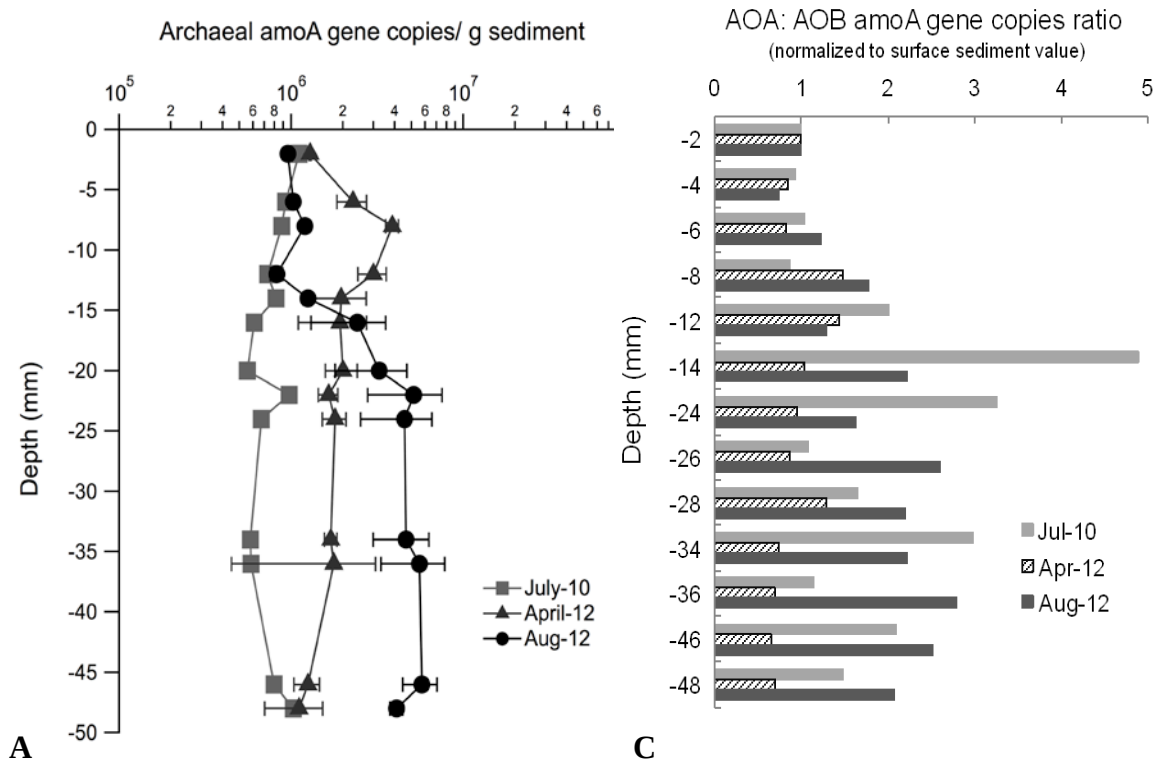


Figure 3.3 Vertical profiles of geochemical variables in CR sediments collected in July 2010 (squares), and April (triangles) and August 2012 (circles): A) oxygen (grey) and E_h (black), B) ammonium and C) nitrite and nitrate. Error bars represent standard deviation from mean of duplicate cores.



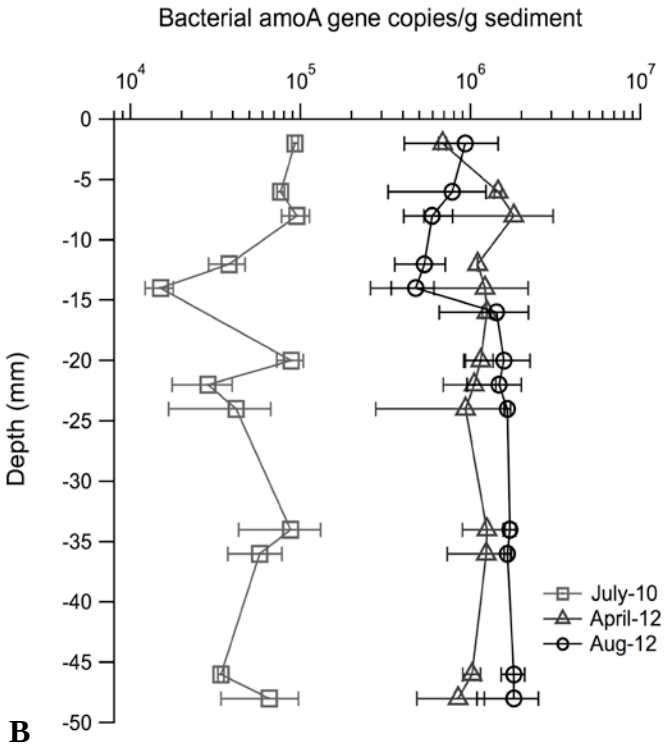
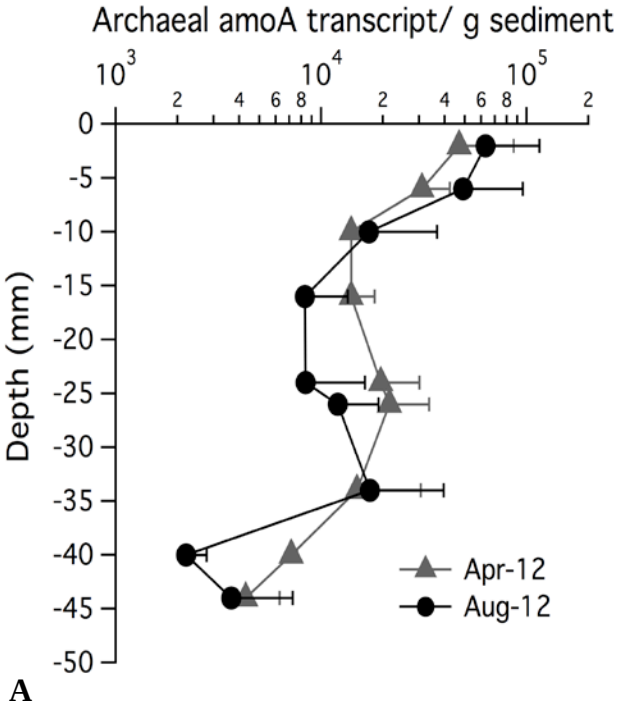
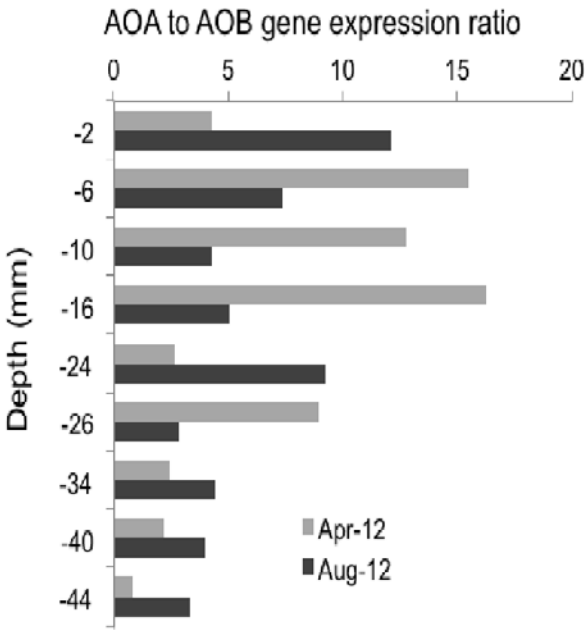


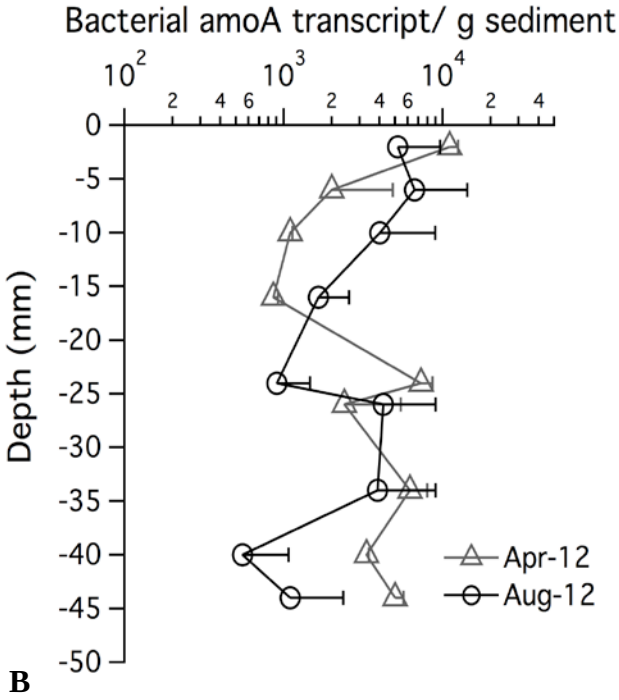
Figure 3.4 Vertical profiles of *amoA* gene abundance in CR sediments collected in July 2010, and April and August 2012: A) archaeal *amoA* gene abundance B) bacterial *amoA* gene abundance. &C) AOA: β-AOB *amoA* gene abundance ratio normalized to the values of surface sediments. . Error bars represent standard deviation from mean of duplicate cores, DNA extractions and PCRs.



A



C



B

Figure 3.5 Vertical profiles of *amoA* expression in CR sediments collected in April 2012 and August 2012: A) archaeal *amoA* expression; B) bacterial *amoA* expression; C) AOA: β-AOB *amoA* gene abundance ratio. Error bars represent standard deviation from mean of duplicate RNA extractions and PCRs.

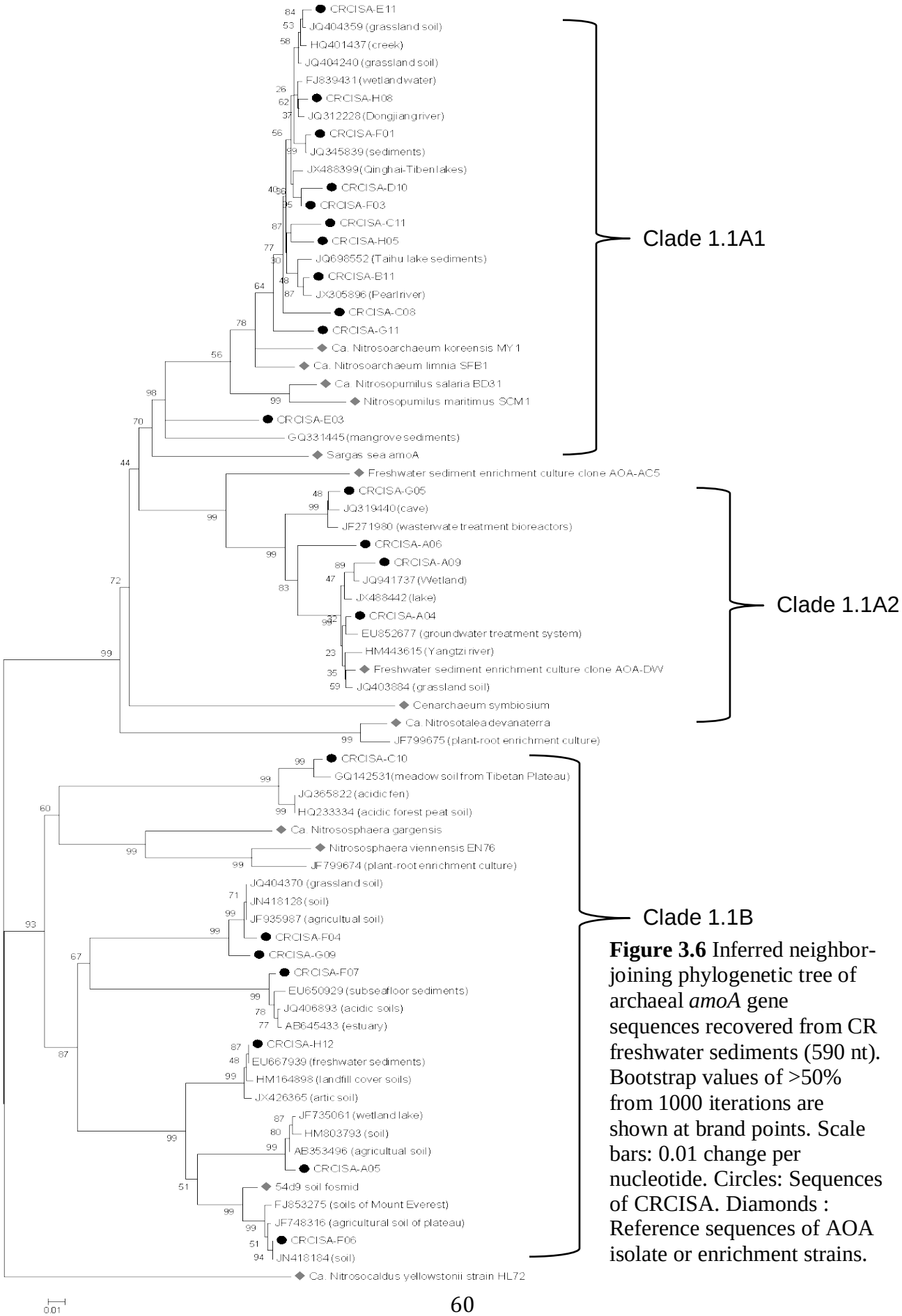


Figure 3.6 Inferred neighbor-joining phylogenetic tree of archaeal *amoA* gene sequences recovered from CR freshwater sediments (590 nt). Bootstrap values of >50% from 1000 iterations are shown at brand points. Scale bars: 0.01 change per nucleotide. Circles: Sequences of CRCISA. Diamonds : Reference sequences of AOA isolate or enrichment strains.

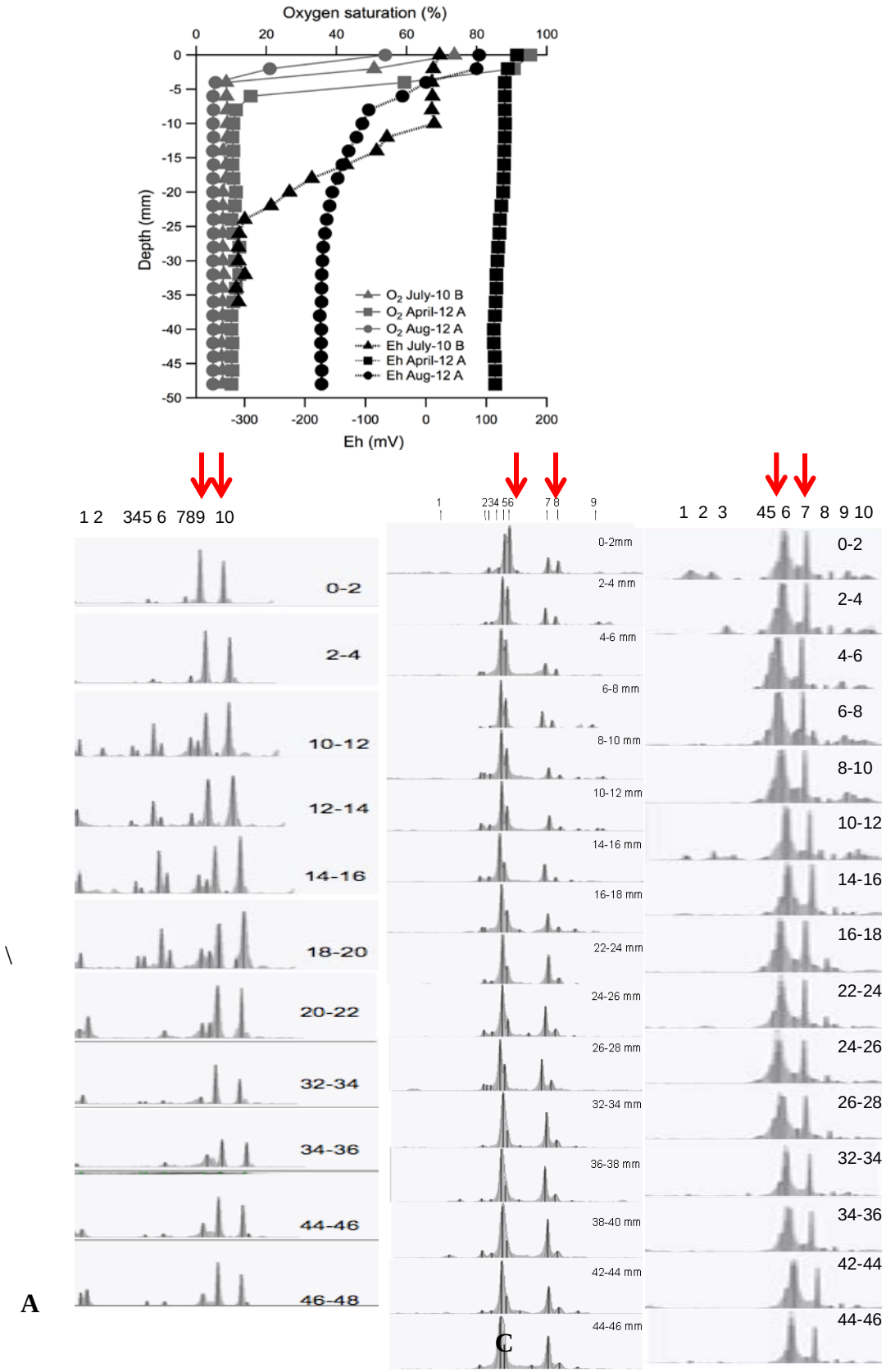


Figure 3.7 PCR-SSCP profiles of June-2010, April-2012 and Augst-2012 cores (A, B, C), showing archaeal *amoA* gene diversity with sediment depth. Different peaks (numbered 1-10) indicate unique archaeal *amoA* gene conformers. Corresponding E_h and oxygen profiles of each core are shown above.

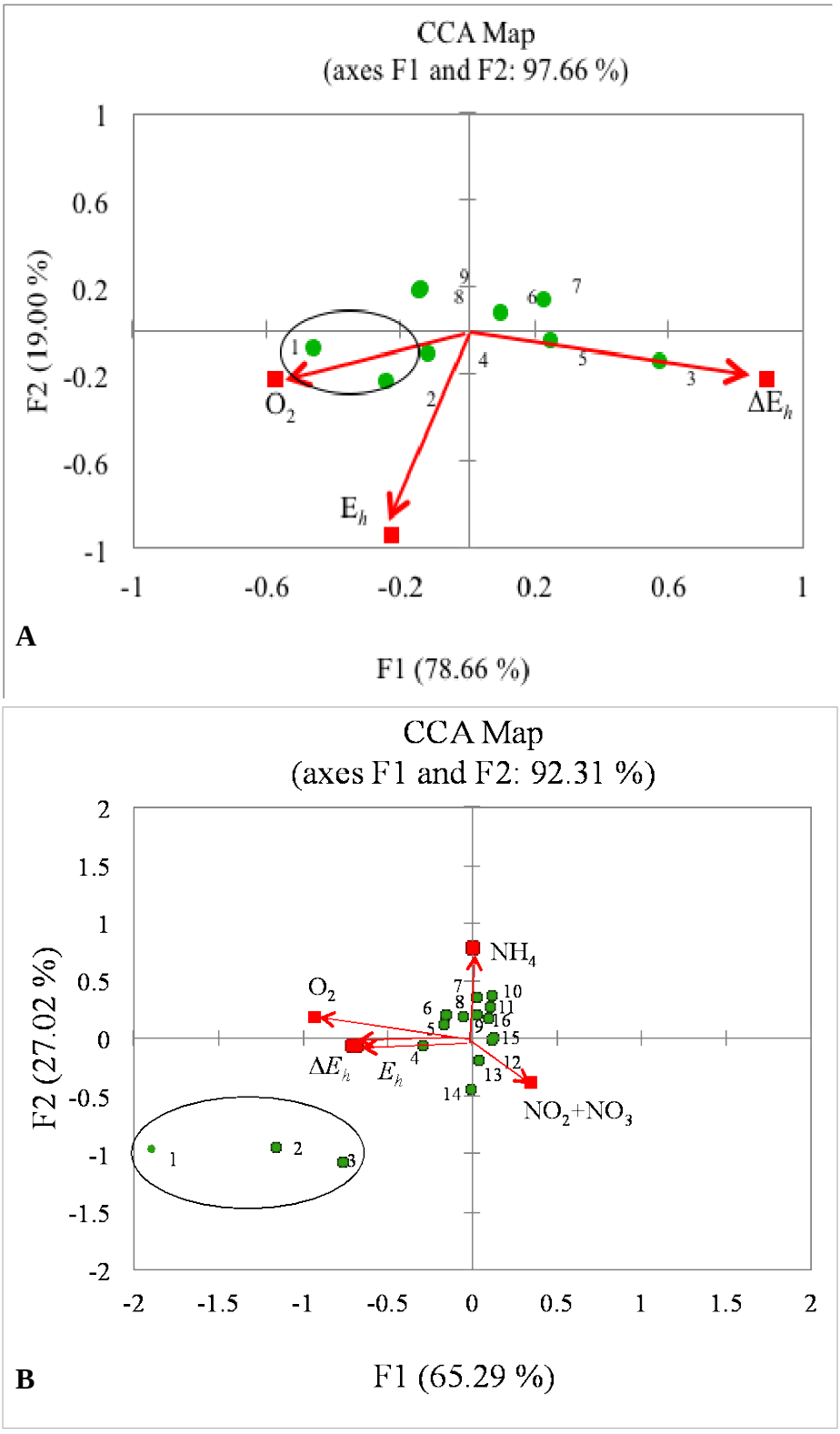


Figure 3.8 Canonical correspondence analysis of AOA diversity and environmental variables on the representative sediment cores of July 2010 (A) and August 2012 (B). Green dots represent AOA communities from selected depths (1 to 16: top to bottom sediment fraction). Red squares represent environmental variables.

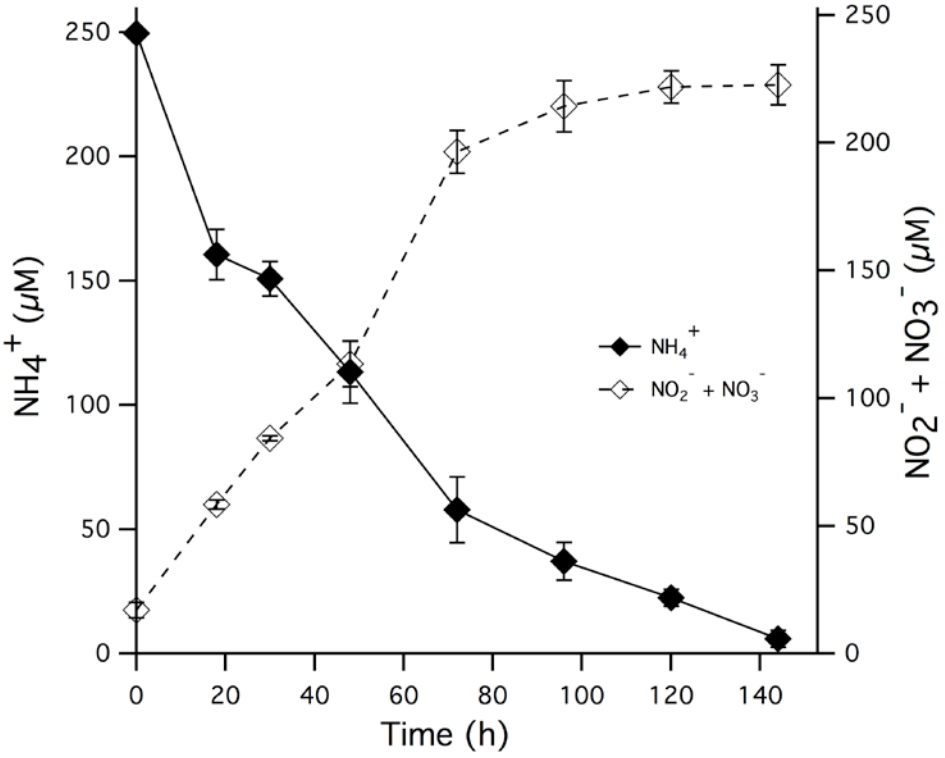


Figure 3.9 Ammonium and nitrite plus nitrate dynamics in incubations for potential nitrification rates with CR sediment slurries. Error bars indicate standard errors from the means of duplicate incubations.

CHAPTER 4

AMMONIA OXIDIZING POTENTIAL OF ARCHAEA AND BACTERIA IN FRESHWATER SEDIMENT INCUBATIONS

4.1 Introduction

Nitrification, the microbial oxidation of ammonia to nitrate, is an essential part of the global nitrogen cycle, linking remineralized nitrogen to the ultimate removal of Nr to N_2 gas via dentrification and anammox (Galloway et al., 2004; 2008). Ammonia oxidation is the first and rate-limiting step in nitrification. This process is known to be carried out by ammonia oxidizing bacteria (AOB) in the β- and γ-Proteobacteria phylum (Purkhold et al., 2000; Kowalchuk and Stephen, 2001), and the newly recognized ammonia oxidizing archaea (AOA) (Treusch et al., 2005; Venter et al., 2004; Könneke et al., 2005). The recent discovery of ammonia oxidation within the *Archaea* domain has dramatically transformed our bacteria-centered view on the nitrogen cycle, and led to the proposal of a new archaeal phylum- *Thaumarchaeota* (Brochier-Armanet et al., 2008; Spang et al., 2010; Brochier-Armanet et al., 2011a; 2011b; Gupta and Shami, 2011; Pester et al., 2012).

The gene encoding subunit A of the ammonia monooxygenase enzyme (*amoA*) in archaea has been found to be ubiquitous in almost every environment on earth (Francis et al., 2005; Leininger et al., 2006; Mincer et al., 2007; He et al., 2007; Pouliot et al., 2009; Beman et al., 2007; la Torre et al., 2008; Hatzenpichler, 2012; Park et al., 2008; Herrmann et al., 2008; Xu et al., 2012). Quantification of *amoA* gene abundance has shown that AOA are extremely abundant, and often outnumber AOB in many aquatic (Hallam et al., 2006a; Konstantinidis et al., 2009; Mincer et al., 2007; Santoro et al., 2010; Beman et al., 2008) and terrestrial (Leininger et al., 2006; Pratscher et al., 2011; Zhang et

al., 2012; Lehtovirta-Morley et al., 2011) habitats. Furthermore, measured *amoA* gene transcript abundance was also higher in archaea compared to bacteria in several studies (Leininger et al., 2006; Herrmann et al., 2008; Nicol et al., 2008), mostly in oligotrophic environments, suggesting AOA have important roles in nitrification and occupy unique ecological niches.

Despite the frequent detection of archaeal *amoA* genes and gene transcripts in diverse environments, the metabolic diversity and physiological properties of AOA strains were not well understood. To date, there are only two cultured representatives of *Thaumarchaeota*: the marine isolate *Nitrosopumilus maritimus* from marine sediment (Könneke et al., 2005), and the soil isolate *Nitrososphaera viennensis* obtained from garden soil (Tourna et al., 2011). Autotrophic growth on ammonia as the sole energy source has been demonstrated in *Thaumarchaeota* pure and enrichment cultures, indicating that many of them are autotrophs, acquiring energy from autotrophic ammonia oxidation (Könneke et al., 2005; la Torre et al., 2008; Hatzenpichler et al., 2008; Kim et al., 2012; Tourna et al., 2011; Mosier et al., 2012c). In addition, genes encoding the near complete 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) CO_2 fixation pathway were identified in a number of thaumarchaeal genomes, indicative of their autotrophic potential (Hallam et al., 2006a; Walker et al., 2010; Blainey et al., 2011; Spang et al., 2012). On the other hand, heterotrophic or mixotrophic metabolisms by some *Thaumarchaeota* have also been suggested by a number of genomic investigation and incubation studies (Hallam et al., 2006b; Blainey et al., 2011; Spang et al., 2012; Muβmann et al., 2011; Xu et al., 2012). The incorporation of organic carbon such as amino acids was observed in marine planktonic archaea (Ouverney and Fuhrman, 2000;

Muβmann et al., 2011; Ingalls et al., 2006; Teira et al., 2006; Pérez et al., 2003). Recently, the soil isolate *N. viennensis* was shown to achieve a high growth rate in the presence of pyruvate, and also to be able to utilize urea as sole energy source, confirming mixotrophic or heterotrophic potential by some *Thaumarchaeota*.

In addition to the lack of physiological characterization, the relative contribution of AOA to nitrification remains largely unclear, especially in terrestrial environments. Most of the environmental studies have focused on the quantification of the *amoA* gene abundance and distribution, while *in situ* nitrification activities have not often been measured in tandem. Archaeal nitrification appeared to be significant in the open ocean, where their high AOA abundance was positively correlated with nitrification activity (Wuchter et al., 2006; Beman and Francis, 2006; Herrmann et al., 2008; Santoro et al., 2010). In terrestrial environments, the significance of archaeal nitrification is still under debate. In some studies, the growth of AOB rather than AOA was positively correlated with nitrification activities (Mertens et al., 2009; Jia and Conrad, 2009; Di et al., 2010), while in other studies, mostly in oligotrophic lakes and acid soils, archaea were the dominant autotrophic ammonia oxidizers (Offre et al., 2009; Zhang et al., 2010; Gubry-Rangin et al., 2010). With respect to freshwater systems, where anthropogenic input of Nr is substantial, nitrification activity and related microbial players were poorly studied.

Nitrification inhibitors have frequently been applied to agricultural soils in order to prevent nitrogen loss, by suppressing nitrification activity (Subbarao et al., 2006). Inhibitors have also been used to differentiate ammonia oxidation activity of AOA and AOB in incubation studies, including acetylene (Hyman and Arp, 1992; Offre et al., 2009; Gubry-Rangin et al., 2010), dicyandiamide (DCD) (Zacherl and Amberger, 1990;

Subbarao et al., 2006), nitrapyrin (Kim et al., 2012; Jung et al., 2011) and allylthiourea (ATU). ATU has been used to successfully differentiate the relative contribution of AOA and AOB to nitrification (Santoro and Casciotti, 2011; Taylor et al., 2010; Mosier et al., 2012c). ATU was shown to inhibit bacterial ammonia oxidation at low concentration (8–86 μM), likely by chelating copper at the active site of the ammonia monooxygenase enzyme (Ginestet et al., 1998; Hofman and Lees 1953; Bédard and Knowles, 1989). In contrast, AOA appear to be less sensitive to ATU, although the mechanism of ATU inhibition on AOA remains unknown.

In this study, we investigated the potential nitrification activity of AOA and β-AOB in freshwater sediments, and attempted to explore the metabolic strategies of *Thaumarchaeota* by using sediment incubations with organic and inorganic amendments. The freshwater sediments were collected from the Columbia River, and incubated in the filtered site water (or synthetic freshwater medium) with different amendments, including ATU, antibiotics, organic carbon and nitrogen. We aimed at addressing three major questions: (*i*) whether a nitrification inhibitor can be used to successfully differentiate the nitrification contribution of AOA from β-AOB in freshwater sediment incubations; (*ii*) whether there are additional carbon utilization strategies for AOA other than autotrophy; and (*iii*) whether organic nitrogen and remineralized nitrogen can be utilized for nitrification.

4.2 Material and methods

4.2.1 Sampling site and sediment properties

Freshwater sediment and river water samples were collected at Cathlamet Island (46.172 °N, 123.355 °W) in the Columbia River during April 2012 and March 2013.

Surface sediment samples were collected using 50 mL sterilized tubes, and site water samples were collected in 1 or 2 liter sterile bottles. Temperature and pH of site water were recorded at the time of sampling. pH of the site water ranged from 7.6–7.9, and temperatures ranged from 6 °C to 21 °C. The sediment texture was silt loam, containing 39.3 % sand, 54.3% silt and 6.4% clay, respectively.

4.2.2 Media preparation for sediment incubations

The media used to incubate ammonia oxidizers from freshwater sediments were either site water filtered through 0.22 micron filters (Millipore Corp., Medford, MA), or synthetic freshwater *Crenarchaeota* medium described in Chapter 2 (Könneke et al., 2005; la Torre et al., 2008).

4.2.3 Potential nitrification activity coupled growth assay

Homogenized sediment samples (20 gram) were inoculated into 200 mL of the medium in duplicate, immediately upon arrival in the laboratory. All sediment incubations were conducted in 500 mL flasks at 22 °C, in the dark without shaking. Aerobic conditions were maintained by continuously bubbling air through a 0.2 micron sterile Millex Vent Filter Unit (Millipore Corp., Billerica, MA) by an aquarium pump. We examined the effects of different factors on ammonia oxidation and the growth of AOA and AOB, including the medium selection, different ATU concentrations, combined antibiotic and ATU treatments, organic nitrogen, organic carbon, and remineralization of organic nitrogen. In each of these treatments, a controls were prepared by incubating sediments in filtered site water with added 250 μM NH_4Cl. During incubation, a small amount of sample (around 600 μL) was collected daily and filtered through a 0.2 micron filter for the measurements of ammonium, nitrite and nitrate

concentrations. The archaeal and bacterial *amoA* genes and gene transcripts in sediment were quantified weekly by qPCR.

Effect of medium

To examine the effect of medium on potential nitrification activity and the growth of AOA and AOB, freshwater sediments were incubated for one week in synthetic freshwater media at pH 7 and 7.9, compared with incubations in filtered site water.

Effect of ATU and antibiotics

To investigate the inhibitory effect of ATU on ammonia oxidation and the growth of AOA and β-AOB, ATU at different concentrations (0, 1, 25 and 86 μM) was tested in 14-day incubations. Both *amoA* genes and gene transcripts were quantified along with nitrification activity. Antibiotics (100 μg/mL streptomycin and carbencillin) were added in the incubations in addition to 86 μM ATU, for the purpose of completely inhibiting β-AOB in longer incubations (3 weeks).

Effects of organic substrate

Sediment incubations were amended with either 125 μM urea or 250 μM NH_4Cl for the purpose of assessing whether AOA and β-AOB could utilize urea as a substrate for ammonia oxidation.

In addition, sediment incubations were amended with pyruvate at concentrations of 50 and 500 μM, in the absence or presence of 86 μM ATU, respectively, to examine the effects of organic carbon amendments on the growth of ammonia oxidizers.

Nitrification from remineralized nitrogen vs. exogenously *inorganic nitrogen addition*

To assess nitrification activity from remineralization of indigenous organic matter and from exogenously added inorganic ammonium, freshwater sediments were incubated in filtered site water for four weeks with either 250 μM NH_4Cl or no NH_4Cl.

4.2.4 Chemical measurements

Ammonium concentrations were determined by colorimetric measurements of absorbance at 340 nm with the Sigma-Aldrich Ammonia Assay Kit (St. Louis, MO). Nitrite and nitrate concentrations were determined by photometric measurement of absorbance at 540 nm, with a Nitrite/Nitrate Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI) (Green et al., 1982; Nims et al., 1995). Detection limits for nitrite, nitrate and ammonium were 2 μM, 2.5μM and 10 μM, respectively.

4.2.5 Nucleic acid extraction, purification and quantification

Total DNA was extracted from 5 mL homogenized sediment slurry (approximately 0.5 g) using the Fast-DNA spin kit for soil (MP Biomedicals, Solon, OH). Total RNA was extracted from 5 mL homogenized sediment slurry using a modified phenol-chloroform method with CTAB extraction buffer (Smith et al., 2010; Griffiths et al., 2000). Extracted RNA was digested with *DNase* to remove genomic DNA using Ambion's TURBO DNA-free™ kit (Ambion, Austin, TX), and then purified with Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacture's protocol. DNA and RNA concentrations were quantified with PicoGreen and RiboGreen dye (Invitrogen Corp., Carlsbad, CA), respectively, on a Nanodrop fluorometer (Thermo Scientific, Wilmington, DE).

4.2.6 Quantitative PCR

Archaeal and bacterial *amoA* genes were amplified from sediment slurry total DNA using 353F/487R and amoAF1/amoAR2 primer sets, respectively. qPCR was conducted in a MyiQ real-time qPCR detection system (Bio-Rad Laboratories, Inc, Hercules, CA) using the protocol described in chapter 3. PCR threshold value and the amplification efficiency of individual wells (82.4% to 98.6%) were calculated using the LinReg PCR program (Ramakers et al., 2003). Inhibition was tested for by serial dilution of templates, and was not observed. Archaeal and bacterial *amoA* gene standards were generated with linearized plasmids extracted from sequenced sediment clones. The linear range for amplified standards was from 10^6 to 10^1 template copies with R^2 values of the standard curves higher than 0.9 for all assays.

4.2.7 qRT-PCR

Total purified RNA was converted to cDNA with oligo dT primers using the SuperScript III First-Strand Synthesis Supermix for qRT-PCR, according to the manufacturer's protocol (Invitrogen Corp., Carlsbad, CA). cDNA was amplified using the same qPCR protocol for DNA.

4.2.8 Stable Isotope Probing

DNA- Stable Isotope Probing (DNA-SIP) was conducted on the sediment incubations with 50 µM ^{13}C- labeled pyruvate. SIP was performed with the addition of ^{13}C carrier DNA enriched from *Halobacterium salinarum* (strain *ATCC* 29341/DSM 671/R1), using the protocol previously described by Gallagher et al (Gallagher et al., 2005). The *H. salinarum* strain used for enhanced visualization of ^{13}C-DNA was grown in an enriched medium containing ^{13}C-labeled ISOGRO powder growth medium (Isotec, Miamisburg,

OH) and Van Niel's medium amended with 25% NaCl. The culture was incubated aerobically at 25°C for approximately three weeks before cells were harvested.

After DNA extraction, approximately 300 ng of sediment slurry DNA and 300 ng of ^{13}C carrier DNA were added to 500-µl filter sterilized CsCl (1 g/ml) in TE buffer (pH=8) containing 20 µg ethidium bromide. After balancing the tubes, ^{12}C and ^{13}C fractions were separated by ultracentrifugation on a Beckman Coulter ultracentrifuge (Palo Alto, CA) at 72k rpm for 24 hours. The separated bands were visualized under UV light, and ^{12}C-DNA and ^{13}C-DNA fractions were withdrawn sequentially into different tubes using 20 µl pipette tips. The recovered DNA was suspended in 200 µl of 10 mM Tris-HCl buffer with 0.5 mM EDTA, and purified using Amicon Ultra centrifugal filter units (Millipore Corp., Germany) according to the manufacturer's protocol. Archaeal and bacterial *amoA* genes were quantified with 353F/487R and amoAF1/amoAR2 primer set, respectively, from DNA recovered from light and heavy fractions using qPCR described above.

4.2.9 Statistical analysis

All data analysis was performed using Igor Pro 6 software (Wavemetrics, Lake Oswego, OR).

4.3 Results

4.3.1 Potential nitrification activity in different media

Freshwater sediment slurries were incubated aerobically in either filtered site water or synthetic freshwater medium amended with 250 µM NH_4Cl. In the incubations with site water, ammonium was rapidly converted to nitrite and nitrate in a near-stoichiometric fashion in approximately seven days (Figure 4.1). Calculated potential nitrification rate was 9.4 ± 1.3 µg N $d^{-1} g^{-1}$ sediment, which was within the range reported in other

freshwater sediments (Henriksen et al., 1981; Dodds and Jones, 1987). Ammonia oxidation was accompanied by the significant increases ($p<0.1$) of both archaeal and bacterial *amoA* gene abundance (Table 4.1), with bacteria having a higher fold-increase than archaea (5.0 vs. 1.9).

In the incubations with synthetic freshwater medium at pH 7.0, nitrification occurred as well, but was slower and incomplete by the end of the 1-week incubation (Figure 4.1). The fold increase of bacterial *amoA* gene abundance in the synthetic freshwater medium was half of what it had been in the site water (2.5 vs. 5), and the archaeal *amoA* gene abundance did not increase appreciably (0.5 fold) (Table 4.1). By adjusting the pH of synthetic freshwater medium to 7.9 (pH of site water), similar nitrate production was achieved (Figure 4.1). However, the archaeal and bacterial *amoA* gene copy numbers were still lower than those in the incubations with site water. Quantification of *amoA* genes suggested that both groups of ammonia oxidizers grew better in the filtered site water, indicating that site water might provide an essential growth requirement(s), or some components in synthetic freshwater medium inhibited growth.

4.3.2 Urea utilization

Sediment slurries were amended with urea as a replacement for inorganic ammonium. In 7-day incubations with urea amendment, nitrite and nitrate concentrations increased to the same level as the controls with ammonium chloride (Figure 4.2), although the rate of increase was significantly lower than that in control (5.8 ± 0.4 μg N $d^{-1}g^{-1}$ compared to 9.9 ± 1.8 μg N $d^{-1}g^{-1}$). Ammonium was not detected in the incubations with urea (data not shown). Nitrate accumulation was likely resulted from nitrification using urea rather than oxidation of remineralized organic nitrogen, because in other experiments,

remineralization did not occur until the second to third week of incubations. Both AOA and β-AOB *amoA* gene copies increased in the incubations with urea. β-AOB had higher fold increase than AOA. In addition, the fold increases in both AOA and β-AOB were slightly lower than in corresponding controls with ammonium chloride (Table 4.2), although the ratio of AOA to β-AOB fold-increase was similar between the two treatments. These results suggested that urea could be utilized but might not be preferred by ammonia oxidizers in CR sediments.

4.3.3 Inhibition studies with ATU

It has been reported that AOA and AOB have different sensitivity to the ammonia oxidation inhibitor ATU (Taylor et al., 2010; Santoro and Casciotti, 2011; Mosier et al., 2012c). At the concentration of 86 μM, ATU was shown to completely inhibit AOB, but only partially inhibit AOA (Ginestet et al., 1998; Taylor et al., 2010; Santoro and Casciotti, 2011; Mosier et al., 2012c). To differentiate the nitrification activities of AOA and β-AOB, and to examine the inhibitory effects of ATU on both, ATU (0, 1, 25 and 86 μM) was added to the incubations, and nitrification activity coupled growth of ammonium oxidizers was monitored. With the addition of ATU, nitrification activity was inhibited, indicated by longer "lag phase" with the low or no production of nitrite and nitrate, compared to the control (Figure 4.3A). The length of the lag phase increased with increasing ATU concentration. Nevertheless, the inhibitory effects appeared to be gradually alleviated with time, and nitrification activity was fully recovered after 2 weeks in 1 and 25 μM ATU incubations (Figure 4.3A). In incubation with 86 μM ATU, the inhibition was so substantial that only a slight increase of nitrate and nitrite was observed

after 2 weeks (Figure 4.3), but the increasing trend appeared to indicate the onset of nitrification.

The inhibition of nitrification activity was also accompanied by the suppressed growth of β-AOB (Table 4.3B). Similar to nitrification activity, the extent of inhibitory effects on β-AOB growth also positively corresponded to the concentrations of ATU. At day 7, the fold change of β-AOB abundance relative to day 0 was 3.2 in incubation with 1 μM ATU, compared to 4.8 in control. In the presence of 25 and 86 μM ATU, the number further decreased to 0.9 and 0.5, respectively (Figure 4.3). The fold change of β-AOB abundance recovered to 5.4 at day 14 in the incubation with 25 μM ATU, but remained low at 0.8 in the incubation with 86 μM ATU. On the other hand, the inhibitory effects of ATU on AOA growth appeared to be less substantial, because AOA *amoA* gene abundance only slightly decreased at day 7 compared to the control and there was no increasing inhibitory effect observed at higher ATU concentrations. (Table 4.3A).

Quantification of bacterial *amoA* gene transcripts showed similar trends as gene abundance (Table 4.4). In 7-day incubations, β-AOB *amoA* expression increased in incubations with 1 μM ATU and in controls, but was inhibited by 25 and 86 μM ATU (Table 4.4). It is interesting to note that archaeal *amoA* gene expression was enhanced with the addition of ATU, although no AOA growth was observed in these sediment incubations (Table 4.3A & 4.4), suggesting that these archaea might be actively expressing *amoA* genes but not actively growing.

4.3.4 Effects of pyruvate amendment

Sediment slurries were amended with pyruvate with/without inhibitor to investigate the effects of organic carbon on potential nitrification activity and on the growth of AOA

and AOB. Pyruvate was selected because it is an important intermediate in organic carbon metabolism, and was shown to stimulate the growth of the soil isolate "*N. viennensis*" (Tourna et al.,2011). In the absence of ATU, the nitrification rate of the incubation with 50 μM pyruvate was not significantly different from the control, but the onset of nitrification was approximately one day earlier with the addition of pyruvate (Figure 4.4). At the same time, the pyruvate amendment slightly stimulated the growth of both AOA and AOB, compared to the control (Table 4.5). In the presence of 86 μM ATU—the concentration shown previously to completely inhibit AOB in the sediment slurries— nitrification activity was almost completely inhibited, and was accompanied by inhibition of the growth of AOB as well. AOA growth was not significantly affected by 500 μM pyruvate addition in the presence of inhibitor, but was slightly enhanced by 50 μM pyruvate amendment. It was noted repeatedly that there was small amount of nitrate accumulated towards the end of two-week incubations regardless of pyruvate (Figure 4.4), suggesting that the inhibitory effects of ATU at higher concentration might be alleviated in the longer incubations.

4.3.5 Inhibition and recovery of nitrification activity over time

To fully capture the inhibition and recovery process of nitrification over time, the same experiment was repeated for three weeks with 86 μM ATU. To completely inhibit β-AOB, sediment slurries were also amended with both antibiotic and 86 μM ATU, as a comparison to ATU alone. In the presence of 86 μM ATU, the onset of nitrification activity in sediment incubations was observed at day 16, and the production of nitrate was comparable to that in the controls without ATU after approximately three weeks. Concurrent with the recovery of nitrification activity, the growth of β-AOB was also

observed, again with a fold increase of *amo*A abundance similar to that of the controls (Table 4.6). In the incubations with antibiotics and ATU, neither nitrification activity nor growth of β-AOB was observed over the course of the three-week period. Interestingly, 86 μM ATU appeared to have a stimulatory effect on AOA growth in the longer incubations. After three weeks, AOA abundance increased 2.4-fold in the controls, whereas higher increases (4.2- and 5.2-fold) were observed in the incubations with 86 μM ATU (Table 4.6). Together these results suggest that the β-AOB growth corresponded to the nitrification activity. The role of AOA in nitrification was unclear, however, since their growth occurred much more slowly than for β-AOB and did not directly correspond to the potential nitrification activity measured in sediment incubations.

4.3.6 Nitrification of exogenously added ammonium *vs.* remineralized nitrogen

Freshwater sediments were incubated in the site water in the presence or absence of exogenously added ammonium chloride, for the purpose of assessing nitrification activity of added inorganic ammonium and remineralized nitrogen. In the absence of added NH_4Cl, oxidation of remineralized ammonia in the sediment incubations was not observed until after 8 days (Figure 4.6A). By 4 weeks, nitrate production was up to about 0.6 mM (Figure 4.6B). The accumulation of ammonium from remineralization, however, was not detected (data not shown), most likely because the released ammonium was immediately consumed by nitrifiers. The oxidation of ammonium released from remineralization occurred in incubations with added exogenous ammonium as well, but was delayed until at least three-weeks of incubation (Figure 4.6B). Quantification of archaeal and bacterial *amoA* genes indicated that β-AOB growth was stimulated by

oxidation of remineralized nitrogen. In incubations with added ammonium, β-AOB abundance increased by 5-fold in 7 days (Table 4.7), probably due to the oxidation of ammonium chloride; in incubations without added ammonium, β-AOB abundance did not rise in 7 days (0.9-fold), but increased by 9-fold (Table 4.7B) after two weeks of incubation, when nitrate began to accumulate (Figure 4.6B). Meanwhile, AOA abundance increased more slowly under both conditions, and the remineralization process did not appear to significantly stimulate their growth in sediment incubations (Table 4.7A).

4.3.7 DNA-SIP

To assess whether AOA or/and AOB could incorporate organic carbon, 50 μM labeled ^{13}C-pyruvated was added to the sediment incubations, and compared with the controls. Archaeal and bacterial *amoA* gene abundance were both measured in the light and heavy fractions. Quantification of *amoA* genes in the heavy fractions showed that the incorporation of labeled pyruvate was too low to be detected (data not shown). The result is not necessarily indicating that AOA or/and β-AOB did not assimilate organic carbon. The failure to detect labeled pyruvate might be because that the added concentration was too low to be detected. Meanwhile, there might be other heterotrophs competing with nitrifiers for pyruvate. Another possible explanation is that AOA might preferentially assimilate other organic compounds that were not tested in this study.

4.4 Discussion

4.4.1 Inhibitory effects of ATU

Unlike the case for AOB, little is known about the mechanism of ATU inhibition on AOA. Several studies have examined the effects of ATU on thaumarchaeal strains

incubated in pure and enrichment cultures (Hatzenpichler et al., 2008; Jung et al., 2011; Santoro and Casciotti, 2011; Kim et al., 2012; Mosier et al., 2012c; T. Shen et al., 2013; Lehtovirta-Morley et al., 2013). The findings suggest that various strains have different sensitivities to ATU, but all are generally less susceptible compared to AOB. For example, the moderately thermophilic "*Ca.* Nitrososphaera gargensis" and the low salinity strain "*Ca.* Nitrosoarchaeum limnia" SFB1 were only partially inhibited by ATU at the concentration (~ 100 μM) known to completely inhibit AOB growth (Hatzenpichler et al., 2008; Mosier et al., 2012c). As for soil enrichment strain "*Ca.* Nitrosoarchaeum koreensis" MY1 and JG1, higher tolerance of ATU (up to 500 μM) was reported (Jung et al., 2011; Kim et al., 2012). Recently, the inhibitory effect of ATU was investigated over a range of concentrations (from 0 to 500 μM) on the soil isolate *N. viennensis*, and results also suggested that this strain was less susceptible to ATU than typical AOB (T. Shen et al., 2013). The half maximal effective concentration of ATU to *N. viennensis* was 193.3 ±16.0 μM (Shen et al., 2013). For comparison, the effective concentrations of ATU on various AOB strains were much lower (1- 10 μM) (Ginestet et al., 1998; Hooper and Terry, 1973; Bédard and Knowles, 1989).

In my freshwater sediment incubations, ammonia oxidation activity accompanied by the growth of β-AOB was inhibited by 86 μM ATU in two-week incubation, while AOA growth was less affected by ATU, supporting the idea that AOB were more sensitive to ATU inhibition. However, in longer incubations, the inhibition on β-AOB growth was eventually alleviated, and nitrification activity was restored. The inhibitory mechanism of ATU on AOB is postulated to involve chelation of the copper at the active site of AMO, possibly entailing a reversible reaction of CS_2 with a nucleophilic amino acid near the

active site copper on AMO (Hooper and Terry, 1973; Hyman et al., 1990). Therefore, it is reasonable to hypothesize that the recovery of activity and growth over time in the presence of ATU was due to this reversible binding to AMO, or, alternatively, to the breakdown of ATU. In addition, it is also possible that some AOB strains in freshwater sediments have higher tolerance to ATU, which has not been discovered yet. Furthermore, a recent study of AOA enrichment from acid soils reported contrasting inhibitory effects of ATU on "*Ca.* Nitrosotalea devanaterra" in liquid culture and soil microcosms. ATU of 50-100 μM significantly reduced the growth of "*Ca.* Nitrosotalea devanaterra" in liquid culture, but did not affect the thaumarchaeal growth in soil microcosms (Lehtovirta-Morley et al., 2013). The lack of inhibition by ATU on AOA in some soils was explained by the protection by the soil matrix, a lower diffusion rate and possible degradation of the inhibitor in soils (Lehtovirta-Morley et al.,2013; Taylor et al., 2010). Therefore, nitrification inhibitors are likely to behave very differently in pure culture and in complex environments, especially in soils and sediments in which the environmental matrix are complex and heterogeneous. Additionally, when applying nitrification inhibitors to incubation studies, it is necessary to monitor the growth coupled nitrification activity in long-term incubations, in case that the inhibitory effects are alleviated over time .

4.4.2 Potential ammonia oxidation activity by AOA and β-AOB from freshwater sediments

Strong evidence from this study indicated that the dominant ammonia oxidizers in my sediment slurry incubations were β-AOB, rather than AOA. First, in all incubations without ATU addition, the growth rate of AOA was significantly slower than β-AOB, despite higher AOA abundance at day 0. In addition, strong concurrency between the

restoration of nitrification activity and β-AOB growth was observed in all incubations with various concentrations of ATU. Although AOA abundance appeared to be even slightly enhanced by ATU by day 14—likely due to inhibition on β-AOB—their growth did not result in appreciable ammonium oxidation. It is also interesting to note that the AOA *amo*A expression was significantly enhanced by ATU to the level that was even higher than that of β-AOB during active nitrification, but again the enhancement did not translate to substantial ammonium oxidation. It almost appears to be that AOA growth was inert to all the variables tested here. It is possible that the incubation conditions within the time frame tested were not optimal for identifying nitrification activity by AOA because β-AOB appeared to grow much faster than AOA to cause majority of nitrification activity. In fact, AOA might be able to use trace amount of ammonium to support their slower growth, given their high affinity for the substrate (Hatzenpichler et al., 2008; Martens-Habbena et al., 2009; Kim et al., 2012). Incubations that last longer than 3 weeks might allow AOA to grow to a significant level to perform detectable nitrification. But at the same time, β-AOB growth must be inhibited for the period of incubation, which did not occur in my sediment incubations even with 86 μM ATU.

My finding that β-AOB dominated the measured potential nitrification activity in laboratory incubations, along with their relatively higher growth rate compared to AOA, was consistent with the results from other recent studies involving freshwater sediment incubations. A study of freshwater lake sediment microcosms incubated with 1 mM ammonium showed that the dominant autotrophic ammonia oxidizers were AOB, and significant autotrophic growth of AOA occurred only at warmer temperature (37 °C), as assessed by DNA-SIP (Wu et al., 2013). In another freshwater sediment enrichment

culture, AOA almost always grew more slowly than AOB (French et al., 2012). However, the differences between laboratory incubation studies and in situ conditions should be noted. In our sediment incubations, the amount of ammonium supplied to the incubations was relatively high compared to natural conditions. Therefore, the potential nitrification activity measured in the sediment incubations cannot be directly related to nitrification activities of AOA and β-AOB in the environment. Recall the results that were shown in chapter 3. In the CR freshwater sediments, higher AOA genes and gene transcripts, compared to β-AOB, were observed in most depths of the sediment and across different seasons, which was the opposite of what was shown in the incubations. Compared to those results, the AOA abundance in sediment incubations was still around the level similar to that in natural environment, but β-AOB grew 5 to 10 times higher in incubations without inhibitors. It is also interesting to note that during long incubations the β-AOB abundance usually dropped significantly when added ammonium was completely consumed (Table 4.6B). These observations together suggested that it is possible that AOA in natural sediments are more resistant to changes in substrate availability, and compete with AOB by incorporating trace levels of substrate below the threshold accessible to β-AOB. Alternatively, AOB may be more susceptible to environmental changes, indicated by their rapid increases and decreases in abundance with different amendments, resulting in less overall abundance in natural sediments.

Furthermore, in natural freshwater sediments, AOA and AOB often coexist, and both maintain a relatively high abundance (Z. Liu et al., 2011; French et al., 2012; Wu et al., 2013). The reported AOA: AOB ratio is not as high as that in oligotrophic environments, such as acid soils and the open oceans. This might be because the ammonium

concentration is higher in rivers and lakes (Z. Liu et al., 2011; FH Liu et al., 2009; French et al., 2012; Wu et al., 2013), compared to those nitrogen-limiting environments (Gubry-Rangin et al., 2011; Wuchter et al., 2006), which favors AOB growth. As a result, in the freshwater sediments with relatively high ammonium, the archaeal role in nitrification might not be as significant as that in the oceans. To elucidate the overall contribution of AOA to nitrification in freshwater sediments, measuring in situ activity will be informative.

4.4.3 Metabolic strategies of ammonia oxidizers in freshwater sediments

The inconsistency between AOB dominating potential nitrification activity in sediment incubations and the more abundant AOA in situ might be explained by another tempting hypothesis that some *Thaumarchaeota* may not drive energy from autotrophic ammonia oxidation, but utilize alternative metabolic strategy, e.g., heterotrophic or mixotrophic metabolism. The incubations with urea or pyruvate were supposed to address this question, but the results did not appear to support this idea. Despite slight stimulation of AOA growth, no preferential growth of AOA over AOB in the presence of either urea or pyruvate was observed, and AOB still dominated potential nitrification activity. This might be because, as discussed earlier, the incubation conditions were not optimal for AOA. Another possible reason is because of the complexity of the microbial consortium in the sediments, which might compete for organic substrates with AOA.

Although the incubations with organic nitrogen or carbon did not show direct evidence of heterotrophic or mixotrophic metabolisms in AOA, this hypothesis is still very plausible based on previous literature findings, and is potentially important in shaping the ecological niches of AOA and AOB in natural environment. Heterotrophy

and/or mixotrophy in some *Thaumarchaeota* has been suggested by genetic investigations and metabolic characterizations (Ouverney and Fuhrman, 2000; Herndl et al., 2005; Teira et al., 2006; Tourna et al., 2011; Alonso-Sáez et al., 2012). Genes predicted to encode the oxidative tricarboxylic acid cycle, as well as a number of transporters for amino acids, urea and cyanate were identified in some thaumarchaeal genomes (Hallam et al., 2006b; Walker et al., 2010; Blainey et al., 2011; Spang et al., 2012), indicating their genetic potential for assimilating small organic compounds. Uptake of amino acid and other organic carbon compounds by marine planktonic archaea has been observed in several studies using microautoradiography, and from measuring the natural distribution of radiocarbon in archaeal membrane lipids (Ouverney and Fuhrman, 2000; Herndl et al., 2005; Ingalls et al., 2006; Agogué et al., 2008).

Furthermore, it has been reported that the soil isolate *N. viennensis* required the addition of pyruvate to achieve a high growth rate, and was able to use urea in place of NH_4Cl as the sole energy source (Tourna et al., 2011). In an acid soil, nitrification activity and AOA growth was not affected by inorganic nitrogen amendment, but was stimulated by the addition of organic nitrogen (Levičnik-Höfferle et al., 2012). Their findings also suggested that some *Thaumarchaeota* preferred to oxidize ammonia generated from remineralization of organic matter, which could be explained by their growth requirements of organic nitrogen and carbon, indicative of heterotrophic/mixotrophic metabolisms, or by their potential physical association and functional interactions with other heterotrophs in soil. In addition, a study of *Thaumarchaeota* in wastewater treatment plants (WWTPs) suggested that some *amoA*-containing archaea might not be autotrophic ammonia oxidizers, because the energy

generated from autotrophic ammonia oxidation alone could not theoretically sustain the extremely high abundance of *Thaumarchaeota* observed (Muβmann et al., 2011). It is interesting to note that the highly abundant archaea measured in WWTPs belonged to the group 1.1b (Muβmann et al., 2011), whose abundance was often not linked to autotrophic nitrification activity, indicating that some group 1.1b archaea might employ heterotrophic and/or mixotrophic metabolisms. Although the assimilation of organic substrate using labeled organic carbon was not detected in these studies, it is possible that the right organic substrates had not been tested (Tourna et al., 2011; Muβmann et al., 2011). Recently, our study of a plant-root enrichment suggested that some *Thaumarchaeota* might be heterotrophic nitrifiers, similar to those heterotrophic species of bacteria that are capable of oxidizing a variety of nitrogen compounds (Robertson and Kuenen, 1990; Xu et al., 2012). They do not appear to derive energy from ammonia oxidation, but use this reaction for the dissipation of excessive reductant (Robertson and Kuenen, 1990; Wood, 1990).

In summary, our data show that the AOB were the dominant ammonia oxidizers in freshwater sediment incubations, with the amendment of ammonium chloride, urea or in the absence of exogenously added nitrogen. Interestingly, AOB appeared to have higher growth rate than AOA in the sediment incubations, while AOA often outnumbered AOB in the natural freshwater sediments, suggesting that AOA may have different metabolic strategies to compete with AOB, possibly by heterotrophic or mixotrophic metabolisms. Therefore, in future studies, it will be necessary to measure in situ nitrification activity to fully assess the relative contribution of AOA to nitrification in freshwater sediments.

4.5 Tables and Figures

Table 4.1 Change in archaeal and bacterial *amoA* gene copy number in sediment incubations with filtered site water (SW) or synthetic freshwater (FW) medium[a].

Culture condition	Day 0		Day 7		Fold change[b]	
	Archaeal *amoA*	Bacterial *amoA*	Archaeal *amoA*	Bacterial *amoA*	Archaeal *amoA*	Bacterial *amoA*
FW medium (pH=7)	1.6×10^6 (1.3×10^4)	1.1×10^6 (4.9×10^4)	7.9×10^5 (3.0×10^5)	2.6×10^6 (2.8×10^5)	0.5	2.4
FW medium (pH=7.9)	1.7×10^6 (4.0×10^5)	1.1×10^6 (4.9×10^4)	1.3×10^6 (8.1×10^4)	3.1×10^6 (2.2×10^5)	0.7	2.8
Filtered SW	1.4×10^6 (3.4×10^4)	1.1×10^6 (3.5×10^5)	2.7×10^6 (3.1×10^5)	5.5×10^6 (1.9×10^5)	1.9	5.0

[a] Means and standard deviations (in parenthesis) per gram are shown; values were estimated from duplicate incubations (tested in replicate PCRs).
[b] That is, the fold change in abundance over 7 days of incubation.

Table 4.2 Change in archaeal and bacterial *amoA* gene copy number in sediment incubations with urea or ammonium chloride [a].

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Culture condition	Day 0		Day 7		Fold change[b]	
	Archaeal *amoA*	Bacterial *amoA*	Archaeal *amoA*	Bacterial *amoA*	Archaeal *amoA*	Bacterial *amoA*
Control	4.6×10^5 (1.4×10^5)	5.8×10^5 (1.1×10^5)	7.2×10^5 (3.4×10^5)	2.2×10^6 (8.2×10^5)	1.6	3.8
Urea	6.1×10^5 (1.1×10^5)	5.6×10^5 (5.0×10^4)	7.7×10^5 (2.1×10^5)	1.9×10^6 (2.8×10^5)	1.3	3.4

[a] Means and standard deviations (in parenthesis) per gram are shown; values were estimated from duplicate incubations (tested in replicate PCRs).
[b] That is, the fold change in abundance over 7 days of incubation.

Table 4.3 Change in archaeal (A) and bacterial (B) *amoA* gene copy number in sediment incubations with 0, 1, 25 or 86 μM ATU[a].

A

Culture condition	Archaeal *amoA* gene abundance			Fold change[b]	
	Day 0	Day 7	Day 14	D7/D0	D14/D0
Control	1.6×10^6 (1.8×10^5)	1.9×10^6 (4.2×10^5)	2.0×10^6 (3.0×10^5)	1.2	1.3
1 μM ATU	1.6×10^6 (3.0×10^5)	1.5×10^6 (4.8×10^5)	1.7×10^6 (3.3×10^5)	0.9	1.1
25 μM ATU	1.9×10^6 (4.0×10^5)	1.6×10^6 (4.4×10^5)	2.7×10^6 (5.0×10^5)	0.9	1.5
86 μM ATU	1.7×10^6 (4.8×10^5)	1.5×10^6 (4.2×10^5)	2.3×10^6 (4.6×10^5)	0.9	1.4

B

Culture condition	Bacterial *amoA* gene abundance			Fold Change[b]	
	Day 0	Day 7	Day 14	D7/D0	D14/D0
Control	1.1×10^6 (1.6×10^5)	5.3×10^6 (1.2×10^5)	4.4×10^6 (1.5×10^6)	4.8	4.0
1 μM ATU	1.5×10^6 (1.4×10^5)	4.7×10^6 (6.4×10^5)	3.8×10^6 (9.0×10^5)	3.2	2.6
25 μM ATU	1.3×10^6 (2.5×10^5)	1.2×10^6 (3.3×10^5)	6.9×10^6 (1.7×10^6)	0.9	5.4
86 μM ATU	1.2×10^6 (4.5×10^5)	5.7×10^5 (2.5×10^5)	9.2×10^5 (6.3×10^5)	0.5	0.8

[a] Means and standard deviations (in parenthesis) per gram are shown; values were estimated from duplicate incubations (tested in replicate PCRs).
[b] That is, the fold change in abundance over 7 days and 14 days of incubation.

Table 4.4 Change in archaeal and bacterial *amoA* gene transcript number in sediment incubations with 0, 1, 25 or 86 μM ATU[a].

Culture condition	Day 0		Day 7		Fold change[b]	
	Archaeal *amoA* transcript	Bacterial *amoA* transcript	Archaeal *amoA* transcript	Bacterial *amoA* transcript	Archaeal *amoA* transcript	Bacterial *amoA* transcript
Control	8.9×10^4 (1.1×10^3)	5.1×10^4 (1.2×10^4)	1.6×10^5 (2.9×10^4)	1.4×10^5 (2.0×10^5)	1.8	2.8
1 μM ATU	6.5×10^4 (3.4×10^4)	3.3×10^4 (2.3×10^4)	2.0×10^5 (1.8×10^5)	1.2×10^5 (2.8×10^4)	3.1	3.7
25 μM ATU	4.2×10^4 (8.3×10^3)	4.2×10^4 (8.6×10^3)	2.8×10^5 (2.3×10^5)	3.6×10^4 (1.3×10^4)	6.7	0.9
86 μM ATU	7.4×10^4 (4.0×10^3)	4.1×10^4 (3.4×10^3)	2.8×10^5 (2.1×10^5)	1.0×10^4 (2.4×10^3)	3.8	0.2

[a] Means and standard deviations (in parenthesis) per gram are shown; values were estimated from duplicate incubations (tested in replicate PCRs).
[b] That is, the fold change in *amoA* expression over 7 days of incubation.

Table 4.5 Change in archaeal (A) and bacterial (B) *amoA* gene copy number in sediment incubations with pyruvate and ATU[a].

A

Culture condition	Archaeal *amoA* gene abundance			Fold change[b]	
	Day 0	Day 7	Day 14	D7/D0	D14/D0
Control	1.8×10^6 (2.8×10^5)	1.8×10^6 (1.9×10^5)	2.2×10^6 (1.8×10^5)	1	1.2
50 μM pyruvate	1.4×10^6 (1.5×10^5)	2.1×10^6 (1.5×10^5)	2.2×10^6 (3.5×10^5)	1.5	1.6
86 μM ATU	1.8×10^6 (2.5×10^5)	1.1×10^6 (2.8×10^5)	2.6×10^6 (6.1×10^5)	0.7	1.6
50 μM pyruvate + 86 μM ATU	1.3×10^6 (7.7×10^5)	2.0×10^6 (7.3×10^6)	2.2×10^6 (5.5×10^6)	1.6	1.8
500 μM pyruvate + 86 μM ATU	1.5×10^6 (1.9×10^5)	1.0×10^6 (3.7×10^5)	2.3×10^6 (4.3×10^5)	0.7	1.5

B

Culture condition	Bacterial *amoA* gene abundance[a]			Fold change[b]	
	Day 0	Day 7	Day 14	D7/D0	D14/D0
Control	1.3×10^6 (2.5×10^5)	6.7×10^6 (1.7×10^6)	6.9×10^6 (2.7×10^6)	5.0	4.2
50 μM pyruvate	1.3×10^6 (2.8×10^5)	7.3×10^6 (3.5×10^6)	7.0×10^6 (2.3×10^6)	5.8	5.6
86 μM ATU	1.6×10^6 (1.1×10^5)	4.8×10^5 (2.5×10^5)	7.0×10^6 (2.2×10^5)	0.3	0.4
50 μM pyruvate + 86 μM ATU	1.3×10^6 (8.6×10^5)	9.5×10^5 (3.0×10^5)	5.3×10^5 (1.7×10^5)	0.8	0.4
500 μM pyruvate + 86 μM ATU	1.5×10^6 (1.4×10^5)	1.9×10^6 (5.0×10^5)	1.3×10^6 (1.7×10^5)	1.3	0.9

[a] Means and standard deviations (in parenthesis) per gram are shown; values were estimated from duplicate incubations (tested in replicate PCRs).
[b] That is, the fold change in abundance over 7 days and 14 days of incubation.

Table 4.6 Change in archaeal (A) and bacterial (B) *amoA* gene abundance in sediment incubations with 86 μM ATU and/or antibiotics (100 μg/mL carbencillin and streptomycin)[a].

A

Culture condition	Archaeal *amoA* gene abundance				Fold change[b]		
	Day 0	Day 7	Day 14	Day 21	D7/D0	D14/D0	D21/D0
Control	3.0×10^6 (4.4×10^4)	3.2×10^6 (6.7×10^5)	5.1×10^6 (8.0×10^5)	7.2×10^6 (1.8×10^6)	1.1	1.7	2.4
86 μM ATU	2.8×10^6 (1.8×10^5)	4.1×10^6 (6.8×10^5)	7.3×10^6 (2.7×10^5)	1.2×10^7 (2.9×10^6)	1.4	2.5	4.2
86 μM ATU + antibiotics	2.9×10^6 (5.5×10^5)	4.1×10^6 (5.8×10^5)	5.6×10^6 (1.9×10^6)	1.5×10^7 (4.4×10^6)	1.4	1.9	5.2

B

Culture condition	Bacterial *amoA* gene abundance				Fold change[b]		
	Day 0	Day 7	Day 14	Day 21	D7/D0	D14/D0	D21/D0
Control	3.1×10^6 (7.5×10^5)	1.4×10^7 (5.0×10^6)	8.8×10^6 (3.7×10^5)	5.0×10^6 (1.6×10^6)	4.6	2.8	1.6
86 μM ATU	3.0×10^6 (6.1×10^5)	2.9×10^6 (1.7×10^5)	2.7×10^6 (2.0×10^5)	1.3×10^7 (2.1×10^5)	1.0	0.9	4.2
86 μM ATU + antibiotics	3.0×10^6 (6.2×10^5)	3.3×10^6 (1.2×10^6)	2.9×10^6 (1.2×10^6)	2.4×10^6 (8.5×10^5)	1.1	1	0.8

[a] Means and standard deviations (in parenthesis) per gram are shown; values were estimated from duplicate incubations (tested in replicate PCRs).
[b] That is, the fold change in abundance over 7 days, 14 days and 21 days of incubation.

Table 4.7 Change in archaeal (A) and bacterial (B) *amoA* gene abundance in sediment incubations with or without exogenously added ammonium[a].

A

Culture condition	Archaeal *amoA* gene abundance					Fold change[b]			
	Day 0	Day 7	Day 14	Day 21	Day 28	D7/D0	D14/D0	D21/D0	D28/D0
Control	1.8×10^6 (2.8×10^5)	1.8×10^6 (1.9×10^5)	2.2×10^6 (1.8×10^5)	4.1×10^6 (9.9×10^5)	6.4×10^6 (9.9×10^5)	1.0	1.2	2.3	3.6
No NH_4Cl	1.5×10^6 (2.0×10^5)	2.0×10^6 (4.1×10^5)	2.7×10^6 (1.3×10^6)	4.0×10^6 (1.3×10^6)	6.2×10^6 (4.8×10^5)	1.4	1.9	2.7	4.3

B

Culture condition	Bacterial *amoA* gene abundance					Fold change[b]			
	Day 0	Day 7	Day 14	Day 21	Day 28	D7/D0	D14/D0	D21/D0	D28/D0
Control	1.3×10^6 (2.5×10^5)	6.5×10^6 (1.7×10^6)	5.5×10^6 (2.7×10^6)	1.1×10^7 (1.4×10^6)	1.1×10^7 (2.6×10^6)	5.2	4.2	8.6	8.5
No NH_4Cl	1.0×10^6 (3.1×10^5)	9.0×10^5 (2.1×10^5)	9.0×10^6 (2.9×10^6)	1.1×10^7 (1.3×10^6)	1.2×10^7 (2.0×10^6)	0.9	9.0	11.0	12.0

[a] Means and standard deviations (in parenthesis) per gram are shown; values were estimated from duplicate incubations (tested in replicate PCRs).
[b] That is, the fold change in abundance over 7 days, 14 days, 21 days and 28 days of incubation.

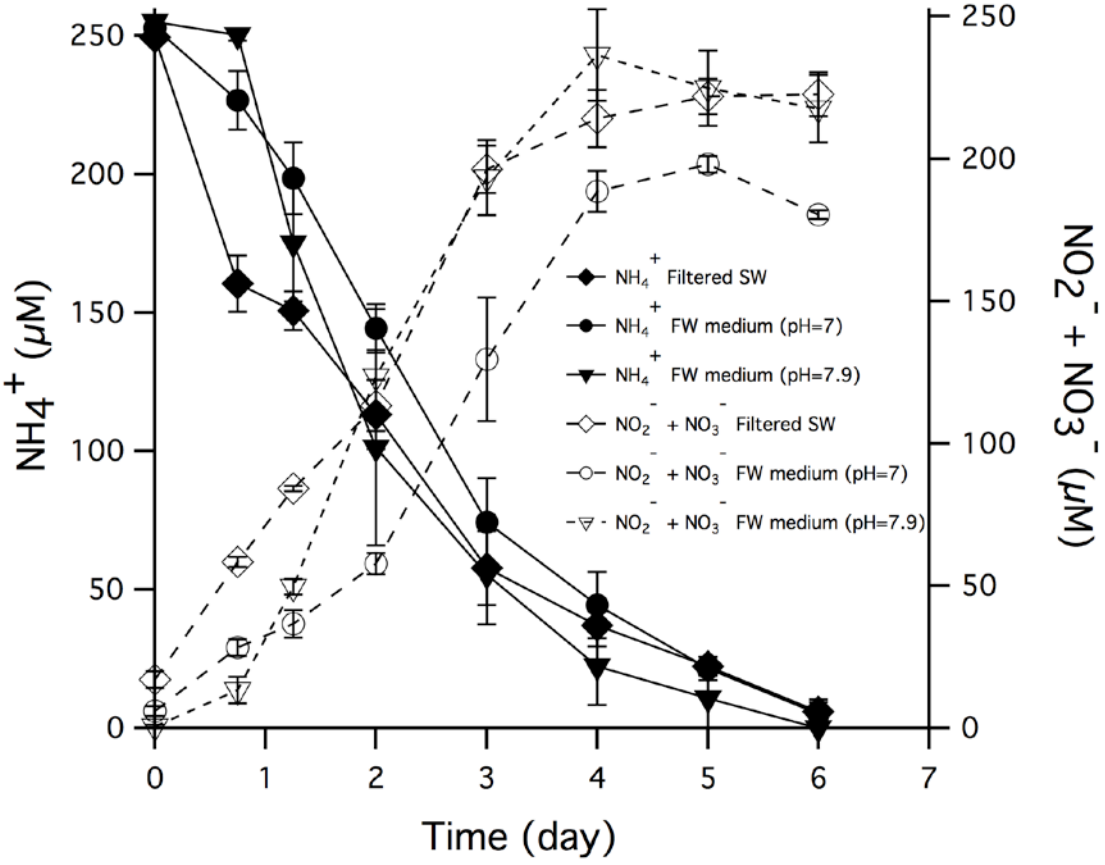


Figure 4.1 Change in ammonium, nitrite and nitrate concentrations in sediment slurry incubations with Filtered site water (SW) and Freshwater (FW) media. Error bars represent standard error from duplicate incubations.

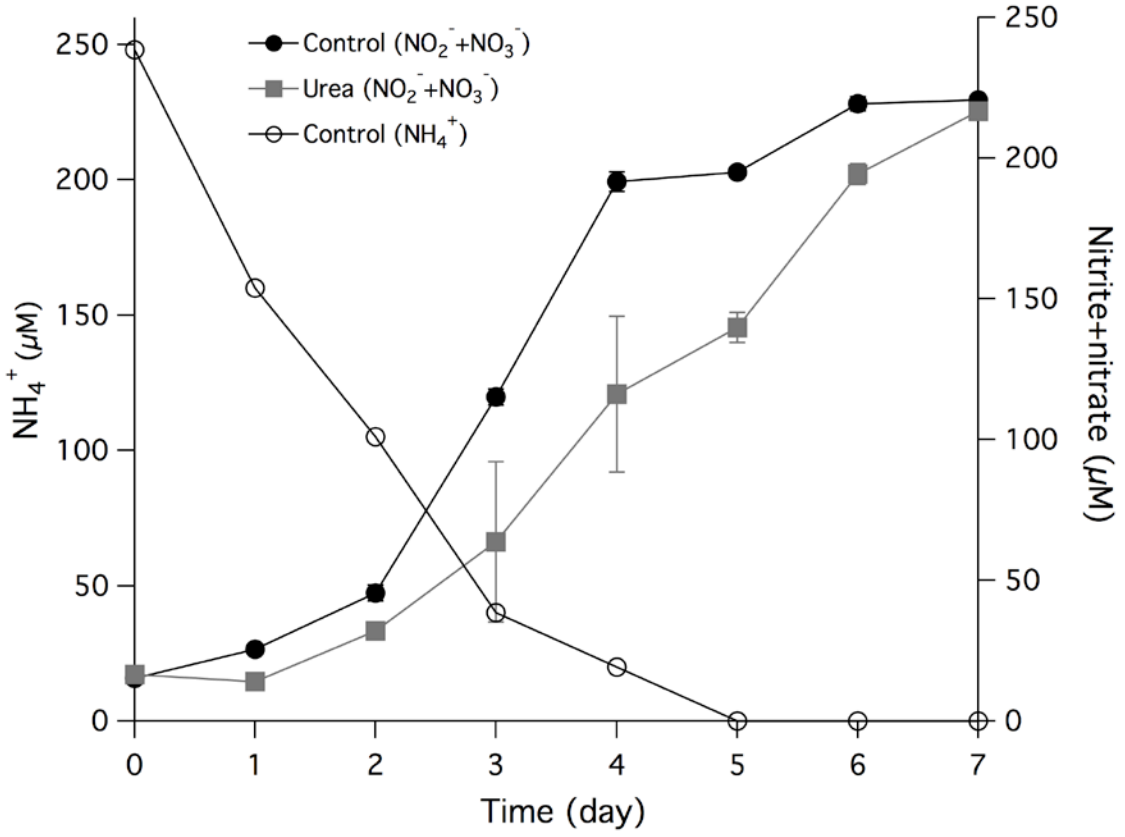


Figure 4.2 Change in ammonium, nitrite and nitrate concentrations in sediment slurry incubations with added ammonium chloride or urea. Note: ammonium concentration was not detectable in the incubations with urea. Error bars represent standard errors from duplicate incubations.

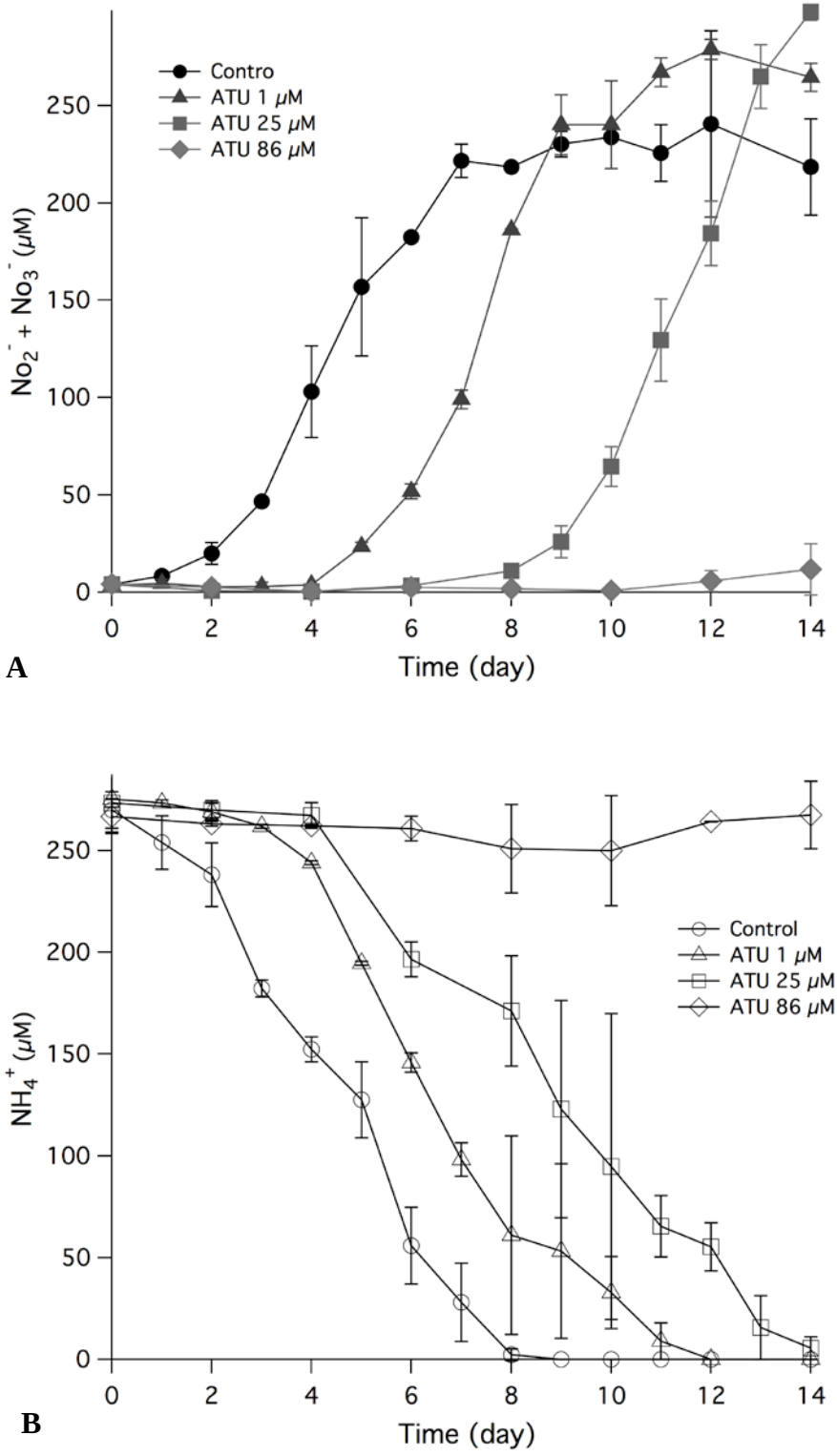


Figure 4.3 Nitrite and nitrate production (A) and ammonium utilization (B) in incubations containing 0, 1, 25 or 86 μM ATU. Error bars represent standard errors from duplicate incubations.

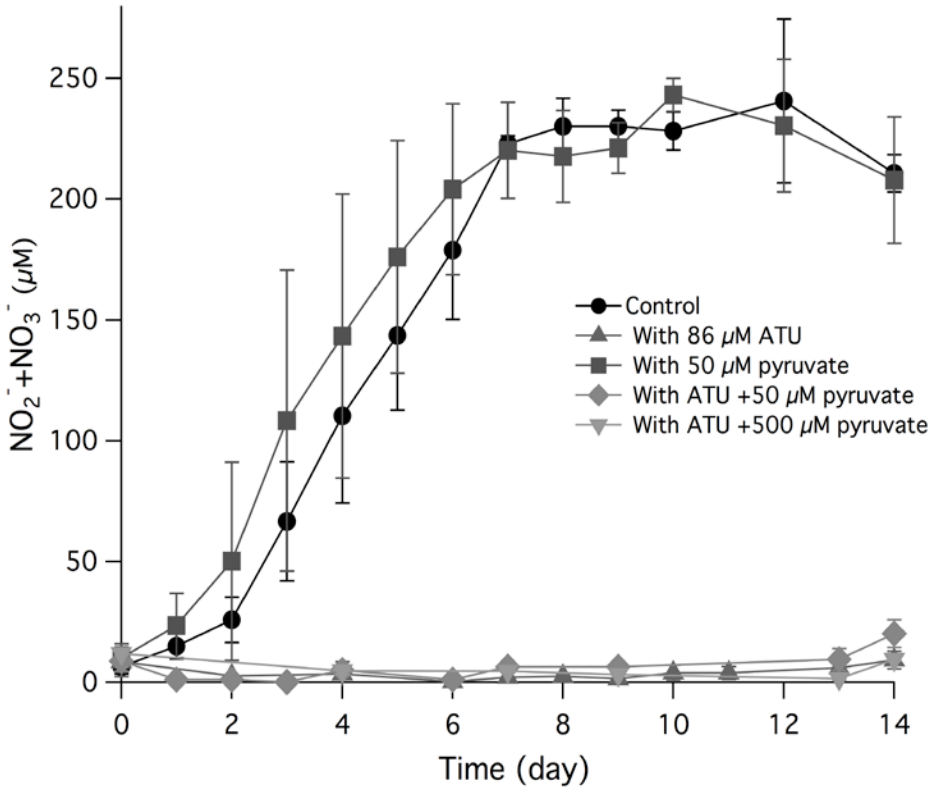


Figure 4.4 Production of nitrite and nitrate in incubations with added ammonia oxidation inhibitor or pyruvate. Error bars represent standard errors from duplicate incubations in two independent experiments.

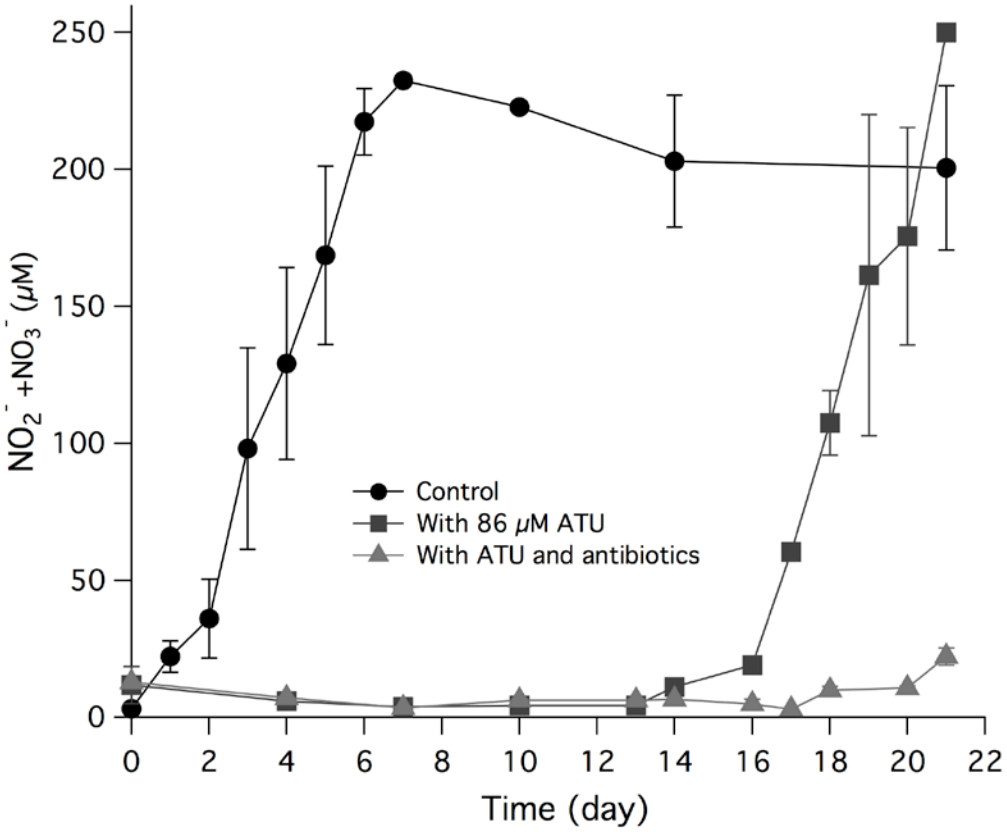
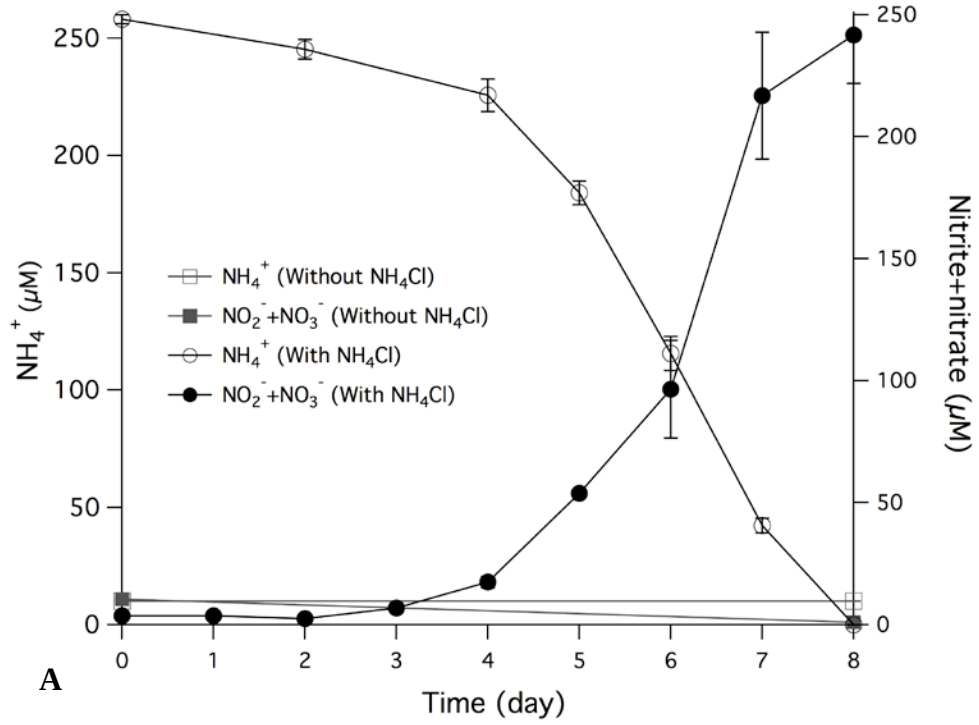


Figure 4.5 Production of nitrite and nitrate in sediment slurry incubations with the addition of the ammonia oxidation inhibitor, ATU, in the presence or absence of antibiotics (100 μg/mL carbencillin and streptomycin). Error bars represent standard errors from duplicate incubations.



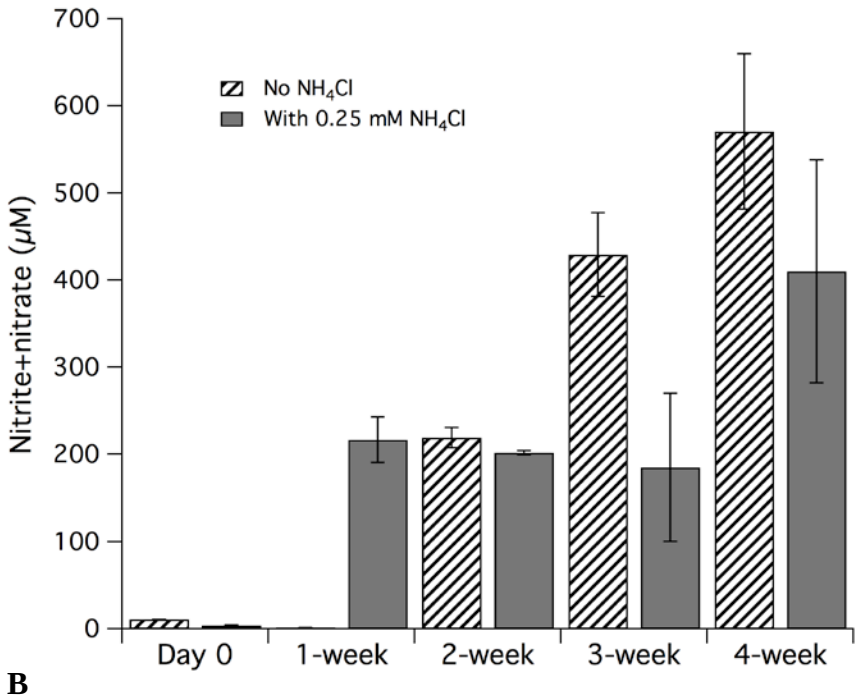


Figure 4.6 Change in ammonium, nitrite and nitrate concentrations measured in relatively short sediment slurry incubations with or without the addition of NH_4Cl. (A). The production of nitrite and nitrate in longer sediment slurry incubations (B). Error bars represent standard errors from duplicate incubations.

[1]CHAPTER 5

COMPARATIVE ANALYSIS OF 16S rRNA AND *amoA* GENES FROM ARCHAEA SELECTED WITH ORGANIC AND INORGANIC AMENDMENTS IN ENRICHMENT CULTURE[1]

5.1 Introduction

The realization that newly discovered and apparently ubiquitous members of the domain Archaea carry out the process of ammonia oxidation (Francis et al., 2007; Hallam et al., 2006a) is one of the most remarkable recent findings from research in environmental microbiology. The ammonia oxidation reaction is the first, and rate-limiting step in the two-step process of the microbial oxidation of ammonia to nitrate (nitrification). Beginning in 1992 with two reports on the recovery of archaeal 16S ribosomal RNA (rRNA) gene sequences from marine plankton (DeLong, 1992; Fuhrman et al.,1992), numerous studies in conventional marine and terrestrial habitats established high abundance and incidence of a novel taxon of archaea, considered at the time to place within the division Crenarchaeota. This, in itself, was a remarkable finding because the division was comprised exclusively of thermophilic and hyperthermophilic archaea. Just as unexpected was the recovery from temperate, oxygenated environments, more than a decade later, of genes encoding the putative archaeal ammonia monooxygenase enzyme that were linked to 16S rRNA genes of these mesophilic Crenarchaeota (Venter et al., 2004; Treusch et al., 2005). Previously, aerobic ammonia oxidation was known for only a

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few genera of bacteria from the *βeta*- and *Gammaproteobacteria* lineages (Purkhold et al., 2000).

There have been several reports of enrichments of mesophilic Crenarchaeota and AOA (Simon et al., 2005; Hallam et al., 2006a; Wuchter et al., 2006; la Torre et al., 2008; Hatzenpichler et al., 2008; Blainey et al., 2011), and a large number of surveys have shown widespread distribution and high copy numbers of the gene (*amoA*) putatively encoding the active subunit of the archaeal ammonia monooxygenase enzyme (Francis et al., 2005; Leininger et al., 2006; Wuchter et al., 2006; Mincer et al., 2007). In fact, AOA *amoA* gene copies were found to outnumber those of bacterial ammonia oxidizers (AOB) in many marine and soil habitats (Francis et al., 2007; Leininger et al., 2006). Two isolates (one marine, one soil) of ammonia oxidizing archaea (AOA) have been reported (Könneke et al., 2005; Tourna et al., 2011), with some notable differences in their growth requirements. While the marine "*Candidatus* Nitrosopumilus maritimus" strain SCM1 achieves 10^7 cells ml^{-1} growing autotrophically on bicarbonate and NH_4^+ (Könneke et al., 2005; Martens-Habbena et al., 2009), the soil "*Candidatus* Nitrososphaera viennensis" strain EN76 requires supplementation with organic carbon, i.e. pyruvate, to achieve continuous growth and reasonably high cell numbers (Tourna et al., 2011). Strain EN76 tolerates higher concentrations of NH_4^+ than SCM1, and can use urea in place of NH_4^+ as the sole energy source. Kinetic studies on strain SCM1 indicated that the organism has a very high specific affinity for its substrate and high specific oxidation rates at low ammonium concentrations found in open oceans (Martens-Habbena et al., 2011). Together with results from studies quantifying the uptake of inorganic carbon using ^{13}C (Wuchter et al., 2003) and ^{14}C (Herndl et al., 2005; Kirchman

et al., 2007) bicarbonate tracer experiments and the natural distribution of radiocarbon in archaeal membrane lipids (Ingalls et al., 2006), these results suggest autotrophic ammonia oxidation by marine archaea is substantial.

Studies in terrestrial ecosystems, however, have been less conclusive. Although archaeal *amoA* genes are ubiquitous in soils and, similar to marine habitats, frequently outnumber bacterial *amoA* genes (Leininger et al., 2006; Jia & Conrad, 2009; Di et al.,2009), relating abundance of AOA to ammonia oxidation activity has been challenging. From some studies, AOA appear to be active in natural samples, based on quantification of *amoA* gene expression in soil microcosms, (Treusch et al., 2005; Nicol et al., 2008), while in other studies AOA gene abundance did not increase with soil nitrification activity (Mertens et al., 2009; Di et al., 2009; Jia & Conrad, 2009; Ying et al., 2010). Furthermore, archaeal members of the clade referred to as Crenarchaeota group 1.1b or Group 1.1b (DeLong, 1998), also referred to as "soil lineage" are by far the most abundant and ubiquitous in soil (Ochsenreiter et al., 2003), but linking the actual growth of AOA to nitrification activity in soil has been reported (Tourna et al., 2008; Offre et al., 2009; Lehtovirta-Morley et al., 2011) only for group 1.1a archaea (DeLong, 1998), also referred to as the "marine lineage," occurring there at much lower abundance. There is also evidence suggesting that AOA may have a selective advantage at low ammonium concentrations, possibly exploiting mineralization as a major source of ammonium (Martens-Habbena et al., 2009; Stopnisek et al., 2010).

Our previous work (Simon et al., 2005) showed selection and growth of group 1.1b archaea in enrichment cultures using root extract as substrate, and others have shown that marine (Pérez et al., 2003; Teira et al., 2006; Ingalls et al., 2006; Kirchman et

al., 2007; Varela et al., 2008) and soil (Tourna et al., 2011) archaea can take up and utilize organic carbon or low amounts of CO_2 (Muβmann et al., 2011) independent of ammonium addition. These results suggest that the capacity for mixotrophic and/or heterotrophic metabolism provides a possible explanation for their abundance in soils worldwide (Bates et al., 2011). We hypothesized that some of these archaea may be classical heterotrophs, others may be heterotrophic ammonia oxidizers, which do not derive energy from the reaction and oxidize ammonia at low rates that are difficult to detect, and still others might be capable of mixotrophic ammonia oxidation. We previously used 16S rRNA gene analysis to document selection of a specific group 1.1b clade from the roots of *Lycopersicon esculentum* (tomato) plants grown in Wisconsin soil, using root extract as a growth substrate in culture. Clones from this clade were designated TRC, for *t*omato *r*oot *C*renarchaeota (Simon et al., 2000), and TREC, for *t*omato *r*oot *e*nrichment *C*renarchaeota (Simon et al., 2005). With this report, we further explore the diversity and metabolic potential of root-colonizing soil archaea by examining the AOA assemblage selected in enrichment culture. We document selection of different clades of group 1.1 archaea, including putative AOA, in incubations with organic and inorganic amendments. Our data suggest that as a whole, the group 1.1b archaea are metabolically diverse, with additional strategies besides autotrophic ammonia oxidation. These results are consistent (i) with previous studies in finding that growth of members of group 1.1b may be uncoupled from ammonia oxidation activity (Jia & Conrad 2009; Mertens et al., 2009; Di et al., 2010; Offre et al., 2009; Zhang et al., 2011; Pratscher et al., 2011; Muβmann et al., 2011), and (ii) with the newly-established requirement of organic carbon for growth of "*Ca.* Nitrososphaera viennensis" strain EN76 (Tourna et al., 2011).

5.2 Material and methods

5.2.1 Inoculum and root extract preparation

Tomato plants were grown and root inoculum was prepared as described previously (Simon et al., 2005), except that the plants were grown in an outdoor plot instead of in a growth chamber. Briefly, plants were grown in Oregon silt loam soil (24.9% sand, 60.8% silt, 14.3% clay) containing 4.8% organic matter. Root inoculum was prepared from tomato plants by rinsing roots thoroughly in sterile-distilled H_2O to remove soil particles prior to bath sonication for 30 sec in sterile Milli-Q H_2O. The roots were rinsed in a similar manner for root extract preparation, but instead of sonication, they were ground with a mortar and pestle under liquid N_2.

5.2.2 Culture conditions

The recyclostat was operated on intermittent chemostat/batch cycles such that medium replacement in the 3-liter vessel occurred approximately every 2 months. Temperature was maintained at 22˚C by water circulation through the jacket of the reactor. Oxygen levels were maintained under 10% by sparging the culture vessel intermittently (twice weekly) with a gas mixture containing 2% O_2 and 0.035% CO_2, with the balance as N_2. The "SC batch" subculture was inoculated from the recyclostat with a 1:10 dilution into 500 ml synthetic freshwater Crenarchaeota medium containing 500 μM NH_4Cl, 1 mM $NaHCO_3$, 10 mM HEPES, 1 g l^{-1} NaCl, 0.4 g l^{-1} $MgCl_2 \cdot 6H_2O$, 0.1 g l^{-1} $CaCl_2 \cdot 2H_2O$ and 0.5 g l^{-1} KCl (with other medium components as described in Könneke et al.,2005. Before inoculation into the flask, the supernatant was removed and the cells were washed gently 3 times with fresh medium. The culture was maintained in the dark at 22 °C, in semi-continuous batch mode in a 1-liter spinner flask (Bellco Glass Inc.,

Vineland, NJ) with ports for transport of medium into and out of the vessel. The measured pH was 6.8 and did not change during experiments.

5.2.3 DNA/RNA extraction, purification and quantification

Genomic DNA was extracted using the Fast DNA spin kit for soil (ThermoFisher Scientific, Waltham, MA). For gene expression analysis, total DNA and RNA were extracted from the same homogenized sample in a 1:9 volume ratio, respectively. Total RNA was isolated using a modified phenol-chloroform method (Smith et al.,2010). RNA was treated with DNase (Promega, Southampton, UK) and purified with the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Purified RNA was examined by PCR to ensure no DNA contamination was present. DNA and RNA concentrations were quantified using PicoGreen and RiboGreen dye (Invitrogen Corp., Carlsbad, CA), respectively, on a Nanodrop fluorometer (Thermo Scientific, Wilmington, DE), according to the manufacture's protocol.

5.2.4 Reverse-transcription PCR

Total RNA was converted to cDNA with random hexamers using SuperScript III First-Strand Synthesis Supermix for qRT-PCR, according to the manufacturer's protocol (Invitrogen Corp., Carlsbad, CA).

5.2.5 PCR, rRNA gene cloning, and sequencing.

Total extracted DNA from enrichments was amplified by PCR using 16S rRNA gene-specific primers as previously described (Simon et al., 2005), PCR was performed in separate reactions with the archaea-biased forward primer 109F (5´–ACKGCTCAGTAACACGT–3´) (Großkopf et al., 1998), or 133F (5´–TGTTGACTACGTGTTACTGAG–3´) (Simon et al., 2000), and the multi-domain

reverse primer 1492R (5´–GGYTACCTTGTTACGACTT–3´) (Lane 1991). According to the method of Francis *et al.*,(Francis et al., 2005), the *amoA* gene-specific primers Arch-amoAF (5´–STAATGGTCTGGCTTAGACG–3´) and Arch-amoAR (5´–GCGGCCATCCATCTGTATGT–3´) were used to generate *amoA* gene fragments with the following PCR protocol: 95°C for 5 min; followed by 30 cycles consisting of 94°C for 45 s, 53°C for 60 s, 72°C for 60 s; and finally 72°C for 15 min. PCR products were cloned by ligation using the pGEM-T vector system (Promega Corp., Madison, WI), employing blue-white colony screening. Inserts were confirmed by PCR amplification, sequencing, and sequence analysis. Partial sequences were generated by bidirectional sequencing of cloned products using the vector primers SP6 and T7. Sequencing reactions were carried out according to the OHSU DNA Services Core standardized protocols (http://www.ohsu.edu/xd/research/research-cores/index.cfm) and were read on an ABI 3130xl, capillary fluorescence instrument.

5.2.6 Phylogenetic analysis

16S rRNA gene sequences were aligned in ARB using the Silva website (Pruesse et al.,2007). The number of added neighbors was limited to 40 while generating the alignment and the auto reverse/complement option was selected. Regions of ambiguous positional homology were removed and additional manual corrections were made using MEGA version 4.0 (Tamura et al., 2007) to create an alignment of 79 sequences containing 1,183 homologous nucleotide positions (the alignment of OREC sequences by themselves consisted of 1,299 homologous nucleotide positions). The CLC Genomics Workbench program (CLC bio, Aarhus, Denmark) was used to align *amoA* gene sequences, using the MUSCLE algorithm with default settings, which yielded an

alignment of 49 sequences containing 590 homologous nucleotide positions, and a translated amino acid alignment of 49 sequences containing 196 homologous amino acid positions.

Phylogenetic analysis was carried out for all data sets in MEGA 4.0, CLC Genomics Workbench, and PhyML (version 2.4.5) (Guindon & Gascuel 2003). Non-parametric bootstrapping was carried out with 1,000 replicates in all cases. Neighbor-joining (NJ) trees were constructed in CLC Genomics Workbench. Minimum evolution (ME) trees were constructed in MEGA using close neighbor interchange (CNI) with a search level of 2. For ME, nucleotide trees were generated using the maximum-composite-likelihood substitution model, including transitions and transversions, and amino acid trees were generated using the JTT matrix substitution model. Maximum-parsimony (MP) trees were constructed in MEGA using CNI with a search level of 3, and initial trees were constructed by radon addition (10 repetitions). Maximum likelihood (ML) trees were constructed in PhyML. For ML, nucleotide trees were made using the GTR model with base frequency estimates set to ML and estimating for transition-to-transversion (ts/tv) ratio, the proportion of invariable sites, and gamma distribution; amino acid trees were constructed with the Blosum62 model estimating for the proportion of invariable sites and gamma, using the JTT matrix substitution model. Trees were rooted with multiple outgroups and beautified in Dendroscope (Huson et al., 2007) for visual clarity.

5.2.7 Primer design for quantitative PCR

The primers used for quantitative PCR (qPCR) with 16S rRNA genes were: 669F, 5´-CGACGGTGAGGGATGAAAG-3´ (Simon et al., 2005) and 88611GR, 5´-CCAGGCGGCAGACTTAAC-3´ (88611GR contains an A to G modification at position

11 in 886R (Simon et al., 2005). Three gene-specific primer sets targeting individual *amoA* clades were also designed using CLC Genomics workbench software. Primers specificity was tested by PCR with sequenced clones, and the primers were found to amplify only the targeted clade. Primer sequences and cycling parameters are listed in Table 5.7.

5.2.8 qPCR

qPCR was performed as described for 16S rRNA genes (Simon et al., 2005), with modifications for *amoA* genes as described below. In each experiment, three independent master mixes were prepared for each culture, and two independent master mixes were prepared for standard DNA and tested in duplicate within a single microtiter plate. Control reactions (with water in place of template) were also included in each experiment. PCR was conducted in a MyiQ Real-Time qPCR detection system (Bio-Rad Laboratories, Inc, Hercules, CA). Reactions were performed in a volume of 25 μL containing 1× iQ SYBR green supermix (Bio-Rad) and 0.2 μM concentration of each primer at optimized annealing temperature (Table 5.7). Each template DNA or cDNA was amplified using the following cycling conditions: 95.0 °C for 4 min; 40 cycles of denaturation at 95.0 °C for 30 s, annealing at 59.0 °C for 45 s and elongation at 72.0 °C for 45 s; followed by a final extension step at 72 °C for 4 min. A melting curve protocol began after amplification and consisted of 1 min at 95 °C, followed by 1 min at 55 °C and 80 10-s steps with a 0.5 °C increase in temperature at each step. The PCR threshold value and the amplification efficiency of individual wells (82.4% to 98.6%) were calculated using the LinReg PCR program (Ramakers et al., 2003). Inhibition was tested for by serial dilution of templates and was not observed. Standard curves were generated by serial dilutions of

linearized plasmids. The linear ranges were from 10^7 to 10^0 template copies with R^2 values of the standard curves ≥0.99 for all assays. The results were normalized for starting template concentration. Mean values and standard deviations were calculated using Microsoft Excel, Version 12.2.8.

5.2.9 PCR–SSCP

PCR– single strand conformation polymorphism analysis (PCR-SSCP) was conducted by using the Crenarchaeota–biased primers 133F/248R5P (Sliwinski & Goodman 2004). Template DNA was PCR-amplified in a 75 μL reaction mixture containing 37.5 μL IQ Supermix (Biorad Laboratories Inc., Hercules, CA), 33 μL PCR water, 0.75 μl of each primer (200 nM final concentration) and 3 μL template DNA. The reaction mixtures were subjected to touchdown PCR as follows: 1 min at 94 °C; followed by 20 touchdown cycles consisting of 30 s of denaturation at 94 °C, 1.5 min annealing at 65 °C, and 1.5 min of elongation at 72 °C. During touchdown, the annealing temperature was reduced every two cycles by 0.5 °C, until the final annealing temperature of 55 °C was reached. This was followed by 15 cycles of 30 s at 94°C, 1.5 min at 55°C and 1.5 min at 72°C, with a final extension of 5 min at 72 °C. After amplification, the PCR products were purified using the Promega Wizard SV gel and PCR purification kit (Promega Corp., Madison, WI) according to the manufacture's protocol. PCR products were then incubated with 15 U of Lambda Exonuclease in 1× buffer (New England Biolabs, Beverly, MA) at 37 °C for 1.5 h, followed by heat inactivation at 65 °C for 10 min. The recovered products were desalted using Sephadex G50 microspin columns (Aldrich Chemical Co, Milwaukee, WI). After purification, each PCR product was combined with 15 μL SSCP stop solution (Lonza, Basel, Switzerland), denatured at

95 °C for 3 min, and immediately transferred onto ice. The products were electrophoresed on 1× MDE (Lonza Group Ltd, Basel, Switzerland) polyacrylamide gels at 300 V for 22 h at 17 °C. The gels were scanned on a Typhoon variable mode imager (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and images were analyzed using GelCompar software II (Applied Maths, Austin, TX). Peak height cutoffs ranged from 5 to 20 relative fluorescence units, depending on signal intensity.

5.2.10 TOC and TKN measurements

Total Kjeldahl nitrogen (TKN) and organic carbon (TOC) were measured at TestAmerica Laboratories, Inc. (Portland, OR) using standard U.S. Environmental Protection Agency methods (US EPA 351.2 & 4 15.2). TKN was determined by semi-automated colorimetry with sulfuric acid digestion. TOC determination was based on the method of UV–promoted persulfate oxidation, converting organic carbon to carbon dioxide.

5.2.11 Nitrification activity assays

Recyclostat biomass was resuspended 1:10 in 150 ml synthetic freshwater Crenarchaeota medium as described for SC batch cultures except 250 μM NH_4Cl and 2 mM $NaHCO_3$ were used. Assays were incubated aerobically in the dark, at 22 °C. Potential ammonia oxidation/nitrification activity was monitored by measuring changes in the concentrations of ammonium, nitrite and nitrate. Ammonium concentrations were determined by colorimetric measurements of absorbance at 340 nm (Mondzac et al.,1965) using a Sigma-Aldrich Ammonia Assay Kit (St. Louis, MO). Alternatively, for low concentrations of ammonium present in root extract, a fluorometric method (Kérouel & Aminot 1997; Holmes et al., 1999) was used. Nitrite+nitrate concentrations were

determined by photometric measurement of absorbance at 540 nm, using a nitrite/nitrate colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) in a two-step process (Green et al., 1982; Nims et al., 1995). Colorimetric and fluorometric measurements were made using a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA). The detection limits for nitrite and nitrate were 2 μM and 2.5 μM, respectively. The detection limit for ammonium was 1.5 nM, and 10 μM for fluorescence and colorimetric assays, respectively.

5.2.12 Nucleotide sequence accession numbers

All sequences have been deposited in the GenBank database with accession numbers JF799588 – JF799670 (16S rRNA genes) and JF799671 – JF799755 (*amoA* genes).

5.3 Results

5.3.1 Enrichment culture

Enrichment culture was used to examine the selection of rhizosphere archaea in response to different substrates and growth conditions. Sonicate prepared from the surface of tomato roots grown in Oregon silt loam soil was used to simultaneously inoculate duplicate batch cultures and a semicontinuous biomass recycle reactor, referred to here as a recyclostat (van Verseveld et al., 1984; Konopka et al., 1996). The cultures were incubated in parallel in a defined medium containing 1 mM NH_4Cl, to which 1× root extract (RE; 5 g liter^{-1}) was added. 1× RE contained 32.6 ± 10.1 mg organic carbon liter^{-1}, 3 μM ammonium, and no detectable nitrate or organic nitrogen (detection limits of 2.5 μM and 1.25 mg liter^{-1}, respectively). The proportion of archaea to bacteria was estimated to range from 10-30% using a semiquantitative PCR method, with analysis by

fluorescence *in situ* hybridization (FISH) and microscopy supporting this assessment (data not shown).

5.3.2 16S rRNA gene phylogeny

DNA isolated from batch and recyclostat cultures was used in PCR with a forward primer biased broadly toward archaea 109F, (Großkopf et al., 1998), or more specifically toward mesophilic soil Crenarchaeota 133F, (Simon et al., 2000), and the reverse primer 1492R (Lane 1991) hybridizing broadly to archaeal and bacterial 16S rRNA genes to generate gene fragments for the construction of libraries. Sequence analysis was performed on ≥30 randomly chosen clones from libraries constructed separately with DNA from the recyclostat and from one batch culture. Similar results were obtained with both forward primers (data not shown). Recovered clones were designated "OREC," for ORegon Enrichment Culture. The clones recovered from recyclostat were more diverse than those from the batch culture, clustering into 3 vs. 2 clades, respectively. The majority of OREC sequences recovered from both cultures clustered in the same two major groups, one containing TRC and TREC clones, referred to herein as the "root" clade, and another designated as the "water-associated subsurface" clade (WaS; Figure 5.1A). In all, from batch and recyclostat cultures, 46 (35 non-identical) sequences grouped within the root clade and 32 (30 non-identical) sequences grouped within the WaS clade (Table 5.5). Root clade sequences clustered closely (98.1% to 100% sequence identity over 1,183 nucleotides [nt]) with TRC and TREC clones recovered directly from tomato roots grown in Wisconsin (WI) soil (e.g., accession no. AF227642, (Simon et al.,2000)), and from enrichment cultures inoculated with WI-grown tomato roots (e.g., accession no. AY487103, (Simon et al., 2005), respectively. This clade also contained

sequences recovered from sediments dominated by the marsh grass *Spartina alterniflora* (e.g., accession no. FJ655668 (Nelson et al., 2009)) and soils from a fallow agricultural site in Wisconsin (e.g., accession no. U62811(Bintrim et al., 1997)) and the Austrian Central Alps (e.g., accession no. DQ278118).

WaS clade sequences from recyclostat and batch cultures shared high sequence identity (Table 5.5), and were most closely related (98.0% to 99.0% identity over 1,183 nt) to biofilm samples from an underground lava cave in Hawaii (e.g., accession no. EF032794), deep subsurface water samples (e.g., accession no. EF562195, (et al., 2007)) and "*Ca.* Nitrososphaera viennensis" strain EN76 (Tourna et al.,2008) (Figure 5.1A). These sequences were also closely related to clones recovered from moderate and high-temperature subsurface water samples (e.g., accession no. AB113626, ≥97% identity over 1,183 nt) and the archaeal ammonia oxidizer, "*Ca.* Nitrososphaera gargensis," enriched from a moderately thermophilic hot spring (≥96% sequence identity (Hatzenpichler et al., 2008)).

Six (5 nonidentical) OREC clones grouped within a separate cluster (Figure 5.1A) most closely related (≥99.6% sequence identity over 1,183 nt) to clones recovered from rice paddy fields (e.g., accession no. AB243809, (Sakai et al., 2007), deep subsurface waters (e.g., accession #AB050208 (Takai et al., 2001)), and the rhizosphere of the freshwater macrophyte *Littorella uniflora* (e.g., accession no. EU309869 (Herrmann et al., 2008)). This sequence group was recovered only from the recyclostat and clustered with group 1.1a, in a clade associated (88.8% identity over 1,183 homologous nucleotides) with the "marine" group (DeLong, 1998; Ochsenreiter et al., 2003). Others (Nicol et al., 2008; Ying et al., 2010; Lehtovirta-Morley et al., 2011) have referred to this clade, which

contains sequences retrieved from freshwater, soils, sediments and the subsurface, as 1.1a-associated. In keeping with this idea, we refer to it here as the "marine-associated" (Ma) clade. The marine clade itself contains sequences of the archaeal ammonia-oxidizer "*Ca.* Nitrosopumilus maritimus," SCM1 isolated from aquarium sediments (Könneke et al., 2005), the sponge symbiont "*Ca.* Cenarchaeum symbiosum" (Preston et al., 1996), and "*Ca.* Nitrosoarchaeum limnia" SFB1, enriched from low-salinity sediments of San Francisco Bay (Blainey et al., 2011). Upon comparing the three groups recovered from enrichment cultures, the root and WaS clade sequences shared higher identity, both placing into group 1.1b, whereas the Ma clade sequences were more divergent from the others and, as mentioned above, clustered with group 1.1a (Table 5.5).

5.3.3 Relative abundance of 16S rRNA gene sequence clades under different growth conditions

The relative abundance of the different clades was determined in the recyclostat in the presence of 1× RE, or 10-times lower concentration (0.5 g liter^{-1}, referred to here as 0.1× RE), using PCR-SSCP analysis. Bands corresponding to individual clades were identified by co-migration on polyacrylamide gels with sequenced controls. For the root clade, two different sequence types (sharing 98% identity over the 147-bp amplified region) were identified, and corresponded to separate bands on PCR-SSCP gels, whereas sequences from the other clades each produced a single band (Figure 5.2). Incubation with 0.1× RE resulted in dominance of the WaS clade (represented by a single band on PCR-SSCP gels) over the root clade (Figure 5.2 and Table 5.1). The root clade (represented by 2 bands on PCR-SSCP gels) was dominant, however, when 1× RE was used as substrate. The WaS clade alone was detected in a semi-continuous batch (SC batch) culture inoculated with

biomass from the recyclostat, and incubated for 4 months with $NaHCO_3$ as sole carbon source (Figure 5.2 and Table 5.1). The Ma clade was not detected under any conditions using this approach.

5.3.4 Selection and growth of the root clade with root extract incubations

To determine whether root-extract-dependent growth of archaea occurred, abundance was measured by qPCR, using the 669F/886-11GR primer set biased broadly toward the 16S rRNA genes of soil crenarchaeotes (Simon et al., 2005). Total archaeal abundance (inferred from 16S rRNA gene copies and based on an estimation of one copy per genome) was 1 to 2 orders of magnitude higher in the recyclostat compared to batch cultures when both were incubated with 0.1× RE for approximately 3.5 months, at which time the batch cultures were supplemented with 1× RE. Over ~one month after the addition, archaeal abundance in the batch culture increased 2 orders of magnitude and reached levels similar to those present in the recyclostat (Figure 5.3). Similar to the results for the recyclostat (Figure 5.2), strong selection for the root clade was observed in batch culture after supplementation with 1× RE (relative abundance of the clade increased from 45% to 72%, Figure 5.3), determined by PCR-SSCP analysis (data not shown).

5.3.5 *amoA* gene phylogeny

The Arch-amoAF and Arch-amoAR primers, biased toward AOA *amoA* genes, were used to generate clone libraries from the enrichment cultures as described previously (Francis et al., 2005). Recovered clones were designated "ORECA," for ORegon Enrichment Culture *amoA*. Similar to results from the 16S rRNA gene libraries, *amoA* sequences recovered from the recyclostat were more diverse, clustering into 3 clades,

while those from the batch culture grouped into a single clade. All 43 ORECA sequences retrieved from batch culture clustered with the *amoA* gene from "*Ca.* Nitrososphaera viennensis" strain EN76 (Tourna et al.,2011) (Figure 5.1B, Table 5.6) in the WaS *amoA* clade, corresponding to the WaS 16S rRNA gene sequence clade. Sequences recovered from the recyclostat clustered mainly into two groups (Figure 5.1B): (i) the WaS clade (12 clones) and (ii) a clade of 28 sequences most closely related (97.6% identity over 590 nt) to *amoA* sequences recovered from marine and estuarine sediments (e.g., accession no. FJ227708), and acidic soils (e.g., accession no. FJ517347, (Ying et al., 2010)). Analogous to the Ma 16S rRNA gene sequence clade, sequences in this *amoA* clade were highly identical (93.6% identity over 590 nt) to *amoA* sequences recovered from the roots and rhizosphere of the macrophyte *L. uniflora*, and were associated with group 1.1a. Thus, we refer to this as the Ma *amoA* clade (Table 5.6). Two additional sequences were recovered from the recyclostat that clustered most closely with *amoA* gene sequences recovered from activated sludge (e.g., accession no. EU860279, (Zhang et al., 2009)) and a wastewater treatment plant (e.g., accession no. DQ304880) and, therefore these are referred to as the "wastewater (WW)" *amoA* clade (Figure 5.1B, Table 5.6). Amplification was not detected for AOB *amoA* genes with amoA-1F and amoA-2R primers (Rotthauwe et al., 1997) in multiple tests by either endpoint or qPCR (data not shown).

5.3.6 Abundance and expression of *amoA* genes under different growth conditions

Primers were designed to differentiate the three *amoA* clades detected by sequence analysis. The primers were used in qPCR and reverse transcription–qPCR analyses to quantify the abundance of *amoA* genes and gene transcripts, respectively, representing

each clade. WaS clade *amoA* genes were approximately 3- to 5- fold more abundant in 1× RE incubations compared to incubations with 0.1× RE, while *amoA* gene copies were 2- to 4- fold more abundant in 0.1× RE incubations for both Ma and WW clades compared to cultures with 1× RE (Table 5.2).

WaS clade *amoA* genes were the most abundant under all conditions tested, and the difference was greatest for incubations with 1× RE (2-3 orders of magnitude more WaS clade gene copies compared to WW and Ma clades, respectively (Tables 5.2 and 5.3). Overall, *amoA* gene copies decreased by more than two orders of magnitude in the SC batch culture (with $NaHCO_3$ as sole carbon source), but were still 10-fold higher for the WaS clade than for the WW clade (Table 5.3). The Ma clade dropped below the level of detection in the SC batch culture. Expression of *amoA* genes was observed only for the WaS clade in both recyclostat and SC batch cultures (Table 5.3). Due to observations of cell clumping during incubation in the SC batch culture, gene and transcript abundance was estimated per ng of total DNA or RNA, respectively.

5.3.7 Nitrification activity and change in abundance of *amoA* clades

Potential nitrification activity was measured by incubating resuspended recyclostat biomass in a defined medium containing 260 μM NH_4Cl and 2 mM $NaHCO_3$ and determining the loss of ammonium and production of nitrite and nitrate over time (Figure 5.4). Although a small amount of nitrite and nitrate was produced and accumulated in these experiments, it was never equivalent to the ammonium lost, except during the first few days of incubation (e.g., after 3 days, 25.5 ± 4.0 μM NH_4Cl lost vs. 28.8 ± 11.3 μM nitrite/nitrate accumulation). Incubations carried out for longer periods resulted in greater losses of ammonium and decreasing concentrations of measured nitrite and nitrate,

indicating assimilation by the enrichment culture consortium (e.g., after 6 days of incubation, a decrease of ~100 μM ammonium was observed, but accumulation of nitrite and nitrate was only ~30 μM). Longer incubations always resulted in lower accumulations of nitrite and nitrate, and the addition of root extract to the assay yielded the same general trend, but increased the variability (data not shown). Comparison of *amoA* gene copy numbers in the starting inoculum to gene copies after 4 days incubation showed small increases for two of the three clades (Table 5.4).

5.4 Discussion

Recent discoveries of novel, mesophilic Crenarchaeota and the ability of at least some members of this group to oxidize ammonia have transformed our understanding of microbial nitrogen cycling. These organisms are so different from other cultivated archaea, in fact, that comparative analysis with the few available genomes led to a proposal (Brochier-Armanet et al., 2008) and support (Spang et al., 2010; Gupta & Shami 2011) for a new archaeal phylum, the *Thaumarchaeota*.

5.4.1 Metabolic diversity revealed by selection and growth of specific clades with organic and inorganic amendments

Similar to an earlier study on archaea from Wisconsin soil (Simon et al., 2005) we showed here the root extract-dependent selection and growth, in culture, of members of the root clade from Oregon soil. Root clade sequences alone were recovered from previous batch enrichments (Simon et al., 2005), while sequences recovered in this study were more diverse; however, more clones were also analyzed. For the first time, putative AOA were also identified in root extract enrichments by recovery of *amoA* gene sequences. Two different types of enrichments were compared in the recyclostat

(semicontinuous replenishment of medium components) and batch (medium components depleted over time) cultures fed with the same root extract-containing medium. The root clade was dominant in the recyclostat culture supplied with 1× RE, and was highly enriched in batch culture upon amendment with fresh 1× RE after 3 months of incubation. The WaS clade was dominant in the recyclostat at the lower RE concentration and in batch cultures after depletion of RE or upon supplementation with bicarbonate as sole carbon source. However, the WaS clade was also more abundant in cultures containing RE than in those without. These results and others (Hatzenpichler et al., 2008; Tourna et al., 2011) suggest a potential mixotrophic lifestyle for members of the WaS clade. There was no apparent selection by root extract for the Ma clade. In fact, the Ma clade was not detected at all in the cultures by PCR–SSCP, probably due to a combination of low abundance (revealed by qPCR) and a single-nucleotide mismatch to Ma clade sequences in both forward and reverse SSCP primers (not shown). While the mismatch did not affect detection of the control (Figure 5.2), relatively low representation in a complex DNA mixture most likely made detection by this method more difficult (Sliwinski & Goodman 2004).

5.4.2 Autotrophic and/or mixotrophic ammonia oxidation indicated for the WaS clade

Expression analysis revealed transcriptional activity from the WaS clade *amoA* genes only, regardless of culture conditions. These results are consistent with results showing autotrophic ammonia oxidation by the terrestrial hot springs crenarchaeon "*Ca.* N. gargensis" (Hatzenpichler et al., 2008), possessing genes with high identity to WaS clade sequences. The WaS clade's increase in abundance when incubated with 1× RE, however,

is consistent with genomic evidence suggesting that AOA may have the ability to take up organic carbon compounds to supplement growth (Hallam et al., 2006b; Walker et al., 2010) and with direct evidence for an organic carbon requirement for growth of "*Ca.* Nitrososphaera viennensis" strain EN76 (Tourna et al., 2011), also closely related to members of the WaS clade. Furthermore, Chen *et al.*,(Chen et al., 2008) reported higher abundance of AOA in the rice paddy rhizosphere compared to non-rhizosphere soil, presumably due to root exudates.

Results from experiments examining potential nitrification activity suggested that ammonia oxidation occurred with concomitant growth of archaea from the WaS clade (Figure 5.4, Table 5.4). Isolation in pure culture, or ^{13}C-CO_2 labeling studies will be necessary, however, to determine whether these organisms actually grow by oxidizing ammonia. Such a result would be consistent with reports showing growth of AOA isolates under nitrifying conditions for similar time periods (Martens-Habbena et al., 2009; Tourna et al., 2011). Documentation of autotrophic and/or mixotrophic ammonia oxidation and growth of group 1.1b archaea in culture (Hatzenpichler et al., 2008) raises the question of why it has been so difficult to link ammonia oxidation activity to growth of members of this clade in soil. A possible explanation is that, although these organisms are selected for in cultivation experiments`, they are not representative of the most abundant archaeal clades in soil (Bates et al., 2011), making it difficult to detect their activity due to their low numbers. Consistent with this idea, a recent study on nitrification in soil microcosms (Xia et al., 2011) found that relatively low numbers of "*Ca.* Nitrososphaera gargensis" – like *amoA* and 16S rRNA genes were labeled, compared to AOB genes, in ^{13}C-CO_2 uptake experiments. If the majority of AOA in soil are

heterotrophic nitrifiers, correspondingly lower rates of ammonia oxidation may also be harder to detect.

Although their numbers were smaller, the fold increase for the WW clade was even greater than for the WaS clade during the nitrification assays (Table 5.4). Taken together with reduced numbers of this clade in the presence of higher concentrations of root extract, this result may indicate an ability to oxidize ammonia autotrophically, although we did not detect *amoA* gene transcripts from this group. Their relatively low numbers may have made such detection more difficult, however. Further research using ^{13}C-CO_2 labeling experiments may shed more light on the metabolic capabilities of this group.

5.4.3 Are members of the root clade heterotrophs?

Phylogenetic analysis of clone libraries indicated correspondence between 16S rRNA and *amoA* genes of both WaS and Ma clades but not between the 16S rRNA gene of the root clade and the *amoA* gene of the WW clade. Furthermore, the WW clade *amoA* genes were detected in the SC batch culture, while the root clade 16S rRNA genes were not. In total, our data suggest that either our primers did not detect an existing *amoA* gene for the root clade, or one does not exist. In our reading of the literature, we found no direct evidence for an *amoA* gene corresponding to this clade, although results from one study comparing 16S rRNA and *amoA* clones retrieved from the same soil samples were somewhat suggestive (Nicol et al., 2008). It is possible that such a gene(s) may be revealed by additional sequence analysis in the future. In fact, the *amoA* primer set used in the present study was shown to have mismatches with *amoA* sequences from, for example, "*Ca.* Nitrosocaldus yellowstonii", "*Ca.* Nitrosopumilus maritimus", and "*Ca.* Cenarchaeum symbiosum" (Konstantinidis et al., 2009). Our failure to recover the

corresponding 16S rRNA gene for the WW *amoA* clade may have been due to a primer mismatch(es), or to the fact that at the time of cloning we appeared to be near the limit of detection for this clade, recovering only two clones.

Given the preferential selection of the root clade by root extract and its absence in incubations with bicarbonate as sole carbon source, it is tempting to speculate that the root clade is heterotrophic and that members of this group utilized organic matter in the root extract for growth. Although we did not discover one, it is also possible they possess an as-yet undetected *amoA* gene, and are, in fact, capable of heterotrophic ammonia oxidation. Some heterotrophic bacteria, for instance, perform ammonia oxidation by a process that is analogous to that used by chemoautotrophic AOB (Crossman et al., 1997). Organic nitrogen was not detected in the root extract (detection limit, 1.25 mg/L), and while it is formally possible that the root clade responded to inorganic ammonium in the root extract in batch culture experiments, this is unlikely to have been the case in the recyclostat, because the concentration of ammonium in the incubation medium was much higher (1 mM) than the ammonium in root extract (3 μM), making organic carbon seem more likely as a driver of the selection. Of course, it is possible that members of the root clade responded to some component of root extract other than organic carbon and/or nitrogen or to detritus from increased biomass and turnover of the enrichment consortium as a whole. It is interesting that the response was similar to root extract produced from plants grown in different soils and locations. Experiments are planned to determine directly whether root-derived carbon is taken up by archaea.

Based on somewhat controversial data (Verstraete 1975; Focht & Verstraete 1977), heterotrophic nitrification is thought to be less important than autotrophic nitrification in

the environment. However, many widespread and abundant heterotrophic bacteria and fungi have the ability to oxidize a variety of nitrogenous compounds (Focht & Verstraete 1977; Castignetti & Hollocher 1984). In addition, several heterotrophic species of bacteria previously considered to be "poor" nitrifiers actually nitrify and denitrify simultaneously, giving a falsely low impression of their nitrification potential (Robertson et al., 1988; Robertson & Kuenen 1990). In some cases, the heterotrophic nitrification rates are less than a factor of 10 lower than rates for autotrophs (Jetten et al., 1997). Heterotrophic nitrifiers do not appear to derive energy from ammonia oxidation, but may instead use nitrification for the dissipation of excess reductant (Robertson & Kuenen 1990). The fact that growth rates of heterotrophs tend to be higher than those of autotrophs, and that heterotrophic nitrifiers can be present in considerably higher numbers than autotrophic nitrifiers (Focht & Verstraete 1977), suggest that the ecological significance of heterotrophic nitrification is not well understood. Together with the fact that the high abundance of AOA in many soil environments has not been linked to (generally higher rates of) autotrophic ammonia oxidation, the idea that heterotrophic (and/or mixotrophic) nitrification is carried out by archaea appears to be a viable hypothesis, and is supported by the requirement for organic carbon by "*Ca.* Nitrososphaera viennensis" strain EN76, and its ability to oxidize organic nitrogen (Tourna et al., 2011).

The hypothesis is additionally compelling in light of evidence that marine Thaumarchaeota take up some forms of organic carbon (Pérez et al., 2003; Ingalls et al., 2006; Teira et al., 2006; Kirchman et al., 2007; Varela et al., 2008). Although the focus on archaeal nitrification has largely been on autotrophic ammonia oxidation, analysis of

mesophilic Thaumarchaeota and AOA in natural environmental samples and microcosms indicates potential for mixotrophy or heterotrophy. Isotopic analysis of archaeal lipid biomarkers suggested that ca. 17% of organisms in mesopelagic zones of the ocean may assimilate organic carbon (Ingalls et al., 2006). Research also indicated that Thaumarchaeota take up amino acids in deep ocean waters (Pérez et al., 2003; Teira et al., 2006; Varela et al., 2008), and one report described assimilation of protein and diatom extracellular polymers in both surface and deeper waters of the Western Arctic Ocean (Kirchman et al., 2007). Furthermore, the genome sequences of "*Ca.* Nitrosopumilus maritimus" SCM1 (Walker et al., 2010) and "*Ca.* Cenarchaeum symbiosum" revealed several putative ABC-type transporter systems for organic carbon acquisition (Hallam et al., 2006b). Genes predicted to encode a nearly complete oxidative tricarboxylic acid cycle were identified, which is consistent with the consumption of organic carbon and the production of intermediates for amino acid and cofactor biosynthesis. Evidence for organic carbon consumption was also uncovered in the genome sequences of "*Ca.* Nitrosoarchaeum limnia" SFB1 (Blainey et al., 2011), and "*Ca.* Nitrosoarchaeum koreensis" MY1, enriched from rhizosphere soil (Kim et al., 2011). Finally, the well-studied autotrophic ammonia oxidizing AOB, *Nitrosomonas europaea*, was recently shown to be a facultative mixotroph, utilizing fructose and pyruvate in the absence of CO_2, its preferred carbon source (Hommes et al., 2003). In conclusion, our results suggest that mesophilic soil archaea harbor a diverse set of metabolisms. Although autotrophic ammonia oxidation by AOA seems clearly important in marine environments and potentially so in soil habitats, it may turn out that the ability of these organisms to

carry out mixotrophic and/or heterotrophic forms of metabolism largely explains their high abundance in soils.

5.5 Tables and figures

Table 5.1 Relative abundance of 16S rRNA gene sequence clades in cultures with organic and inorganic amendments.

	% peak abundance (SE)[a]		
Clade	1× Recyclostat	0.1× Recyclostat	SC batch
Root	65.9 (1.3)	15.6 (3.4)	ND
WaS	34.1 (1.4)	84.4 (3.4)	100 (0)
Ma	ND	ND	ND

[a] Values represent the mean relative percentage of each individual clade's peak area to total peak area for 3 replicates per treatment in PCR-SSCP assays. ND, not detected

Table 5.2 Comparison of *amoA* gene copy numbers in cultures with 1× and 0.1× root extract.

	1× RE[a]		0.1× RE	
Clade	Copy no.	SD	Copy no.	SD
Per ml				
WaS	6.2×10^5	2.2×10^4	3.7×10^5	5.1×10^3
Ma	3.0×10^2	2.8×10^1	2.0×10^3	1.5×10^1
WW	5.5×10^3	4.0×10^2	4.1×10^4	1.3×10^3
Per ng				
WaS	3.7×10^4	1.3×10^3	1.1×10^4	1.7×10^3
Ma	1.8×10^1	1.6×10^0	5.8×10^1	5.2×10^0
WW	3.3×10^2	2.4×10^1	1.2×10^3	4.4×10^2

[a] RE, root extract

Table 5.3 Abundance of *amoA* genes and gene transcripts in cultures with organic (recyclostat) and inorganic (SC batch) amendments.

D ND

[a] Values represent the mean and standard deviation, per ng total DNA or RNA, for three replicate PCRs (tested in duplicate). ND, not detected, or detection only after 35 PCR cycles.

Table 5.4 Change in archaeal *amoA* gene copy number during nitrification activity assays[a].

	Day 0		Day 4		Fold
Clade	Copy no.	SD	Copy no.	SD	change[b]
WaS	2.0×10^3	1.1×10^2	4.3×10^3	2.5×10^2	2.2
Ma	2.2×10^0	5.2×10^{-1}	ND	ND	
WW	2.2×10^1	1.4×10^0	1.2×10^2	1.7×10^1	5.5

[a] Means and standard deviations per ml are shown; values were estimated from replicate PCRs (tested in duplicate) for each clade. ND, not detected, or detection of signal only after 35 PCR cycles.
[b] That is, the fold change in abundance over 4 days of incubation.

Table 5.5 Percent sequence identity, over 1299 nt, of OREC clones.

Clade	Root	WaS	Ma
Root	≥98.1	≥94.2≤95.2	≥86.5≤87.3
WaS	≥94.2≤95.2	≥99.2	≥86.5≤87.3
Ma	≥86.5≤87.3	≥86.5≤87.3	≥99.5

Table 5.6 Percent sequence identity, over 590 nt (196 aa), of ORECA clones.

Clade	WaS	Ma	WW
WaS	≥98.7 (≥96.9)	≥70.6≤71.9 (≥73.5≤78.6)	≥72.5≤74.2 (≥88.3≤90.8)
Ma	≥70.6≤71.9 (≥73.5≤78.6)	≥98.0 (≥99.4)	≥69.6≤71.1 (≥74.5≤79.1)
WW	≥72.5≤74.2 (≥88.3≤90.8)	≥69.6≤71.1 (≥74.5≤79.1)	≥94.3 (≥98.5)

Table 5.7 PCR primer sequences and annealing temperatures used in this study.

Target gene	Primer	Sequences (5'–3')	Annealing temperature	Citation
Archaeal *amoA*	Arch-amoAF	STAATGGTCTGGCTTAGACG	53 °C	Francis *et al.* (2005)
	Arch-amoAR	GCGGCCATCCATCTGTATGT		
	Was-amoAF	TCGTGGTGTTCTCCGTATC	59 °C	This study
	Was-amoAR	GATTGTGGCGTAGTATGTGG		
	Ma-amoAF	GGTGCGTGGTTCTCCTTAG	59 °C	This study
	Ma-amoAR	GTGTTTGTTTCTCTTTGTTGCC		
	WW-amoAF	ATGGTTCGCTTTGGGTTATC	59 °C	This study
	WW-amoAR	TTCCRACTAGMACACCACCA		
Archaeal 16S rRNA	669F	CGACGGTGAGGGATGAAAG	61.3 °C	Simon *et al.* (2005)
	88611GR	CCAGGCGGCAGACTTAAC		
	109F	ACKGCTCAGTAACACGT	55 °C	Großkopf *et al.* (1998)
	1492R	GGTTACCTTGTTACGACTT		Lane (1991)
	133F	GCGGATCCGCGGCCGCCTCA	55 °C	Sliwinski *et al.* (2004)
	248R	GCCATTACCTCACCAACAGC		

A B

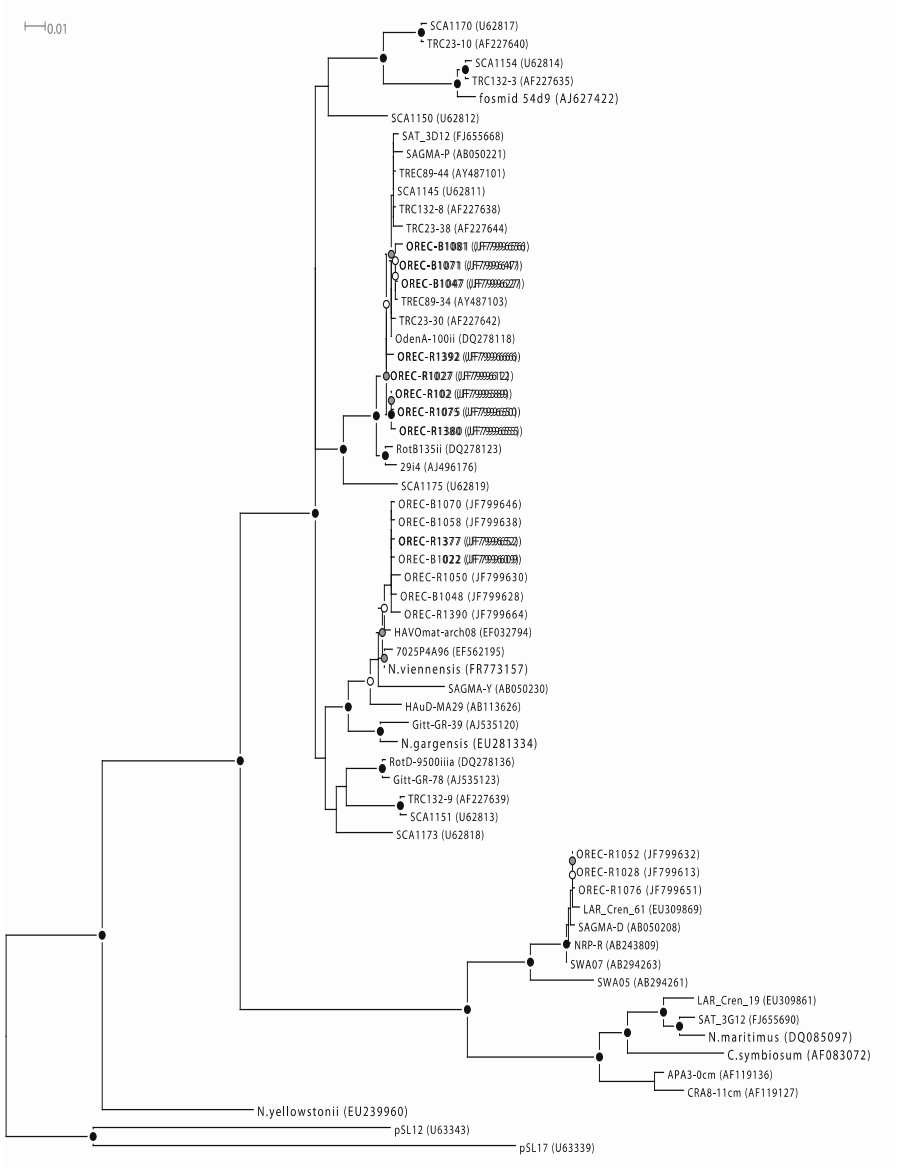

Figure 5.1 Inferred phylogenetic (ML) tree of archaeal (A) 16S rRNA and (B) *amoA* gene sequences recovered from plant root enrichment cultures. OREC and ORECA clones from this study (see text) are shown in boldface type, as are other clones anchoring phylogenetic clades, e.g., "*Ca.* Nitrososphaera gargensis", cloning anchoring phylogenetic clades, including tomato root clones TRC and TREC, from previous studies, are boxed. Clone number prefixes "B" and "R" indicate recovery from batch and recyclostat enrichments, respectively. GenBank accession numbers are listed parenthetically. Branch points supported by bootstrap values of ≥90%, ≥75%, and ≥50% in all MP and ED methods are indicated by filled, grey, and open circles, respectively. Scale bar, 0.01 (A) and 0.1 (B) change per nucleotide.

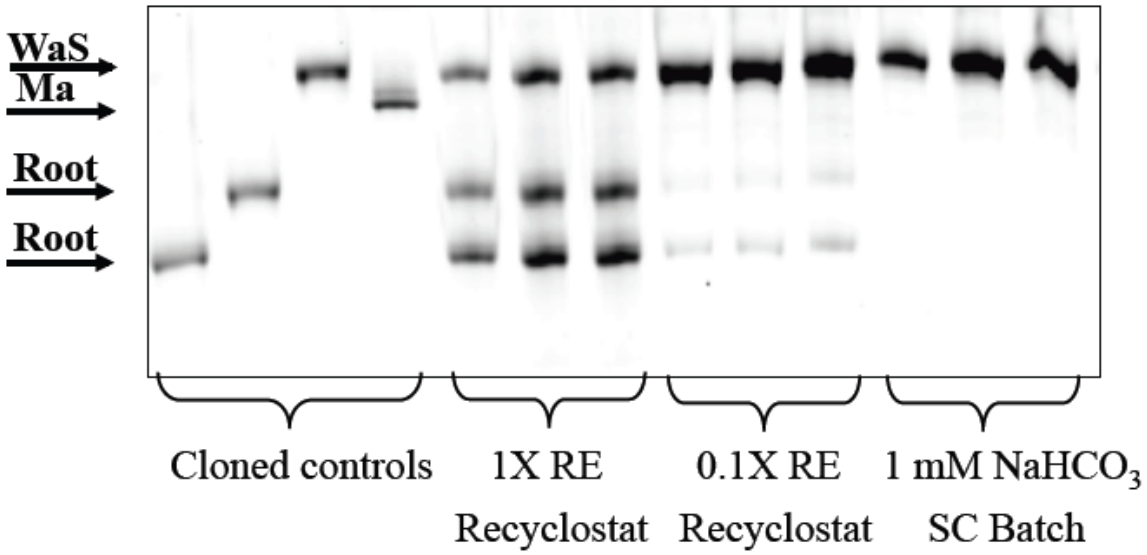

Figure 5.2 Representative PCR-SSCP gels of the archaeal assemblage in cultures with organic and inorganic amendments. DNA fragments (bands) correspond to root, WaS and Ma clades, as indicated by co-migration with cloned and sequenced controls. Culture conditions are indicated across the bottom of the gel.

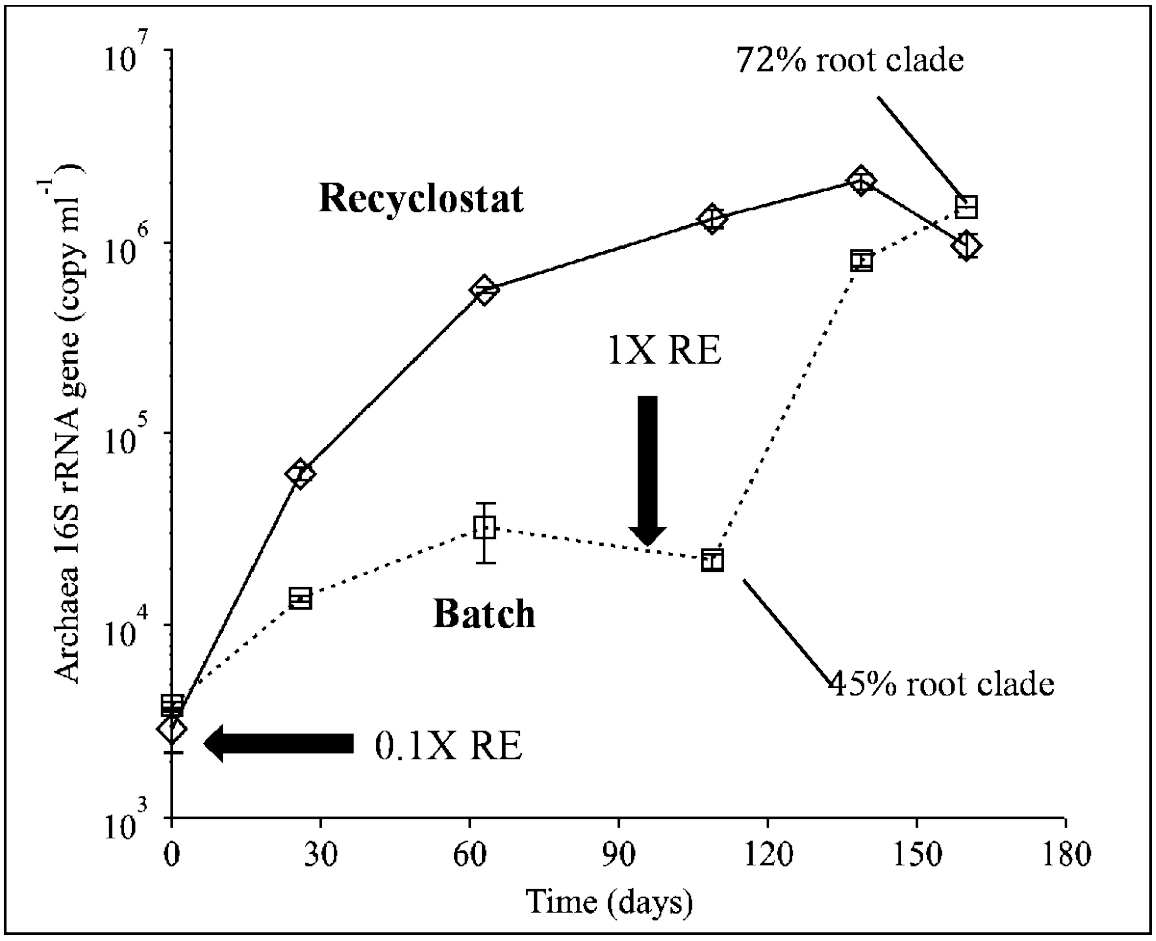

Figure 5.3 Change in 16S rRNA gene copy number for total archaea in the recyclostat (diamonds) and batch (squares) cultures over time. The horizontal arrow indicates the time both cultures were supplemented with 0.1× RE; the vertical arrow indicates time at which batch cultures were supplemented with 1× RE. Representative data are shown. Boxed numbers indicate the change in relative abundance of the root clade in batch culture over the time period indicated (see the text). Point values indicate the mean, and error bars indicate the standard deviation, from three replicate PCRs (tested in duplicate).

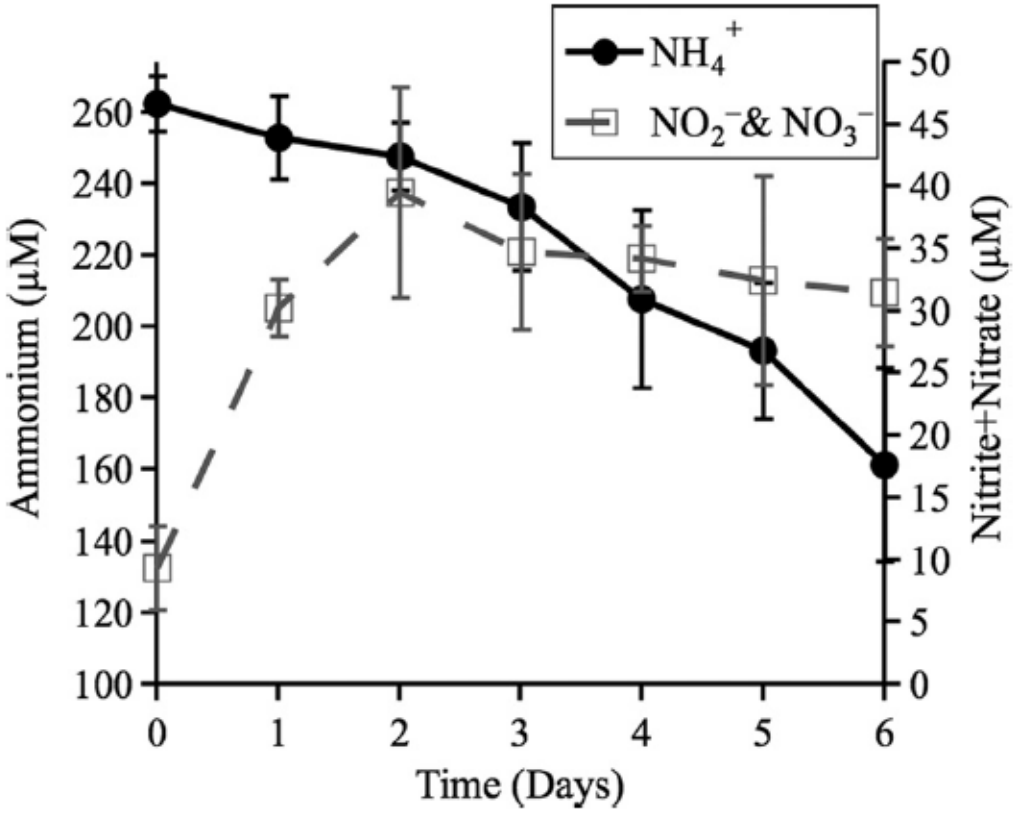

Figure 5.4 Changes in the concentrations of ammonium and nitrite plus nitrate in incubations for nitrification activity. Means and standard deviation values from two representative experiments (with four replications each) are shown.

CHAPTER 6
SUMMARY

6.1 Summary of results

The objectives of the research presented in this thesis were to examine the metabolic properties, phylogenetic diversity, and ecological functions of *Thaumarchaeota* in terrestrial environments using both cultivation-dependent and cultivation-independent techniques. The newly designated ammonia oxidizing *Thaumarchaeota* are ubiquitous and extremely abundant in diverse environments, and potentially play important roles in global nitrogen and carbon cycles. While archaea appear to have dominant roles in marine nitrification, possibly due to their adaptation to nutrient-poor environments, it is not clear whether this is the case in terrestrial environments. The work presented in this thesis focused on the *Thaumarchaeota* and their ecological impacts on nitrification in two terrestrial environments that were poorly characterized: freshwater sediments and the plant rhizosphere.

Freshwater systems are among the environments most impacted by anthropogenic activities, due to the large input of Nr through agricultural runoff (Rabalais, 2002; Galloway et al., 2004). Ammonia oxidizing archaea and bacteria potentially play important roles in controlling the availability of Nr. However, the abundance, diversity and activity of ammonia oxidizers in freshwater systems are not well characterized (Auguet et al., 2012; Hatzenpichler et al., 2012; Vissers et al., 2013). Furthermore, the key environmental factors influencing the distribution of AOA and AOB in freshwater environments are still not clear, although high AOA *amoA* abundance was detected in various rivers and lakes (Santoro et al., 2008; Herrmann et al., 2008; Liu et al., 2011;

Auguet et al., 2012; Hugoni et al., 2013). In addition, recent studies suggested that AOA are potentially important players in nitrification in the plant rhizosphere (Chen et al., 2008; Herrmann et al., 2008; 2009), which is often rich in organic material and characterized by enhanced microbial activities. A prior study using plant root enrichment cultures showed that a particular clade (C1b.A1) of 1.1b *Thaumarchaeota* was associated with plant roots (Simon et al., 2005). Whether the root-colonizing *Thaumarchaeota* could perform autotrophic ammonia oxidation, however, was not investigated previously. Investigating the diversity, distribution and activity of *Thaumarchaeota* from the plant rhizosphere would shed light on their metabolic potential. Taken together, the studies of these two terrestrial environments provide a more complete picture of *Thaumarchaeota* in the global nitrogen cycle.

Thaumarchaeota in CR freshwater sediments

In this thesis, I investigated key environmental factors shaping the distribution of AOA and AOB in Columbia River freshwater sediments, by linking biogeochemical variables with molecular data at a very fine vertical scale. Overall, based on *amoA* gene and gene transcript data, AOA always appeared to be more abundant than AOB regardless of season or depth, suggesting archaea are potentially important in nitrification in these sediments. Strong seasonal fluctuations were observed in ΔE_h and the concentrations of nitrogen species between summer and spring sediment cores. However, seasonal variability was not observed in either *amoA* abundance or expression. It is likely that I did not have enough samples representing different seasons to capture the temporal patterns of ammonia oxidizing assemblages. Nevertheless, a higher AOA to β-AOB ratio of *amoA* gene abundance was only observed in deeper sediments in summer,

potentially suggesting favorable growth conditions for AOA over β-AOB in deeper stratified sediments.

Correlation between molecular abundance and environmental parameters

Furthermore, Pearson's Correlation analysis was performed to examine the relationships among environmental variables and *amoA* gene and transcript abundance in AOA and β-AOB. In summer sediment, oxygen was shown to be positively correlated with *amoA* transcript abundance in AOA but not AOB. However, separation of AOA and AOB along an O_2 gradient—discovered in a few prior studies (Francis et al., 2005; Santoro et al., 2008; Beman et al., 2008; Lam et al., 2009)—was not observed in these sediments. Both AOA and AOB *amoA* transcript abundance decreased with depth in the surface sediments, corresponding to the oxygen depletion, suggesting a requirement of oxygen for expression of *amoA* genes. While several prior studies (Auguet et al., 2011; 2012; Auguet and Casamayor, 2013; S. Liu et al., 2013; Wu et al., 2013) also suggested that ammonium is an important factor influencing the niche diversification of AOA and AOB, ammonium was not observed to affect the distribution of AOA and AOB in CR freshwater sediments, most likely due to its relatively high concentration there. Furthermore, nitrate and nitrite were positively correlated with *amoA* gene abundance but negatively correlated with *amoA* transcript abundance. This finding suggested that using gene or gene transcript data alone to assess nitrification activity is usually not sufficient. Molecular data should be combined with other measurements if possible, such as in situ nitrification assays using nitrification inhibitors, to better assess potential nitrification activity.

Archaeal amoA gene phylogeny in CR freshwater sediments

Analysis of *amoA* phylogeny indicated that sequences from the dominant archaeal clade were similar to sequences recovered from other freshwater systems, and were associated with group 1.1a *Thaumarchaeota*. A member of this clade from freshwater was recently shown to carry out autotrophic ammonia oxidation in enrichment culture (French et al., 2012), indicating a potentially important role in nitrification. It is interesting to note that the group 1.1a *Thaumarchaeota* prevalent in freshwater systems do not appear to be the *Nitrosopumilus*-like archaea that are abundant in ocean waters (Auguet et al., 2011; 2012), but instead grouped together with sequences recovered from the same ecosystems harboring the "freshwater *amoA* ecotype," (Auguet et al., 2011; Cao et al., 2013).

High AOA diversity was associated with steep E_h gradients

Additionally, composition changes in putative AOA populations across steep O_2 and E_h gradients were observed by PCR-SSCP. Both PCR-SSCP results and CCA plots of data from summer sediment samples suggested that the AOA community composition was strongly correlated with ΔE_h. Specifically, higher AOA diversity was associated with steep E_h gradients, indicating that diverse *amoA* phylotypes in sediments were influenced by redox potential. It is tempting to speculate the steep E_h gradients might allow microorganisms occupying different ecological niches to compete for resources (Jones, 1979; Torsvik et al., 2002). For example, the studies of N_2-fixing enzyme (*nifH*) for diverse prokaryotic diazotrophs in microbial mats indicated broad phylogenetic potential for diazotrophs along steep E_h and O_2 gradients (Steepe et al., 1996; Paerl et al., 2000). It suggests that higher metabolic diversity may be present in environments in which rapid

changes in energy or nutrient sources are common, while diversity may be relatively low in stable environments with only a few substrate available.

AOB were the dominant ammonia oxidizers in CR sediment slurry incubations

The results of freshwater sediment slurry incubations suggested that AOB, rather than AOA, were the dominant group responsible for nitrification, which was the opposite of that inferred from the much higher abundance of AOA *amo*A genes and gene transcripts measured in sediment cores. Complete oxidation of ammonium to nitrate was observed with either inorganic or organic nitrogen amendment within 1 week in the absence of the ammonia oxidation inhibitor, ATU. At high ATU concentrations, with which AOB was mostly inhibited during the first 2 to 3 weeks, nitrification was suppressed until AOB growth recovered. AOA grew slightly better in the presence of ATU, as indicated by abundance and expression data, but no corresponding nitrification activity was observed. The significantly different responses of AOA and AOB to inhibitors and nitrification activity suggested that at least some AOA have different requirements for growth and/or ammonia oxidation than autotrophic AOB, and potentially utilize alternative survival strategies, e.g., heterotrophic nitrification. I added organic substrates to the incubations in attempts to test the ability of AOA to assimilate organic compounds. However, none of the results were conclusive for AOA, due to much higher growth rates of AOB. It is possible that under laboratory incubation conditions, AOA requires a much longer time to grow and also requires substantial AOB inhibition in order to show measurable nitrification activity. Furthermore, the results from the experiment examining incorporation of ^{13}C-labeled pyruvate were not conclusive, possibly because the

concentration of pyruvate used was too low, or because there was competition for pyruvate from other heterotrophs.

As mentioned earlier, the potential nitrification activity by AOA and AOB in the sediment incubations could not be directly related to their abundance and distribution in nature. Nevertheless, the comparison of *amo*A abundance data between incubations and sediment core measurements may shed light on potential nitrification activity in nature. In sediment incubations, abundance of archaeal *amo*A genes and gene transcripts was generally within the range measured in natural surface sediment (1–4 x 10^6 copies of the gene; 10^4-10^5 copies of the gene transcript). In contrast, for AOB, abundance of *amo*A genes and gene transcripts increased significantly in incubations in which nitrification occurred, much higher than their typical ranges measured in sediment cores. AOB *amoA* gene abundance measured in natural sediment cores (5 x 10^5~2 x10^6) was at a similar level with the abundance measured in incubations without significant nitrification activity (~1–2 x 10^6). In addition, it was noted earlier that in incubations, the AOB *amo*A gene and transcript abundance changed dramatically in response to ammonium availability (Table 4.6B and Table 4.7B), suggesting that AOB might be more sensitive to environmental changes. As a result, the overall AOB abundance might be relatively low in the environment, whereas AOA grow slowly, more resistant to environmental fluctuations, and maintain relatively high abundance. Nevertheless, AOA may still be important players in nitrification, given their high abundance in the sediments. Furthermore, a recent study of anaerobic toluene biodegradation suggested that using mRNA/DNA ratios of benzylsuccinate synthase (*bssA*) genes may provide more robust evidence for microbial activity, since basal level of *bssA* gene expression was always

detected in the absence of biodegradation activity (Brow et al., 2013). An activity threshold (mRNA/DNA) might also be useful indicator for nitrification activity, which need to be determined separately for AOA and AOB. Based on limited data from my potential nitrification activity assays, if the *amoA* transcript/gene ratio of AOB is smaller than 0.018, there was no corresponding nitrification activity. If this holds true in the environment tested here, the calculated bacterial *amoA* gene transcript/gene ratio in Columbia River sediments (0.001-0.016) suggests that AOB were not highly active when sediment cores were collected (July-2010, April and August-2012), although AOB *amoA* transcripts were detected (10^2-10^4) in all sediment cores.

Root-colonizing Thaumarchaeota in enrichment culture

In the plant-root enrichments, a previous study reported that a specific clade within group 1.1b *Thaumarchaeota* was selected in culture with root extract as a growth substrate (Simon et al.,2005). We further examined the molecular diversity and metabolic potential of soil *Thaumarchaeota* in enrichment culture, and their selective growth in response to organic and inorganic amendments. Clone libraries of archaeal 16S rRNA and *amoA* genes were constructed, sequences were generated and phylogenetic trees were analyzed. No AOB were detected in this system. Three *amoA*-containing clades were identified, while a fourth clade identified by 16S rRNA gene analysis alone, referred to as the "root clade," was associated with 1.1b *Thaumarchaeota* and yielded no corresponding *amoA* gene with the primers used. This is likely because that the primer set does not target root clade sequences, or the root clade does not contain an *amoA* gene. Analysis of archaeal community composition by PCR-SSCP under different culture conditions revealed that the root clade was present in media with organic amendment,

and absent in the incubations with only bicarbonate. Together with the qPCR results showing the selective growth of the root clade by root extract in incubations, these data suggested that the root clade archaea might be heterotrophs, assimilating organic carbon in root extract. With respect to the *amoA*–containing clades, the WaS clade archaea were present in media with either organic or inorganic amendment, and their growth was stimulated by root extract addition. This result suggested potential mixotrophic metabolism in this clade. The possibility of heterotrophy or mixotrophy being carried out by some *Thaumarchaeota* has been supported by both genomic and metabolic investigations (Hallam et al., 2006b; Blainey et al., 2011; Muβmann et al., 2011; Tourna et al., 2011). For example, genes involved in coding the oxidative TCA cycle, as well as a number of transporters for organic compounds, such as amino acids, were identified in several thaumarchaeal genomes (Walker et al., 2010; Blainey et al., 2011; Spang et al., 2012), indicating the genomic potential of *Thaumarchaeota* to assimilate small organic compounds. In addition, the metabolic ability to incorporate amino acids and other organic carbon compounds by some *Thaumarchaeota* has been suggested by results from several studies of archaea in the open ocean and pure culture (Herndl et al., 2005; Ouverney and Fuhrman, 2000; Ingalls et al., 2006; Varela et al., 2011; Tourna et al., 2011).

Potential nitrification activity in root-colonizing Thaumarchaeota

Furthermore, we explored the ability of rhizosphere archaea to perform autotrophic ammonia oxidation. The results from *amoA* gene abundance and expression analyses, together with potential nitrification assays, suggested differential contributions by the different clades to nitrification in our system. In particular, the growth of two 1.1b clades

corresponded to potential nitrification activity while expression of *amoA* genes was observed only for the WaS clade. In contrast, the *amoA*-containing group 1.1a archaea, present in the enrichments in low abundance, appeared to have no nitrification coupled growth. It is also interesting to note that in most studies of terrestrial environments the group 1.1b *Thaumarchaeota*, although often highly abundant in soils, were not the main autotrophic nitrifiers. Instead, the active AOA populations performing autotrophic ammonia oxidation were found to belong to group 1.1a, which is generally less abundant. Taken together, this suggests that at least some of group 1.1b *Thaumarchaeota* are not autotrophic ammonia oxidizers, and as a group, *Thaumarchaeota* have diverse metabolic lifestyles in soil *Thaumarchaeota*.

6.2 Implications and future directions

Correlation with activity

The global distribution of archaeal *amoA* genes, indicated by qPCR studies, suggested the potentially significant involvement of archaea in nitrification. It remains unclear whether the presence of abundant archaeal *amoA* genes indicates *in situ* nitrification activity in all environments. Results from incubation studies suggested that not all of the *Thaumarchaeota* are autotrophic ammonia oxidizers, and they may not be the dominant ammonia oxidizer in some freshwater systems. It is worth noting that the abundance of archaeal *amoA* genes or even gene transcripts could not be directly linked to nitrification activity in CR sediment slurry incubations. To comprehensively assess the functional roles of AOA and AOB, qPCR quantification of *amoA* gene and transcript abundance has to be combined with other approaches, such as stable isotopic labeling techniques and nitrification activity assays. Measuring nitrification activity in sediment microcosms

under conditions of low oxygen and ammonium may accurately reflect AOA activity *in situ*.

Heterotrophic potential

My studies, together with others, suggested that not all *Thaumarchaeota* are autotrophic ammonia oxidizers, and some *Thaumarchaeota* might be mixotrophs, heterotrophs or even heterotrophic nitrifiers. Heterotrophic nitrification is often overlooked due to the low associated nitrification rates. However, considering the potentially high abundance of heterotrophic nitrifiers, heterotrophic nitrification can be significant in organic-rich environments over long time periods (Robertson et al., 1988; Pedersen et al., 1999). Using specific inhibitors for autotrophic nitrification, such as acetylene, may facilitate the quantification of heterotrophic contributions to nitrification.

Characterization of the archaeal AMO enzyme

To elucidate the ammonia oxidation mechanism in archaea, isolation and characterization of the archaeal AMO enzyme is necessary. The biochemical characterization of this enzyme will provide insights into the mechanism of ammonia oxidation in archaea, and possibly shed light on additional functions for AMO. For example, the bacterial AMO enzyme has low substrate specificity, and although it is able to oxidize some organic compounds, does not generate energy from the oxidation (Hooper et al., 1997). It will be interesting to test the substrate range of the archaeal AMO enzyme.

Cultivating novel Thaumarchaeota strains

Furthermore, cultivating novel *Thaumarchaeota* strains from diverse environments is likely to reveal new physiology and metabolic properties, and might even reveal novel

processes involved in global geochemical cycles. To date, only a few strains of freshwater *Thaumarchaeota* have been obtained in enrichments, although AOA appear to be abundant in freshwater systems (French et al., 2012). The *Thaumarchaeota* freshwater ecotype might be important in nitrification, and possess different metabolic properties from marine and soil *Thaumarchaeota*.

Relationships between Thaumarchaeota and other microbes

In addition, *Thaumarchaeota* often coexist with other microorganisms, such as nitrite oxidizing bacteria and other heterotrophs (Mincer et al., 2007; Santoro et al., 2010; Taylor et al., 2010; Levicnik Hofferle et al., 2012). The physical and functional relationships between *Thaumarchaeota* and other microbes might be important to fully appreciate their roles in biogeochemical cycles, which remain to be fully explored.

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