TEMPORAL AND VERTICAL VARIABILITY OF AMMONIA OXIDIZING ARCHAEA IN THE SUBTROPICAL NORTH PACIFIC OCEAN

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ACKNOWLEDGEMENTS

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ABSTRACT

We sought to investigate environmental controls on two phylogenetically distinct Marine Group I (MGI) ammonia-oxidizing *Thaumarchaeae* at Station ALOHA, the field site for the Hawaii Ocean Time-series (HOT) program. Samples were collected throughout the water column (0-4000 m) on near-monthly cruises between 2006 and 2012. We utilized quantitative PCR amplification of ammonia monooxygenase subunit A (*amoA*) genes and gene transcripts to evaluate temporal variability in the abundances, transcriptional activities, and vertical distributions of ammonia-oxidizing MGI at Station ALOHA. We observed three to four orders of magnitude differences in the depth distributions of these organisms, with peak abundances occurring between 125 and 300 m. Our results revealed *amoA* gene abundances underwent moderate increases in the epipelagic waters (0-100 m) during periods of deeper winter mixing. MGI *amoA* genes near the base of the euphotic zone (100-200 m) were elevated in the fall and winter, suggesting seasonal decreases in irradiance in this region of the water column could partly regulate abundances. Examination of MGI *amoA* mRNA revealed peak transcriptional activity near the base of the euphotic zone (125-175 m). Our results provide new information on processes influencing the distributions and physiological activities of microorganisms mediating nitrification.
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CHAPTER 1. INTRODUCTION TO THE BIOGEOCHEMICAL AND
ECOLOGICAL ROLES OF THAUMARCHAEA IN THE MARINE NITROGEN CYCLE

1. Introduction

1.1. Marine nitrogen pools and processes

The growth and metabolism of planktonic marine microorganisms regulates the cycling of numerous bioelements. In large regions of the open sea, nitrogen (N) supply to the well-lit upper ocean regulates net ecosystem productivity and the carbon sequestration potential of these habitats. Hence, elucidating the processes that transform N in the marine environment, and studying the ecology of the organisms mediating N cycle processes are fundamental to our understanding of the functioning and health of ocean ecosystems.

Nitrogen is an essential element for life, required as component of protein, nucleotides, and other complex organic molecules, making N containing compounds required nutritional substrates for plankton growth and production. In the marine environment, N compounds are found in 5 oxidation states ranging from the most oxidized forms such as nitrate (NO$_3^-$; oxidation state $+V$; Figure 1), to reduced forms including organic N and ammonium (NH$_4^+$; oxidation state $-III$; Figure 1). The wide redox potential of N containing substrates makes them reactive components of numerous
metabolic reactions, including those where the N substrates serve as electron acceptors or donors.
Figure 1. Diagram showing various oxidation states of nitrogen and processes governing transformation of N among these redox states (modified from Gruber, 2008).
In the ocean, N is found in both organic and inorganic forms. Dinitrogen (N₂) is the most prevalent of the N substrates (Table 1); however, the stability of the N≡N triple bond renders this gaseous form of N unusable to most organisms except for certain prokaryotic N₂-fixers (called diazotrophs). The largest inventory of fixed N in the ocean is NO₃⁻ (Table 1), an oxidized substrate that can serve as both an essential nutrient to phytoplankton and plays an important role as a terminal electron acceptor during denitrifying chemoorganoheterotrophic metabolisms. Dissolved organic N (DON) comprises the largest reduced pool of N in the sea (Table 1), although the reactivity and turnover of bulk pools DON remains largely unknown. However, DON appears to serve as an important nutritional and perhaps energetic resource for both heterotrophic and autotrophic plankton. Ammonium can be assimilated as a nutrient for phytoplankton, or it may be used as a substrate for various chemolithoautotrophic processes, including ammonia (NH₃) oxidation and anaerobic NH₃ oxidation (Ward, 2008). Similarly, nitrite (NO₂⁻) is assimilated as a nutrient resource, and consumed as an energy source and as a terminal electron acceptor during biological N cycling processes such as NO₂⁻ oxidation and anaerobic NH₃ oxidation (anammox).
Table 1. Nitrogen inventories and ranges in concentrations of various pools of nitrogen at Station ALOHA in the North Pacific Subtropical Gyre. Global inventory estimates from Capone (2000).

<table>
<thead>
<tr>
<th>Species</th>
<th>Oceanic Inventory (Tmol N)</th>
<th>Upper Euphotic Station ALOHA (25 m) (µmol l⁻¹)</th>
<th>Upper Mesopelagic Station ALOHA (200 m) (µmol l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate (NO₃⁻)</td>
<td>4.1 × 10⁴</td>
<td>0.0003 – 0.04</td>
<td>0.9 – 7</td>
</tr>
<tr>
<td>Nitrite (NO₂⁻)</td>
<td>11</td>
<td>0.0002 – 0.009</td>
<td>0.004 – 0.08</td>
</tr>
<tr>
<td>Ammonia (NH₄⁺)</td>
<td>24</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Nitrogen gas (N₂)</td>
<td>7.1 × 10⁵</td>
<td>390*</td>
<td>450*</td>
</tr>
<tr>
<td>DON</td>
<td>5.5 × 10³</td>
<td>3.8 – 7.7</td>
<td>3.6 – 5.7</td>
</tr>
</tbody>
</table>

* Emerson et al., 1995
1.2. Nitrification in the sea

Nitrification describes the aerobic utilization of NH₃ as an energy source by microorganisms, resulting in the production of NO₂⁻ and subsequently NO₃⁻ (Ward, 2008). The first step of nitrification occurs when NH₃ is oxidized to NO₂⁻, which is then independently followed by NO₂⁻ oxidation to NO₃⁻:

\[
\text{NH}_3 + O_2 \rightarrow \text{NO}_2^- + 3H^+ + 2e^- \quad \text{(ammonia oxidation)}
\]

\[
\text{NO}_2^- + H_2O \rightarrow \text{NO}_3^- + 2H^+ + 2e^- \quad \text{(nitrite oxidation)}
\]

The two steps of complete nitrification are made possible by different groups of microbes, including obligate and facultative chemolithoautotrophic Archaea and Bacteria. The aerobic oxidation of NH₃ has a low energy yield (-10.8 kcal mol⁻¹; Morel, 1983), and a major fraction of the energetic demands of cultivated groups of chemolithoautotrophs appears devoted to CO₂ fixation (Forrest and Walker, 1971; Kelly, 1978). To fix one mole of CO₂, approximately 35 mol of NH₃ need to be oxidized (Ward 2008), suggesting that obligate nitrifying microorganisms grow slowly.

Studies on ocean nitrification often indicate rates of ammonia oxidation peak near the base of the euphotic zone (Olson 1981a, b; Dore and Karl, 1996a, b; Ward, 2005; Yool et al., 2007). The base of the euphotic zone at Station ALOHA, defined for this study as the depth where photosynthetically available radiation (PAR) declines to 1% of surface flux, occurs at 106 ± 8 m. Geochemical and immunofluorescent studies show that nitrifiers are active near the base of the euphotic zone and upper regions of the mesopelagic waters, coincident with the depth of the primary nitrite maximum (PNM;
Olson 1981a; Ward et al., 1982; Ward et al., 1989). In the North Pacific Subtropical Gyre (NPSG), rates of ammonia oxidation are estimated to range between 2.2 and 7.3 nmol N L\(^{-1}\) day\(^{-1}\), with peak rates occurring around the PNM (100 – 175 m; Olson 1981a). The upper primary nitrite maxima (UPNM) at Station ALOHA occurred near 110 m, where concentrations of NO\(_2^-\) range from 60 – 200 nM. A secondary, smaller peak in NO\(_2^-\) occurs near 150 m, termed the lower primary nitrite maximum (LPNM). The highest observed rates of nitrification 137 nmol N L\(^{-1}\) day\(^{-1}\) coincided with the LPNM (Dore and Karl, 1996a).

1.3. Nitrogen control of the biological carbon pump

The net transfer of carbon from the surface ocean to the deep sea is a major control on the size of the atmospheric CO\(_2\) reservoir. Thus, the processes that facilitate carbon storage in the ocean’s interior play an important role in regulating Earth’s climate. The biological carbon pump (BCP) describes the collection of processes that result in the production, export, and remineralization of organic material in the oceans. The BCP maintains the strong vertical gradient in dissolved inorganic carbon observed throughout the world’s oceans, hence understanding the processes that influence the strength and efficiency of the pump has direct bearing on the role of the ocean in the global carbon cycle. The supply of growth limiting nutrients appears to be among the most important factors controlling spatiotemporal variability in the BCP. In ecosystems where ocean productivity appears strongly limited by the availability of N, the assimilation of fixed N by plankton appears closely coupled to changes in productivity and growth. Euphotic
zone plankton productivity is fueled by allochthonous (external to the system) and autochthonous (internal to the system) sources of nutrients, with production fueled by allochthonous nutrients termed “new” and production sustained by autochthonous nutrients termed “regenerated” (Dugdale and Goering 1967). Assuming euphotic zone N inventories are in steady state over reasonable time and space scales, new production should be quantitatively balanced by N removal terms (*i.e.* export). Moreover, increases in plankton biomass or net productivity must be sustained by supply of allochthonous nutrients. In the open sea, where the terrestrial input of nutrients is limited, new production is sustained through entrainment of nutrient-enriched waters into the euphotic zone via mixing or upwelling, atmospheric deposition, and N\textsubscript{2} fixation (Dugdale and Goering, 1967). Such processes appear to fuel ~5 – 15% of gross productivity in these systems, with the remaining productivity (85 – 95%) fueled by active nutrient recycling by food web processes within the euphotic zone (Eppley and Peterson, 1979).

Early investigations aimed at quantifying rates of new and regenerated production traced rates of cellular assimilation of different forms of fixed N growth substrates into plankton biomass (Dugdale and Goering, 1967). Such studies considered NO\textsubscript{3} as the major allochthonous nutrient substrate, while NH\textsubscript{4}\textsuperscript{+} and urea were often used as model autochthonous substrates (Dugdale and Goering, 1967; Harrison *et al.*, 1996). These approaches provided initial estimates of new and regenerated production and broad-scale views of the interactions between nutrient supply and plankton productivity. However, a few key limitations and assumptions to these approaches were acknowledged including: 1) ignoring contributions of N\textsubscript{2} fixation in supporting new production, and 2) assuming euphotic zone nitrate was an allochthonous nutrient (and hence was not regenerated
within the euphotic zone). More recent work has questioned both of these assumptions. For example, in ecosystems where vertical supply of N appears limited, N\textsubscript{2} fixation can be a major contributor to new production (Karl et al., 1997, Michaels et al., 2001; Karl et al., 2002). The extent to which NO\textsubscript{3} is regenerated (via the process of nitrification) within the euphotic zone remains unclear. Evidence from a modeling study suggests that nitrogen regenerated in the euphotic zone supports 10 – 25% of primary production (Yool et al., 2007). If plankton assimilate nitrate that has been produced via nitrification within the euphotic zone, more of the production would be supported by regenerated production than predicted from studies that assume NO\textsubscript{3} uptake equates to new production (Figure 2). Moreover, if euphotic zone nitrification occurs at appreciable rates, use of nitrate as a tracer for new production would lead to an overestimation of carbon export by these ecosystems (Dugdale and Goering, 1967; Ward et al., 1989; Dore and Karl, 1996a).
Figure 2. Schematic depiction of new and regenerated production. Boxes represent different pools of N. Arrows represent transformations of N, mediated by phylogenetically diverse groups of microorganisms. Dotted arrows depict unresolved fluxes of nitrogen in the euphotic zone.
1.4. The role of marine Archaea in the N cycle

Application of cultivation-independent approaches to the study of picoplanktonic organisms has revealed that all three major domains of life, Bacteria, Eukarya, and Archaea, inhabit the sea (Giovannoni et al., 1990, DeLong, 1992, Fuhrman et al., 1992). Although Archaea had previously been considered halophiles, thermophiles, methanogens, and thermoacidophiles dwelling in extreme habitats, use of rRNA gene based tools revealed that members of the Archaea were prevalent in the marine environment (Fuhrman et al., 1992; DeLong, 1992). Two major phylogenetically distinct taxa were identified populating ocean waters; these groups are termed marine groups I and II Archaea. The marine group I (MGI) Archaea initially appeared mostly closely related to members of the extreme thermophilic Crenarchaea (DeLong, 1992; Fuhrman and Davis, 1997). Recent comparative genomic analyses provide evidence that the MGI Archaea are in fact phylogenetically distinct from Crenarchaea, comprising a new, deeply divergent phylum of Archaea termed Thaumarchaea (Brochier-Armanet et al., 2008). The marine group II (MGII) Archaea cluster phylogenetically with members of the Euryarchaea (containing methanogens, halophiles, and some thermophiles; DeLong, 1998). A group III Archaea also inhabit in the ocean and seem to be distantly related to Euryarchaea (Fuhrman and Davis, 1997; Lopez-Garcia et al., 2001), but their metabolism and ecological role in the marine environment is unknown and they seem exclusively relegated to the deep sea (Galand et al., 2009). Another distinct taxon also present only in deep ocean waters, Group IV Archaea, seem to cluster more closely with Haloarchaea than with Euryarchaea (Lopez-Garcia et al., 2001).
Archaea are ubiquitous in the marine environment, making them some of the most cosmopolitan members of the ocean plankton. Studies have documented their presence throughout near-shore and oceanic habitats, including in the central Pacific (DeLong et al., 2006; Church et al., 2010), Monterey Bay (DeLong et al., 1999), the Gulf of California (Beman et al., 2008), the Sargasso Sea (Beman et al., 2010), the Arctic Ocean (Christman et al., 2011), the Southern Ocean and Antarctic waters (DeLong et al., 1994; Murray et al., 1998; Massana et al., 1998; Church et al., 2003; Herndl et al., 2005; Alonso-Saez et al., 2011); and the Black Sea (Francis et al., 2005). One approach to enumerating planktonic Archaea involves hybridizing the cells with group-specific fluorescently-labeled nucleotide probes to ribosomal RNA genes and counting total cell abundances by microscopy (DeLong et al., 1999). Adopting this approach, Karner et al. (2001) suggested pelagic Thaumarchaea might represent some of the most abundant cell types in the ocean, with an estimated $10^{28}$ cells comprising about 20% of the total picoplankton in the world’s oceans. Thaumarchaeal abundances vary strongly with depth, often increasing three to four orders of magnitude between the near-surface ocean and the lower mesopelagic zone (DeLong et al., 1999, Karner et al., 2001, Mincer et al., 2007; Church et al., 2010). Thaumarchaeal abundances in the epipelagic often range $\sim 10^7$ cells L$^{-1}$, typically increasing through the lower euphotic zone, and ranging from $\sim 10^5$ to $\sim 10^8$ cells L$^{-1}$ in the meso- and bathypelagic regions of the ocean’s interior (DeLong et al., 1999, Karner et al., 2001). Although less is known about the MGII Archaea, these organisms are also abundant, with elevated abundances often reported in the euphotic zone (Murray et al., 1999; Massana et al., 2000), where the MGII can comprise 2 – 10% of the total picoplankton cells ($\sim 10^7$ to $10^8$ cells L$^{-1}$).
Although early gene surveys provided phylogenetic information about *Archaea*, the ecological and biogeochemical role these organisms played in marine ecosystems remained largely unknown until the early 2000s. Carbon isotope analyses of archaeal lipids (Pearson et al., 2001; Ingalls et al., 2006), incubation-growth experiments (Ouverney and Fuhrman, 2000; Herndl et al., 2005; Teira et al., 2006; Wüchter et al., 2006; Kirchman et al., 2007), and reconstructions of archaeal genomes and metagenomes (DeLong et al., 2006; Hallam et al., 2006a,b; Martin-Cuadrado et al., 2008; Walker et al., 2010; Iverson et al., 2012) have all provided new insights into metabolic strategies employed by these organisms. Such analyses support metabolic strategies including chemolithoautotrophy and chemoorganoheterotrophy (Ouverney and Fuhrman, 2000; Herndl et al., 2005; Hallam et al., 2006b; Kirchman et al., 2007; Varela et al., 2008; Walker et al., 2010; Iverson et al., 2012). Notably, genomic and metagenomic analyses have revealed that MGI *Thaumarchaea* appear to utilize a modified 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycle for CO₂-fixation (Hallam et al., 2006b; Walker et al., 2010; Martin-Cuadrado et al. 2008; La Cono et al., 2013). In addition, these organisms appear to possess various genes that suggest some capacity for organoheterotrophy, including genes encoding transporters for peptides, amino acids, and glycerol (Walker et al., 2010).

In the early 2000’s, two separate studies provided important clues into energy sources supporting chemoaerotrophy by marine and terrestrial *Thaumarchaea*, namely the identification of ammonia monooxygenase (*amoA*) genes. In nitrifying *Bacteria*, the ammonia monooxygenase enzyme catalyzes the aerobic oxidation of ammonia to hydroxylamine, the initial step in the oxidation of NH₃ to NO₂⁻. The protein has three
subunits (α, β, and γ) that are encoded by the amoABC genes, respectively. In 2004, reconstruction of genomic scaffolds from plankton collected in the near-surface waters of the Sargasso Sea revealed an amoA gene on the same scaffold containing a thaumarchaeal 16S rRNA gene (Venter et al., 2004). In a separate soil study, Treusch et al. (2005) reported results from sequencing a large insert from a fosmid library that contained a Thaumarchaea 16S rRNA gene together with amoAB genes. However, the specific pathways utilized for NH₃ oxidation remains unclear; genomic and metagenomic studies indicate the Thaumarchaea lack genes that encode hydroxylamine oxidoreductase (as in bacteria), leading to the hypothesis that the organisms may oxidize NH₃ to nitroxyl (HNO) as an intermediate to the production of NO₂⁻ (Walker et al., 2010; Pester et al., 2011). One hypothesized implication of this finding is that the nitroxyl pathway uses 0.5 mol O₂ per mol NH₃ oxidized (Schleper and Nicol, 2010), potentially allowing Thaumarchaeae to oxidize NH₃ in low oxygen environments (Erguder et al., 2009; Pester et al., 2011). Although historically various groups of Bacteria were considered to be the predominant NH₃ oxidizing microorganisms in the ocean (Ward, 1990); these discoveries provided the first glimpses that Thaumarchaeae might play a previously unrecognized role in N cycling.

Our understanding of the physiologies and biogeochemical influence of MGI Thaumarchaeae has been aided by several important cultivation-based efforts. Despite years of effort, obtaining representative members of the dominant marine Archaea has proven challenging. However, several recent efforts have proven successful and yielded valuable information for improving culturing-based efforts. In particular, a survey of
microorganisms inhabiting the temperate marine sponge *Axinella mexicana* revealed that a member of the MGI *Thaumarchaea*, named *Cenarchaeum symbiosum*, comprised up to 65% of the biomass of the sponges’ resident microbiota (Preston *et al.*, 1996). The enrichment of MGI biomass in this organism enabled a targeted metagenomic reconstruction of the *C. symbiosum* genome (Hallam *et al.*, 2006 a, b). These analyses revealed that the resulting genomic reconstruction represented a composite of two ribosomal variants, termed type A and B (Schleper *et al.*, 1998), indicative of at least two similar, but distinct *C. symbiosum* populations inhabiting the sponge. The resulting genome supported a chemoautotrophic physiology by these organisms, and highlighted both NH\(_3\) and urea as potential energy sources supporting their growth (Hallam *et al.*, 2006 a, b).

Further insights into the physiology of these organisms came from the successful isolation of a member of the MGI *Thaumarchaea*, named *Candidatus Nitrosopumilus maritimus*, from a saltwater aquarium (Könneke *et al.*, 2005). Phylogeny based studies on the *amoA* genes of *N. maritimus* and *C. symbiosum* indicate both these organisms represent a subcluster of *amoA Thaumarchaea* that is distinct from the prominent MGI *Thaumarchaea* often observed in the ocean. Laboratory studies with *N. maritimus* indicated this organism can grow chemolithoautotrophically (at growth rates of \(~0.65 \text{ d}^{-1}\) as an obligate aerobic NH\(_3\) oxidizer, stoichiometrically converting NH\(_3\) to NO\(_2^-\) (Könneke *et al.*, 2005). Further studies on this isolate indicated that this organism demonstrates high NH\(_3\) oxidation rates at low (nanomolar) concentrations of NH\(_3\), with a half saturation constant for growth on NH\(_3\) of \(~130 \text{ nM N} \) and oxidation rates of \(~13 \text{ fmol NH}_3 \text{ cell}^{-1} \text{ d}^{-1}\) (Martens-Habbena *et al.*, 2009). Such results suggest MGI *Thaumarchaea*
are well adapted to growth in oligotrophic environments, and likely compete efficiently with phytoplankton and bacteria at limiting concentrations of NH$_3$. Additional cultivation-based efforts from the Eastern North Pacific Ocean recently yielded three MGI *Thaumarchaeal* enrichment cultures (Santoro and Casciotti, 2011) whose *amoA* gene phylogenies cluster among MGI *Thaumarchaeas* frequently retrieved from marine clone libraries. Like *N. maritimus*, all three enrichments grew chemoautotrophically using NH$_3$ as an energy source albeit at lower growth rates ($\sim$0.15 d$^{-1}$) and lower per cell rates of NH$_3$ oxidation ($\sim$2 fmol NH$_3$ cell$^{-1}$ d$^{-1}$; Santoro and Casciotti, 2011).

These discoveries led to numerous efforts to characterize the diversity and prevalence of *amoA* containing *Thaumarchaeas* in the ocean (Francis *et al.*, 2005; Wüchter *et al.*, 2006; Mincer *et al.*, 2007; Beman *et al.*, 2008; Church *et al.*, 2010). Such studies generally suggest that NH$_3$ oxidizing *Archaea* are more abundant than nitrifying *Bacteria*, and constitute a major fraction of planktonic biomass in the deep sea (DeLong *et al.*, 1999; Karner *et al.*, 2001; Wüchter *et al.*, 2006; Mincer *et al.*, 2007). Development of quantitative polymerase chain reaction (qPCR) based approaches to amplify *amoA* genes has provided support of ecotypic vertical segregation of these microorganisms (Beman *et al.*, 2008). Analyses of *amoA* gene phylogeny revealed at least two vertically segregated groups of *Thaumarchaeas* (termed water column ‘A’ and water column ‘B’; WCA and WCB, respectively), with WCA generally restricted to the lower euphotic zone and upper mesopelagic waters (<500 m) and WCB most prevalent in the lower mesopelagic and bathypelagic waters (Mincer *et al.*, 2007; Beman *et al.*, 2008). In addition, studies examining *amoA* gene expression have provided insight into the transcriptional activities of these microorganisms (Friaz-Lopez *et al.*, 2008; Church *et al.*, 2010).
2010; Santoro et al., 2010). In a study examining spatial patterns of thaumarchaeal distributions and gene expression in the central Pacific Ocean amoA transcript abundances were often low in the well-lit regions of the euphotic zone, increasing more than an order of magnitude into the dimly lit waters of the lower half of the euphotic zone (Church et al. 2010), consistent with reported elevated rates of NH₃ oxidation coinciding with the top of the nutricline (Olsen, 1981a, b; Ward et al., 1989; Dore and Karl, 1996a; Santoro et al., 2010).

1.5. Major objectives of master’s thesis

Despite the rapidly expanding set of measurements and field experiments on the biogeochemistry and ecology of MGI Thaumarchaea, to date we have limited information on the factors controlling the distributions and abundances of these functionally important microorganisms in the open sea. To address this issue, I sought to investigate temporal and vertical variability in NH₃ oxidizing Thaumarchaea at Station ALOHA (A Long-term Oligotrophic Habitat Assessment) (22°45’N, 158°W), the field site for the Hawaii Ocean Time-series (HOT) program, to provide insight on environmental controls on these organisms. Quantitative PCR amplification of amoA genes and transcripts provided insight into temporal variability in the distributions of ammonia oxidizing microorganisms.

By quantifying amoA gene abundances of both the WCA and WCB clades of Thaumarchaea at Station ALOHA, from the near-surface to the bathypelagic waters, I sought to examine temporal changes associated with depth-dependent partitioning of
these organisms. Ammonia oxidizing *Thaumarchaea* are some of the most abundant organisms in the ocean (DeLong *et al.*, 1999; Karner *et al.*, 2001; Wüchter *et al.*, 2006; Mincer *et al.*, 2007; Beman *et al.*, 2008), and examining their depth distributions provides more information regarding their biogeochemical role in the marine environment.
CHAPTER 2. TEMPORAL AND VERTICAL VARIABILITY OF AMMONIA OXIDIZING ARCHAEA IN THE NORTH PACIFIC SUBTROPICAL GYRE

2. Introduction

2.1. Oceanic nitrification

In the world’s oceans, plankton growth and accumulation of biomass is limited by the supply and availability of nitrogen (N). Rapid recycling of bioessential nutrients largely supports plankton productivity in such ecosystems; hence, studying the processes underlying nutrient recycling is critical to our understanding of productivity in the sea. Nitrification is the two-step, microbially mediated process that converts ammonia (NH$_3$) to nitrate (NO$_3^-$). During the first step of nitrification, NH$_3$ is oxidized to nitrite (NO$_2^-$), while the second step oxidizes NO$_2^-$ to NO$_3^-$. The combined nitrification processes are fundamental to the global N cycle, and as such, exert important control on the cycling of other bioelements in the biosphere. Nitrification converts reduced N to an oxidized bi-product (NO$_3^-$), thereby forming a link between organic matter degradation (and concomitant NH$_3$ formation) and N loss processes (annamox and denitrification).

The importance of nitrification to ocean biogeochemistry has resulted in intensive efforts to study both the process and the organisms mediating this process. In the well-oxygenated oceans, the coordinated growth of nitrifying microorganisms results in concentrations of NO$_3^-$ in the deep sea that can be several orders of magnitude greater than observed in the well-lit upper ocean (Capone, 2000; Karl et al., 2008). Nitrification
may also play an important role in global climate; incomplete oxidation of NH$_3$ to NO$_2^-$ can result in production of nitrous oxide (N$_2$O), a potent heat-trapping gas. In large regions of the open sea, nitrification appears to be a major control on air-sea N$_2$O fluxes (Elkins et al., 1978; Kim and Craig, 1990; Dore et al., 1998; Ostrom et al., 2000; Popp et al., 2002; Santoro et al., 2011).

The persistent occurrence of a NO$_2^-$ maxima in well-oxygenated seawater has motivated a number of studies to assess the contribution of NH$_3$ oxidation to this feature (Ward et al., 1982; Dore and Karl, 1996a; Lomas and Lipschultz, 2006). In the ocean, metabolic processes known to produce NO$_2^-$ include: incomplete assimilatory reduction of NO$_3^-$ by photoautotrophs (Vaccaro and Ryther, 1960; Wada and Hattori, 1972; Keifer et al., 1976), dissimilatory reduction of NO$_3^-$ (Brandhorst, 1959; Fiadeiro and Strickland, 1968; Codispoti and Richards, 1976), and NH$_3$ oxidation (Brandhorst, 1959; Olson, 1981b; Ward, 1990). In well-oxygenated waters, rates of denitrification are assumed to be low (Karl et al., 1984); hence, likely sources of NO$_2^-$ in well oxygenated oceanic ecosystems are presumed to be incomplete NO$_3^-$ assimilation and NH$_3$ oxidation (Brandhorst, 1959; Vaccaro and Ryther, 1960; Kiefer et al., 1976; Olson, 1981b; Dore and Karl, 1996a).

### 2.2. Station ALOHA and nutrient cycling in the oligotrophic ocean

The North Pacific Subtropical Gyre (NPSG) is the largest ecosystem on planet Earth (Sverdrup et al., 1946). The region is characterized by persistently oligotrophic conditions in the near surface waters, a condition resulting from stratification of the upper
ocean, together with a perennially high irradiance and rapid plankton growth. In 1988, Hawaii Ocean Time-series (HOT) program began near-monthly measurements on temporal variability associated with pools and fluxes of carbon and other biogenic elements at the open ocean field site Station ALOHA (A Long-term Oligotrophic Habitat Assessment; 22°45’N, 158°W; Figure 3). The resulting HOT program sampling of this region provides a unique view of seasonal and inter-annual variability associated with physical, biogeochemical, and ecological dynamics in the oligotrophic NPSG. The epipelagic waters (<200 m) at Station ALOHA are characterized by warm near-surface waters (ranging ~23°C during winter to ~26°C during summer), relatively shallow mixed layers averaging 87 m (ranging 40 m – 110 m) in the winter and 49 m (ranging 35 m – 78 m) during summer, low concentrations of nitrate in near-surface waters (<10 nM), a persistent deep chlorophyll a (chl a) maximum layer occurring near ~125 m, and low concentrations of plankton biomass (1 – 2 μmol C L⁻¹; Karl, 1999).
Figure 3. Satellite (SeaWIFS) ocean color view of the North Pacific Subtropical Gyre (in red circle). Location of Station ALOHA (22°45’N, 158°W) depicted by the red star. Image obtained from the NASA Goddard Space Flight Center’s SeaWIFS Project.
The persistent oligotrophic upper ocean at Station ALOHA has made it a focal point for studies on oceanic nutrient cycling. Several studies have specifically examined the process of nitrification and nitrifying microorganisms. The moderate to weak seasonal fluctuations in solar irradiance, upper ocean temperatures, and mixed layer depths appear to play important roles in structuring plankton ecology and biogeochemistry in the NPSG. For example, phytoplankton in both the well-lit regions of the upper ocean and in the dimly-lit deep chlorophyll maximum layer appeared to undergo photoadaptive responses to seasonal changes in irradiance. In the upper euphotic zone (0 – 50 m), chlorophyll a (chl a) concentrations increase in the winter and decrease in the summer as part of a photoadaptive response to seasonal changes in surface irradiance (Letelier et al., 1993; Winn et al., 1995; Letelier et al., 2004). In contrast, in the lower euphotic zone (100 – 175 m), chl a concentrations exhibit greater concentrations in the spring, in response to increasing light intensities (Letelier et al., 1993; Winn et al., 1995). Moreover, seasonal changes in the penetration of light to depth appear to directly control vertical distributions of nutrients in the lower epipelagic zone. During winter months, NO$_3^-$ accumulates between 100 – 130 m concurrent with a decrease in the average daily photon flux (Letelier et al., 2004). Concentrations of NO$_2^-$ range from undetectable (<2 nmol N L$^{-1}$) in near-surface waters to as high as 200 nM at the primary nitrite maxima (PNM; ~150 m). Distributions of NO$_2^-$ in the mesopelagic zone are typically low (<5 nM; Dore and Karl, 1996a). Near the persistent oxygen minimum zone (Bingham and Lukas, 1996), an anomalous two-point NO$_2^-$ maximum has been described, occurring between 815 – 825 m; this feature may derive from partial denitrification in lieu of aerobic metabolisms (Dore and Karl, 1996a).
2.3. Characterization of nitrifying Archaea in the sea

Historically, studies on aquatic nitrifying microorganisms have relied on cultivation-dependent approaches to isolate and characterize the physiological activities of these organisms. These early studies suggested that naturally occurring nitrifiers were chemolithoautotrophs, utilizing energy gained from the aerobic oxidation of ammonia or nitrite to fuel cellular synthesis, and fixing inorganic carbon (Winogradsky 1892; Watson, 1965; Koops et al., 1991). Such cultivation-dependent approaches revealed that marine nitrifiers appeared to be slow growing, with generation times reported to be on the order of several days (Kaplan, 1983; Watson et al., 1989). Further research yielded isolates of NH$_3$ oxidizing Bacteria (AOB) belonging to various genera of gamma- and beta- Proteobacteria (Head et al., 1993; Teske et al., 1994; Hiorns et al., 1995; Voytek and Ward, 1995; Hovanec and DeLong, 1996). Based on these studies, a number of approaches were developed to study the distributions and abundances of AOB. Use of immunofluorescent probes to enumerate AOB revealed that these organisms exhibited little vertical structure and were found at very low relative abundances as free-living cells in the marine environment (<1% of total bacteria; Ward, 1982; Ward, 1984).

The application of cultivation-independent approaches has fundamentally changed our understanding of the organisms responsible for oceanic nitrification. The existence of planktonic Archaea in the ocean has been known for more than 20 years (Fuhrman et al., 1992; DeLong, 1992), although the specific roles these organisms play in ocean biogeochemistry only began to emerge in the past decade. Analyses of rRNA
genes retrieved from picoplankton in polar and temperate marine ecosystems revealed members of the Crenarchaeota (later reclassified as Thaumarcheota; Brochier-Armanet et al. 2008) and Euryarchaeota (Murray et al., 1998; Massana et al., 1998; Murray et al., 1999; Massana et al., 2000). These studies indicated that the Thaumarcheota were often abundant (20-30% of 16S rRNA genes or picoplankton cells) inhabitants of meso- and bathypelagic waters (DeLong et al., 1994, Murray et al., 1998; Massana et al., 1998; DeLong et al., 1999).

Use of polynucleotide rRNA gene probes and fluorescence in situ hybridization (FISH) allowed Karner et al. (2001) to examine temporal variability associated with planktonic Bacteria, Euryarchaeota, and Thaumarcheota at Station ALOHA from near-monthly samples collected between September 1997 and December 1998. The abundances of these organisms varied among the different phylotypes investigated; Bacteria dominated the upper 150 m of the water column, representing up to 90% of the total prokaryotic cells, and decreased with depth, comprising 35 – 40% of picoplanktonic cells in the meso- and bathypelagic waters. Euryarchaeota were occasionally present in near surface waters, but typically represented a few percent of the total picoplankton cell abundance, while Thaumarchaeota were sporadically observed in the epipelagic waters, but increased sharply to ~5 × 10^7 cells L^{-1} between 100 – 150 m, and decreased with increasing depth to ~5 × 10^6 cells L^{-1} in the bathypelagic (Karner et al., 2001).

Clues to the potential role of Thaumarchaeota in nitrification emerged during a metagenomic sequencing effort from the surface waters of the Sargasso Sea when Venter et al. (2004) retrieved an ammonia monooxygenase gene occurring on the same scaffold as a thaumarchaeal 16S rRNA gene. Shortly after, Francis et al. (2005) analyzed PCR
amplified thaumarchaeal amoA genes from diverse aquatic environments, and found ubiquitous and diverse assemblages of these organisms. These preliminary genomic results motivated new cultivation-based approaches eventually leading to the first Thaumarchaea ammonia-oxidizing isolate, Candidatus Nitrosopumilus maritimus, which was isolated from a saltwater aquarium (Könneke et al., 2005). This chemolithoautotrophic organism demonstrated a near-stoichiometric conversion of NH₃ to NO₂⁻ (Könneke et al., 2005) when grown on bicarbonate as a sole source of carbon.

Shortly after the first pure culture of an ammonia-oxidizing Archaea was obtained, a North Sea surface water enrichment of pelagic Thaumarchaea were shown to oxidize NH₃ to NO₂⁻ (Wüchter et al., 2006).

These discoveries motivated further efforts to characterize the diversity and abundance of ammonia oxidizing Thaumarchaea in the ocean (Mincer et al. 2007; Beman et al., 2008; Church et al., 2010). Results from quantitative assessments revealed that ammonia oxidizing Archaea constitute a significant amount of picoplanktonic biomass in the deep sea, and are more abundant than nitrifying Bacteria (DeLong et al., 1999; Karner et al., 2001; Wüchter et al., 2006; Mincer et al., 2007). Quantitative PCR (qPCR)-based approaches targeting thaumarchaeal 16S rRNA and amoA genes at Station ALOHA indicated amoA thaumarchaeal gene abundances were >2 orders of magnitude greater than bacteria that possessed the amoA gene (Mincer et al., 2007). Moreover, the estimates of qPCR-derived amoA gene abundances supported previous findings (Karner et al., 2001) that thaumarchaeae are low in abundance in the upper regions of the epipelagic, increasing sharply below 100 m (Mincer et al., 2007). Phylogenetic analyses on archaeal amoA genes revealed two vertically segregated groups of Thaumarchaea.
(termed water column A and B, or WCA and WCB, respectively); WCA tend to predominate in the epipelagic and upper mesopelagic (<500 m), while WCB appear to dominate meso- and bathypelagic waters (Mincer et al., 2007; Beman et al., 2008).

My thesis focuses on the ecology of nitrifying *Thaumarchaea* at Station ALOHA. Specifically, I sought to investigate the temporal and vertical variations in the distributions and physiological activities of WCA and WCB *Thaumarchaea* based on near-monthly time series analyses of *amoA* genes and gene expression. The suite of biogeochemical properties measured by the HOT program (Karl and Lukas, 1996) provided insight into environmental controls on the distributions of these organisms. Examining the vertical distributions of both WCA and WCB phylotypes provided insight into apparent depth-dependent niche-partitioning by these two phylogenetically distinct *amoA* gene-containing *Thaumarchaea*. The near-monthly sampling intervals provided information on both seasonal and inter-annual variability in the distributions and abundances of ammonia oxidizing *Thaumarchaea*.  

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3. Methods

3.1. Sample collection

This project relied on near-monthly sampling at Station ALOHA (22°45’N, 158°W) to quantify *Thaumarchaea amoA* gene abundances and transcripts. Beginning in March 2006, samples for subsequent extraction of planktonic nucleic acids were collected from 16 discrete depths (5, 25, 45, 75, 100, 125, 150, 175, 200, 300, 500, 770, 1000, 2000, 3000, 4000 m). Seawater was collected using 24 ten-liter polyvinyl chloride sampling bottles attached to a Sea-Bird Conductivity-Temperature-Depth (CTD) rosette sampling system (Karl and Lukas, 1996). Seawater was subsampled from the CTD rosette into acid-rinsed 4.5 L polycarbonate bottles or 10 L polyethylene carboys. Two to five liters of seawater were filtered onto inline 25 mm diameter 0.2 µm pore size Supor® polyethersulfone filters using a peristaltic pump. For samples collected between HOT 180 and 239 (March 2006 – January 2012) filters were placed in 2 mL microcentrifuge tubes containing either 500 µL RLT buffer (Qiagen RNeasy) with 1% (v/v) final concentration β-mercaptoethanol and 100 mg 0.1 mm zirconium-silica glass beads (for subsequent RNA extraction), or 500 µL lysis buffer (20 mM Tris-HCl, pH 8.0; 2mM EDTA, pH 8.0; 1.2% Triton X and 20 mg mL⁻¹ lysozyme) and 100 mg 0.1 mm zirconium-silica glass beads (for subsequent DNA extraction). Sample filters collected from HOT cruises 240 – 249 (March 2012 – February 2013) were stored in 400 µL buffer AP1 (Qiagen DNeasy Plant Mini Kit) with a mixture of 100 mg 1:1 by volume; 0.1 mm and 0.5 mm zirconium-silica glass
beads. All DNA and RNA samples were immediately flash frozen in liquid nitrogen and stored at -80°C until processed in the shore-based laboratory.

3.2. DNA extraction procedures

Two different DNA extraction procedures were used during the course of this study. For those samples collected from HOT cruises 180 – 239 (March 2006 – January 2012), DNA was extracted following slight modifications to the manufacturers protocols for the Qiagen DNeasy Blood and Tissue Kit protocol (Figure 4). Briefly, in the shore based laboratory, microcentrifuge tubes containing sample filters and lysis buffers were thawed at room temperature, then homogenized using a Fast Prep machine (Bio101, Carlsbad, CA, USA) and mechanically agitated for 1.5 minutes at 3450 vibrations per minute. Following this bead beating step, samples were placed in a hybridization oven at 37°C for 1 h and vortex mixed at highest speed for 15 seconds every 15 minutes. After this initial incubation, 42 µL of Proteinase K and 334 µL of lysis buffer AL (Qiagen DNeasy Blood and Tissue kit) were added to each sample. Samples were vortexed and placed in a hybridization oven at 70°C for 30 minutes. Following this incubation, 334 µL of 100% ethanol was added to each sample, and the microcentrifuge tubes were vortexed and supernatants were transferred by pipette onto silica gel membrane Qiagen DNeasy spin columns; care was used to ensure that zirconium-silica beads were not added to the spin columns. After centrifugation at 5,900 × g for 60 seconds, 500 µL buffer AW1 (Qiagen) was added and centrifuged at 5,900 × g for 60 seconds. Following that step,
500 µL buffer AW2 (Qiagen) was added and centrifuged at 16,100 × g for three minutes. Residual ethanol was then evaporated at 70°C for 10 minutes. Two hundred microliters of preheated buffer AE (Qiagen) was added to the spin column and eluted into 1.5 mL centrifuge tubes. The purified DNA in buffer AE was then stored at -80°C pending subsequent analyses.

Sample filters for subsequent extraction of DNA collected from HOT cruises 240 – 254 (March 2012 – February 2013) were extracted using a modified Qiagen DNeasy Plant Mini Kit (Figure 4). Microcentrifuge tubes containing sample filters were thawed and homogenized in a bead-beater for 2 minutes at highest bead-beating speed. Forty five microliters of Proteinase K was added to each sample and samples were placed in a hybridization oven at 55°C for 1 hour, vortexing every 15 minutes. Samples were then treated with 4 µL RNase A, and incubated at 65°C for 10 minutes. One hundred thirty microliters buffer P3 (Qiagen) was added to each sample to precipitate proteins and polysaccharides, and samples were centrifuged at 20,817 × g for 5 minutes. Supernatants were applied to a QIAshredder Mini Spin Column and centrifuged at 20,817 × g for an additional 2 minutes. Seven hundred fifty microliters buffer AW1 (Qiagen) was added to the lysate and contents were transferred to a silica gel membrane DNeasy Mini Spin Column and centrifuged at 5,900 × g for 1 minute. Five hundred microliters of buffer AW2 (Qiagen) was added to each column, and the columns were centrifuged at 5,900 × g for 1 minute, followed by an additional wash step with buffer AW2 (containing ethanol), where columns were centrifuged at 20,817 × g for 2 minutes to completely dry the membrane and remove residual ethanol. Spin columns were then transferred to sterile 1.5 mL microcentrifuge tubes.
DNA was eluted through two sequential additions of 100 µL buffer AE (Figure 4). Extracts were stored at -80°C until subsequent analyses. DNA concentrations were determined fluorometrically using the Quant-iT® DNA High-sensitivity assay kit (Invitrogen, Carlsbad, CA, USA) and Perkin Elmer 2030 Multilabel Plate Reader.
Figure 4. DNA samples collected from HOT cruises 180 – 239 (March 2006 – January 2012) were extracted following slight modifications to the manufacturers protocols for the Qiagen DNeasy Blood and Tissue Kit protocol (left). DNA samples collected from HOT cruises 240 – 254 (March 2012 – February 2013) were extracted using a modified Qiagen DNeasy Plant Mini Kit (right).
3.3. RNA extraction and reverse transcription

Extraction of total RNA from planktonic cells followed protocols described in the Qiagen RNeasy Mini Kit. Samples containing filters in RLT lysis buffer for subsequent extraction of total RNA were homogenized for three-30 second intervals with 1.5 minute periods of cooling on ice between each agitation. Two hundred fifty microliters of ethanol (100%) was then added to each sample, and samples were pipetted onto Qiagen RNeasy Mini spin columns. Columns were spun for 15 seconds at 20,817 × g and decanted. Three hundred fifty microliters buffer RW1 (Qiagen) was added to each spin column and centrifuged at 20,817 × g for 15 seconds. Samples were treated with DNase I (to degrade residual DNA) following the Qiagen On-Column DNase I® RNA extraction protocol. Another application of 350 µL buffer RW1 was used to rinse each sample after the DNase treatment. Five hundred microliters of buffer RPE (Qiagen) was added to each column and centrifuged twice to remove residual salts. Samples were then eluted with 50 µL RNase-free water and stored at -80°C until subsequent analyses.
3.4. PCR amplification and cloning of thaumarchaeal amoA genes

Archaeal amoA diversity was examined via the construction of PCR amplified amoA gene clone libraries. Thaumarchaeal amoA gene fragments were amplified by PCR in 50 ml reactions using the following mix (final concentrations): 1X reaction buffer; 0.2 mM dNTPs; 0.2 µM forward and primers (Cren_amoAF and CrenAmoAModR (Table 2); 0.3 µg µL⁻¹ bovine serum albumin (BSA); 2 mM MgSO₄; 1.0 unit Platinum® Taq. Thermal cycling conditions were as follows: 5 minutes initial denaturation at 95°C; followed by 30 cycles of 94°C for 1 minute, 53°C for 1 minute, 72°C for 1 minute; and a 15 minute final extension at 72°C. Amplification products were ligated into pCR®4-TOPO ampicillin-resistant cloning vectors and transformed into Invitrogen™ TOP10 Chemically Competent E. coli. Clones were isolated and plasmids containing the PCR amplified inserts were Sanger sequenced using at the Advanced Studies in Genomics, Proteomics and Bioinformatics sequencing facility (University of Hawaii at Manoa).
Table 2. List of primers used for both PCR and qPCRs. All reactions performed using the CrenAmoAModR reverse primer.

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Sequence (5’ – 3’)</th>
<th>Annealing temp. (˚C)</th>
<th>Expected amplicon (bp)</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CrenAmoAModR</td>
<td>AAGCGGCCATCCATCTGTA</td>
<td>-</td>
<td>-</td>
<td>Mincer et al., 2007</td>
</tr>
<tr>
<td>Cren_amoAF</td>
<td>ATGGTCTGGCTAAGACGTA</td>
<td>53˚C</td>
<td>635</td>
<td>Hallam et al., 2006b</td>
</tr>
<tr>
<td>Arch-amoAFA</td>
<td>ACACCAGTTTGGYTACCWTCGTA</td>
<td>52˚C</td>
<td>125</td>
<td>Beman et al., 2008</td>
</tr>
<tr>
<td>Arch-amoAFB</td>
<td>ACACCAGTTTGGYTACCWTCGTA</td>
<td>52˚C</td>
<td>125</td>
<td>Beman et al., 2008</td>
</tr>
</tbody>
</table>
3.5. qPCR components and thermocycling conditions

WCA and WCB thaumarchaeal amoA gene copy abundances were quantified using previously described qPCR gene primers (Beman et al., 2008). The qPCR assays consist of duplicate 20 µL reactions containing: 10 µL 2× SYBR® Green Master Mix (KAPA Biosystems, Woburn, MA, USA), 6.2 µL of nuclease-free water, 0.4 µL 50× ROX™ high reference dye, 0.6 µL bovine serum albumin (10 mg mL⁻¹ BSA), 0.4 µL (200 nM final concentration) of both forward and reverse primers, and 2 µL of environmental DNA. qPCR primers used to detect the WCA Thaumarchaea were Arch-amoAFA and CrenAmoAModR, while WCB primers were Arch-amoAFB and CrenAmoAModR (Table 2). Quantitative PCR reactions were analyzed using an Applied Biosystems® 7300 real-time thermocycler with the following reaction conditions: 94°C for 15 min, followed by 40 cycles of (94°C for 15 s, 52°C for 30 s, 72°C for 41 s), followed by a final 72°C extension for 1 min. At the completion of the amplification cycles, melt curve analyses were run on the amplified products. Melt curve procedures were: 95°C for 15 s melt, 50°C for 30 s re-annealing and a final denature at 95°C for 15 s. Standards for qPCR reactions consisted of serial 10-fold dilutions of plasmids containing PCR amplified and cloned amoA gene fragments from the WCA and WCB phylotypes, respectively. The efficiency of qPCR reactions was determined by the slope of the standard curves:

\[ \text{qPCR efficiency} \% = (10^{(\text{slope})} - 1) \times 100 \]

Efficiency of the WCA primers averaged 98 ± 5%, while qPCR efficiency for WCB primers averaged 89 ± 5%.
3.6. Reverse transcription of archaeal amoA genes

Thaumarchaeal amoA mRNA transcripts were reverse transcribed from environmental RNA extracts. For these reactions, total RNA samples were reverse transcribed with gene specific (amoA) antisense primers using the SuperScript® III First Strand cDNA synthesis kit (Invitrogen™) following the manufacturer’s recommended protocol. Reactions for cDNA synthesis included (final concentrations): 6 μL total RNA, 1 mmol L\(^{-1}\) dNTPs, 1 × RT buffer, 5 mmol L\(^{-1}\) MgCl\(_2\), 10 mmol L\(^{-1}\) DTT, 40 U RNaseOUT, 200 U SuperScript® III RT, and 0.5 μmol L\(^{-1}\) of the primer CrenAmoAModR (Table 2). Samples were diluted to 60 μL total volume with nuclease-free water, and stored at -20°C until analyzed by qPCR. An identical set of no reverse transcriptase (no RT) samples were used as controls for examining potential contributions of carryover genomic DNA during RT-qPCR amplification of the cDNA.

3.7. Contextual biogeochemical properties

Temperature (°C) was measured using a SeaBird CTD system affixed to the sampling rosette. The depth of the mixed layer was defined by the depth where >0.125 kg m\(^{-3}\) change in potential density occurred relative to the near-surface ocean (Monterey and Levitus, 1997). Shipboard measurements of incident photosynthetically available radiation (PAR; 400 – 700 nm) were obtained using a LI-COR® LI-1000 data logger.
Profiles of downwelling PAR were measured using a HyperPRO bio-optical profiler. Concentrations of chlorophyll a (chl a) were determined from High Performance Liquid Chromatography (HPLC) separation of algal pigments as described in Bidigare et al. (2005). Dissolved oxygen (O₂) was measured based on Winkler titrations as described in Carpenter (1965). Seawater for subsequent determination of nutrient concentrations was collected (without filtration) into acid-cleaned, sample-rinsed, high-density polyethylene bottles (Karl et al., 2001). Samples were frozen in the upright position and stored at -20°C in the dark until analyzed at the shore-based laboratory (Dore et al., 1996). Nitrate + nitrite (NO₂⁻ + NO₃⁻) was measured using a 4-channel continuous flow Bran+Luebbe® Autoanalyzer III. Because surface concentrations of N are below detection limits of the standard autoanalyzer procedures, a high-sensitivity chemiluminescence method was employed for upper ocean (<200 m) nitrate + nitrite concentrations (Dore and Karl, 1996a).

3.8. Statistical analyses

Statistical analyses were performed on seasonally-binned, photosynthetically available radiation (PAR) flux, particulate nitrogen (PN) flux, mixed layer depths, NO₃⁻ + NO₂⁻ and depth-integrated gene copy abundances to evaluate seasonality in the measured properties. Each season was separated by inflection points in Earth’s annual orbital procession; winter was defined by the period between the winter solstice (December 20) to the vernal equinox (March 25), spring was the period between the vernal equinox (March 26) to the summer solstice (June 20), summer was defined by the period from the
summer solstice (June 21) to the autumnal equinox (September 21), and Fall was the period between the autumnal equinox (September 22) to the winter solstice (December 21).
4. Results

4.1. Habitat characteristics of Station ALOHA

By coordinating this study with the near-monthly sampling of Station ALOHA conducted by the HOT program, I was able to evaluate how variability in biogeochemical and physical dynamics influenced the vertical and temporal dynamics of nitrifying Archaea. Samples were collected from the top of the epipelagic (5 m) to the depths of the cold bathypelagic waters (4,000 m) between April 2006 and February 2013.

Consistent with HOT sampling at Station ALOHA, the upper ocean waters were persistently oligotrophic during this study, with low concentrations of inorganic nutrients and chlorophyll $a$, and deep penetration of PAR through the upper ocean. Concentrations of NO$_3^-$ + NO$_2^-$ in the well-lit upper ocean (0 –100 m) ranged near the lower limits of detection (<1 nM) to ~0.2 µM, with concentrations increasing steadily through the lower euphotic zone and into the mesopelagic waters (100 – 1,000 m; Figure 5). Although NO$_2^-$ was not measured as part of this study, time series measurements of NO$_2^-$ concentrations were reported from Station ALOHA based on samples collected from September 1989 to November 1993 (Dore and Karl, 1996a). Based on results of Dore and Karl (1996a), NO$_2^-$ concentrations were consistently near the lower limits of detection (<0.2 nM) in the upper euphotic zone, increasing to 80 – 238 nmol L$^{-1}$ in the primary nitrite maxima (PNM; 115 – 175 m; Dore and Karl, 1996a), before decreasing steadily below the PNM (Figure 5).
Over the course of this study, mixed layer depths varied seasonally, averaging 87 ± 27 m during winter months, and shoaling to 44 ± 20 m (on average) during the spring coincident with warming of the upper ocean (Figure 6). Seasonally-binned mixed layer depths were significantly different from each other (One-way ANOVA; p < 0.01). Incident PAR also varied seasonally throughout the euphotic zone, but demonstrated different patterns in the upper and lower regions of the euphotic zone. In the well-lit regions of the upper ocean, surface PAR was greatest in the summer (42 ± 6 mol quanta m⁻² d⁻¹), decreasing into the late fall and winter (averaging 31 ± 8 mol quanta m⁻² d⁻¹). In contrast, PAR in the lower euphotic zone (100 m) peaked in the spring (0.72 ± 0.13 mol quanta m⁻² d⁻¹), and was lower in the summer and fall (averaging 0.59 ± 0.20 mol quanta m⁻² d⁻¹ and 0.28 ± 0.09 mol quanta m⁻² d⁻¹, respectively). K_{par} throughout this study averaged 0.043 ± 0.003 m⁻¹. Seasonally-binned 100 m PAR fluxes were significantly different from each other (One-way ANOVA; p < 0.01). I also examined seasonal-scale variability in particulate nitrogen (PN) export out of the upper ocean (at 150 m) during this study. PN fluxes varied significantly seasonally (One-way ANOVA, p<0.01), peaking during the late summer (averaging 358 ± 98 µmol N m⁻² d⁻¹), decreasing to 248 ± 75 µmol N m⁻² d⁻¹ during the winter months (Figure 6).
Figure 5. Vertical profiles of NO$_3^-$ + NO$_2^-$ (blue symbols) and NO$_2^-$ (orange symbols), seasonal range of 1% light level (hashed bar), and depth horizon of sediment traps (dash-dot line). Note: Y-axis is plotted on a logarithmic scale.
Figure 6. Monthly-binned PAR at 100 m (panel A), mixed layer depth (panel B), and PN flux at 150 m (panel C) at Station ALOHA from April 2006 to February 2013.
4.2. Vertical and temporal variability in distributions of thaumarchaeal nitrifiers

This study sought to examine vertical distributions and temporal dynamics of archaeal ammonia oxidizers at Station ALOHA. Ammonia monooxygenase (amoA) genes and transcripts were used as molecular biomarkers to investigate the depth-varying abundances, temporal distributions, and transcriptional activities of two clades of ammonia oxidizing Thaumarchea. Using clade-specific oligonucleotide primers, I was able to selectively amplify DNA and reverse-transcribed mRNA from two phylogenetically distinct groups of ammonia-oxidizing Thaumarchea (WCA and WCB, respectively; Beman et al., 2008). The resulting vertical and temporal patterns emerging from these analyses provided insights to how variations in habitat structure influence ammonia oxidizing Thaumarchea in the open sea.

Over the course of this study (2006 – 2013), thaumarchaeal amoA genes demonstrated consistent vertical patterns, with both WCA and WCB phylotypes occurring at low abundances in the upper ocean (<100 m), increasing in abundance through the lower epipelagic and upper mesopelagic waters (Figure 7). However, the two phylotypes demonstrated distinct differences in their vertical distributions, with WCA amoA gene abundances peaking in the dimly-lit regions of the lower epipelagic (150 – 200 m) and declining through the mesopelagic waters. In contrast, WCB phylotypes abundances began to increase in the lower euphotic zone, and continued to increase through the upper mesopelagic waters and remaining elevated throughout the deep meso- and bathypelagic waters (Figure 7). Throughout the epipelagic waters, WCA amoA gene abundances were highly variable in time ranging $4 \times 10^2 – 2 \times 10^5$ gene copies L$^{-1}$ in
near-surface waters, increasing in abundance to $6 \times 10^5 - 4 \times 10^7$ gene copies L$^{-1}$ at 175 m. Through the mesopelagic waters (200 – 1000 m) WCA abundances averaged $3 \times 10^6$ gene copies L$^{-1}$ (ranging $5 \times 10^4 - 3 \times 10^7$ gene copies L$^{-1}$) at 200 m and decreased to $\sim 1 \times 10^4$ gene copies L$^{-1}$ (ranging $8 \times 10^2 - 4 \times 10^4$ gene copies L$^{-1}$) at 1,000 m (Figure 7).

Interestingly, WCA abundances in bathypelagic waters increased slightly, averaging $\sim 4 \times 10^4$ gene copies L$^{-1}$ (ranging $3 \times 10^3 - 1 \times 10^5$ gene copies L$^{-1}$; Figure 7) at 4,000 m. In contrast, the WCB phylotypes tended to be less variable and lower in abundance than WCA in the epipelagic waters, averaging $2 \times 10^3$ gene copies L$^{-1}$ (ranging $2 \times 10^2 - 1 \times 10^4$ gene copies L$^{-1}$) in near-surface waters, with abundances increasing steadily with depth and becoming less variable in time, reaching a maximum of $2 \times 10^6$ gene copies L$^{-1}$ (ranging $1 \times 10^5 - 1 \times 10^7$ gene copies L$^{-1}$) at 300 m. WCB abundances remained relatively constant throughout the lower mesopelagic, but declined nearly an order of magnitude into bathypelagic waters, averaging $4 \times 10^5$ gene copies L$^{-1}$ at 4000 m (ranging $7 \times 10^4 - 1 \times 10^6$ gene copies L$^{-1}$; Figure 7).
Figure 7. Vertical distributions and abundances of WCA and WCB phylotypes of ammonia-oxidizing *Thaumarchaeia* in both the upper ocean (Panels A and C) and through the meso- and bathypelagic waters (panels B and D) at Station ALOHA. Depicted are *amoA* gene abundances analyzed from samples collected from April 2006 to January 2012 (extracted using the Qiagen Blood and Tissue kit).
4.3. Temporal variability in thaumarchaeal nitrifiers

The resulting six-year time series study (2006 – 2012) on the abundances of ammonia oxidizing *Thaumarchaea* demonstrated that these organisms vary both vertically and through time in this oligotrophic habitat. Throughout most of the year, WCA *amoA* gene copy abundances were typically <10$^4$ gene copies L$^{-1}$ in the upper regions epipelagic (<100 m), but during winter months (with the exception of the winter of 2009) WCA *amoA* genes increased approximately 10-fold to ~10$^5$ gene copies L$^{-1}$, coincident with periods of deeper mixing (Figure 8). Seasonal binning of depth integrated WCA gene abundances demonstrated that this wintertime increase in gene abundances coincided with periods of deeper mixing and decreased light flux (Table 3). During spring and summer months, the surface mixed layer shoaled and WCA gene abundances decreased in the upper epipelagic waters (Figure 8). Statistical analyses on the depth-integrated, seasonally binned WCA gene abundances revealed significant seasonal differences (One-way ANOVA; p = 0.005), with upper ocean (0 – 100 m) gene inventories elevated in the winter and lower in the summer. Conversely, the 100 – 200 m seasonally binned WCA gene abundances were not significantly different from each other (One-way ANOVA; p = 0.891). Unlike WCA, WCB phylotype abundances in the epipelagic waters did not demonstrate seasonality (Figure 9, Table 3). WCB *amoA* genes did not demonstrate significant seasonal variability in either the euphotic zone (0 – 100 m) (One-way ANOVA; p = 0.432) or lower epipelagic (100 – 200 m) (One-way ANOVA; p = 0.891).
Figure 8. Time-series of upper ocean (0 – 200 m) WCA (top) and WCB (bottom) gene abundances at Station ALOHA between 2006 and 2012. Depicted are log amoA gene copies L⁻¹; pink dashed line indicates mixed layer depth (based on 0.125 kg m⁻³ change in potential density).
Figure 9. Monthly-binned WCA (upper panel) and WCB (lower panel) amoA phylotype abundances in the upper ocean (0 – 200 m) at Station ALOHA between 2006 and 2012. Depicted are log₁₀ amoA gene copies L⁻¹. Note months arranged to highlight seasonally in WCA abundances.
Table 3. Seasonally-binned downwelling PAR (at 100 m), mixed layer depths, and depth-integrated (0 – 100 and 100 – 200 m) NO$_3^-$ + NO$_2^-$ and WCA and WCB gene abundances. Average and range of Thaumarchaeota gene inventories are provided.

<table>
<thead>
<tr>
<th>Season</th>
<th>PAR (mol quanta m$^{-2}$ d$^{-1}$) 100 m</th>
<th>N flux (µmol m$^{-2}$ d$^{-1}$) 150 m</th>
<th>Mixed layer depth (m)</th>
<th>NO$_3^-$ + NO$_2^-$ (mmol m$^{-2}$) 0 – 100 m</th>
<th>WCA (amoA gene copies m$^{-2}$) 0 – 100 m</th>
<th>100 – 200 m</th>
<th>WCB (amoA gene copies m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter Dec. 22 – Mar. 19</td>
<td>0.43 ± 0.13</td>
<td>248 ± 75.4</td>
<td>87 ± 27</td>
<td>10.0 ± 6.74</td>
<td>1740 ± 388</td>
<td>1 × 10$^{10}$</td>
<td>3 × 10$^9$ – 5 × 10$^{10}$</td>
</tr>
<tr>
<td>Spring Mar. 20 – June 21</td>
<td>0.72 ± 0.13</td>
<td>317 ± 57.0</td>
<td>44 ± 20</td>
<td>6.27 ± 5.62</td>
<td>125 ± 87.6</td>
<td>5 × 10$^{11}$</td>
<td>3 × 10$^9$ – 1 × 10$^{10}$</td>
</tr>
<tr>
<td>Summer June 22 – Sept. 21</td>
<td>0.59 ± 0.20</td>
<td>358 ± 98.5</td>
<td>49 ± 11</td>
<td>4.77 ± 1.87</td>
<td>1233 ± 655</td>
<td>4 × 10$^{11}$</td>
<td>6 × 10$^8$ – 4 × 10$^9$</td>
</tr>
<tr>
<td>Fall Sept. 22 – Dec. 21</td>
<td>0.28 ± 0.09</td>
<td>225 ± 50.3</td>
<td>77 ± 20</td>
<td>6.94 ± 5.24</td>
<td>135 ± 68.3</td>
<td>4 × 10$^{10}$</td>
<td>1 × 10$^9$ – 8 × 10$^8$</td>
</tr>
</tbody>
</table>
4.4. Comparison of DNA extraction procedures

The current study reports seven years (April 2006 to January 2012) of near monthly time series measurements of amoA gene abundances; during this period, planktonic DNA was extracted following a modified version of the Qiagen DNeasy Blood and Tissue kit (see description in Methods). However, while the derived distributions appeared consistent with previous reports of thaumarchaeal amoA genes in this ecosystem, the amoA qPCR procedures employed for the present study appeared to underestimate Thaumarchaeota abundances relative to previous studies that have quantified the abundances of these organisms at Station ALOHA (Karner et al., 2001, Mincer et al., 2007). Direct counts cell counts based on polynucleotide FISH estimated Thaumarchaeal abundances in the epipelagic waters ~10^{7} cells L^{-1} (ranging: 7 \times 10^{5} – 1 \times 10^{8} cells L^{-1}; Karner et al., 2001). Similarly, qPCR based estimates of Thaumarchaeota amoA genes reached abundances up to 6 \times 10^{7} genes L^{-1} (Mincer et al., 2007). Beginning in March 2012, I employed a different procedure for DNA extractions that relied on a modified version of the Qiagen Plant DNeasy kit.

Using the Qiagen Plant DNeasy extraction kit for HOT cruise 240 – 249 (March 2012 – February 2013) yielded increased WCA and WCB amoA gene abundances. During this time period, thaumarchaeal amoA genes demonstrated vertical patterns similar to amoA abundances derived from using the Blood and Tissue kit extracts; with both WCA and WCB phylotypes occurring at low abundances in the upper ocean (<100 m), and increasing in abundance to the lower epipelagic and upper mesopelagic waters, respectively (Figure 10). Throughout the epipelagic waters, WCA amoA gene
abundances averaged $5 \times 10^4$ gene copies L$^{-1}$ (ranging $7 \times 10^2 - 2 \times 10^5$ gene copies L$^{-1}$) in near-surface waters, increasing to $8 \times 10^7$ gene copies L$^{-1}$ (ranging $3 \times 10^7 - 4 \times 10^8$ gene copies L$^{-1}$) at 175 m, converging with earlier studies of *Thaumarchaea* abundances at Station ALOHA (Karner *et al.*, 2001; Mincer *et al.*, 2007). Through the mesopelagic waters (200 – 1,000 m) WCA abundances averaged $5 \times 10^7$ gene copies L$^{-1}$ (ranging $2 \times 10^7 - 8 \times 10^7$ gene copies L$^{-1}$) at 200 m and decreased to ~$2 \times 10^5$ gene copies L$^{-1}$ (ranging $6 \times 10^4 - 4 \times 10^5$ gene copies L$^{-1}$) at 1,000 m (Figure 10). WCA abundances in bathypelagic waters remained relatively constant throughout the rest of the water column, averaging $2 \times 10^5$ gene copies L$^{-1}$ (ranging $4 \times 10^4 - 1 \times 10^6$ gene copies L$^{-1}$; Figure 10).

WCB *amoA* abundances were lower in abundance than WCA in epipelagic waters, averaging $5 \times 10^3$ gene copies L$^{-1}$ (ranging $3 \times 10^2 - 2 \times 10^4$ gene copies L$^{-1}$) in near-surface waters, with abundances increasing steadily with depth, reaching a maximum of $2 \times 10^7$ gene copies L$^{-1}$ (ranging $2 \times 10^5 - 5 \times 10^7$ gene copies L$^{-1}$) at 500 m. WCB abundances remained constant throughout the lower mesopelagic, and declined almost an order of magnitude into bathypelagic waters, averaging $6 \times 10^6$ gene copies L$^{-1}$ at 4000 m (ranging $2 \times 10^6 - 1 \times 10^7$ gene copies L$^{-1}$; Figure 10).
Figure 10. Vertical distributions and abundances of WCA and WCB phylotypes of ammonia-oxidizing *Thaumarchaeota* in both the upper ocean (Panels A and C) and through the meso- and bathypelagic waters (panels B and D) at Station ALOHA. Depicted are *amoA* gene abundances analyzed from samples collected from March 2012 to February 2013.
Figure 11. Ratio of time-averaged WCA and WCB phylotypes extracted using the Qiagen Blood and Tissue kit (Blood) and the Plant kit (Plant) in both the upper ocean (Panels A and C) and through the meso- and bathypelagic waters (panels B and D). Plant kit samples were extracted for cruises between March 2012 and February 2013, while Blood kit samples were extracted between March 2012 and January 2012.
Direct comparisons of the total DNA concentrations and subsequent qPCR *amoA* gene abundances derived from DNA extracts based on use of these kits were conducted on two HOT cruises in March 2012 and September 2013 (HOT 240 and HOT 254). The two extraction procedures were tested on replicate filters collected from depth profiles: 0 – 1,000 m on HOT 240 and 0 – 3,000 m on HOT 254. Subsequent analyses revealed that the two extraction procedures resulted in differences in total DNA concentrations (Figure 12). For these paired depth profiles, there was a statistically significant relationship (Least squares linear regression, $R^2=0.48$, $p<0.05$) between DNA concentrations derived from the two extraction procedures, with the Plant DNA extraction kit resulting in DNA concentrations that were ~7-fold greater than the Blood and Tissue Kit (Figure 13). In addition to comparing DNA yields by these two extraction procedures, we also quantified thaumarchaeal *amoA* gene copy abundances from the DNA extracts to see if the different extraction techniques also resulted in differences in *amoA* gene abundances. Similar to results from based on DNA yields, the two extraction procedures resulted in differences in *amoA* gene abundances for WCA and WCB phylotypes (Figure 12). Throughout the water column, *amoA* gene abundances did vary systematically between the two extraction procedures for WCA (least squares linear regression, $R^2=0.83$, $p<0.01$). Similarly, there was a weak but systematic relationship among the two extraction procedures for the WCB phylotypes (least squares linear regression, $R^2=0.50$, $p<0.05$; Figure 13).

Comparisons between the Karner *et al.* (2001) polynucleotide FISH cell counts and qPCR amplification of *amoA* genes (using both the Blood and Tissue and Plant kits) revealed apparent discrepancies between the different methods for quantifying the
abundances of *Thaumarchaea* (Figure 14). In the upper regions of the epipelagic (<100 m), the time-averaged amoA gene abundances between the two different extraction techniques appeared similar, averaging ~10^4 genes L⁻¹ in the top half of the epipelagic. However, in the lower half of the epipelagic, and throughout the meso- and bathypelagic waters, amoA gene abundances derived from the Blood and Tissue and Plant kits diverge markedly, with the Plant DNA extraction-derived amoA gene copy abundances converging on ~10^7 genes L⁻¹, similar to estimates derived based on FISH cell counts (Figure 14). Together, these results suggest that for much of this time series (2006-2012) DNA extracts obtained using the Blood and Tissue kit based extraction procedure underestimated amoA gene abundances below the euphotic zone.
Figure 12. Comparison of two DNA extraction methods used during this study. Duplicate samples were collected on HOT 240 and HOT 254 and DNA was extracted using the Qiagen DNeasy Plant kit and the Qiagen DNeasy Blood and Tissue kit. Depicted are resulting DNA concentrations (panel A and B, respectively), WCA amoA gene abundances (panel C and D, respectively), and WCB amoA gene abundances (panel E and F, respectively) for the two cruises.
Figure 13. Least squares linear regression analyses describing relationships between seawater DNA concentrations extracted using the Qiagen DNeasy Plant kit and the Qiagen DNeasy Blood and Tissue kit (panel A). Comparison of WCA amoA gene abundances (panel B), and WCB amoA gene abundances (panel C) derived from DNA samples extracted using two different extraction procedures from samples collected on HOT 240 and HOT 254.
Figure 14. Various estimates of thaumarchaeal abundance at Station ALOHA. Blue circles represent polyFISH-derived MGI cell abundances (Karner et al., 2001), red squares represent the sum of WCA + WCB amoA genes L⁻¹ using the Blood and Tissue DNA extraction kit (April 2006 to January 2012 in the present study), and green triangles represent the sum of WCA + WCB amoA genes L⁻¹ using the Plant DNA extraction kit (March 2012 to February 2013 in the present study).
4.5. **Thaumarchaeal amoA transcripts**

We also examined vertical (0 – 1,000 m) and temporal variability in thaumarchaeal *amoA* gene transcripts from samples collected between July 2008 and January 2011 based on RT-qPCR amplification of reverse transcribed *amoA* mRNA transcripts. Thaumarchaeal *amoA* transcripts tended to be less variable with depth than gene abundances. On average, WCA *amoA* transcript abundances increased three-fold between the near-surface waters and the bottom of the epipelagic (Figure 15), before declining an order of magnitude into the lower mesopelagic waters. WCA *amoA* gene transcripts averaged ~1 × 10^6 (ranging 8 × 10^4 – 4 × 10^6) transcripts L^{-1} in the top half of the epipelagic (0 – 100 m), increasing to ~3 × 10^6 (ranging 9 × 10^4 – 3 × 10^7) transcripts L^{-1} in the lower epipelagic (125 – 175 m), before decreasing to ~1 × 10^5 (ranging 1 × 10^4 – 5 × 10^5) transcripts L^{-1} at the bottom of the mesopelagic zone (Figure 15). WCB phylotype transcripts remained relatively constant around 5 × 10^4 (ranging 3 × 10^3 – 5 × 10^5) transcripts L^{-1} throughout the epipelagic (Figure 15), reaching a maximum of ~1 × 10^5 (ranging 1 × 10^4 – 4 × 10^5) transcripts L^{-1} at 500 m, decreasing to ~2 × 10^4 (ranging 3 × 10^3 – 3 × 10^4) transcripts L^{-1} at the base of the mesopelagic (Figure 15). Although there did not appear to be any discernable seasonality with transcript abundances with either WCA or WCB in the epipelagic, there are significant seasonal differences with WCB 100-200 m depth-integrated transcripts (One-way ANOVA; p = 0.0263) (Table 4).
Figure 15. Vertical profiles of amoA gene transcripts at Station ALOHA. Upper ocean WCA and WCB (panels A and C, respectively) and WCA and WCB through the mesopelagic waters (panels B and D, respectively).
Table 4. Seasonally-binned particulate N flux (at 150 m) and depth-integrated (0 – 100 and 100 – 200 m) WCA and WCB amoA transcript abundances. Average and range of *Thaumarchaeia* transcript inventories are provided.

<table>
<thead>
<tr>
<th>Season</th>
<th>N flux (µmol m⁻² d⁻¹)</th>
<th>WCA (amoA transcripts m⁻³)</th>
<th>WCB (amoA transcripts m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>150 m</td>
<td>0-100 m</td>
<td>100-200 m</td>
</tr>
<tr>
<td>Winter</td>
<td>248 ± 75.4</td>
<td>5 × 10¹¹ - 3 × 10¹³</td>
<td>3 × 10¹¹ - 1 × 10¹³</td>
</tr>
<tr>
<td>Dec. 22 – Mar. 19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>317 ± 57.0</td>
<td>5 × 10¹¹ - 2 × 10¹³</td>
<td>2 × 10¹¹ - 4 × 10¹²</td>
</tr>
<tr>
<td>Mar. 20 – June 21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>358 ± 98.5</td>
<td>5 × 10¹⁰ - 3 × 10¹²</td>
<td>2 × 10¹¹ - 5 × 10¹²</td>
</tr>
<tr>
<td>June 22 – Sept. 21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fall</td>
<td>225 ± 50.3</td>
<td>5 × 10¹⁰ - 9 × 10¹¹</td>
<td>2 × 10¹¹ - 3 × 10¹²</td>
</tr>
<tr>
<td>Sept. 22 – Dec. 21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Normalizing WCA and WCB *amoA* transcripts to gene abundances provided insight into depth variability in the transcriptional activities of ammonia oxidizing *Thaumarchaea*. Although uncertainty in the *amoA* gene abundances (stemming from issues related to the DNA extraction procedure) introduces uncertainty in the transcript to gene copy ratios, the observed depth-dependent patterns are presumably robust. WCA *amoA* genes and transcripts followed a similar vertical dynamics; both profiles increased with depth through the epipelagic waters, then decreased throughout the mesopelagic waters. The resulting gene copy normalized *amoA* transcripts were elevated in the well-lit upper ocean and decreased through the lower regions of the epipelagic waters (Figure 16). In the mesopelagic waters, the resulting transcript to gene ratios increased reflecting the proportionally larger decreases in WCA gene abundances than transcripts (Figure 16). In contrast, despite WCB gene copy abundances increased from near-surface waters to the mesopelagic, while WCB *amoA* transcripts remained relatively constant (Figure 16). The resulting WCB transcript to gene copy ratio was elevated in near-surface waters, but decreased sharply throughout the epipelagic waters, becoming relatively low and constant through the mesopelagic waters (Figure 16).
Figure 16. Vertical profiles of thaumarchaeal *amoA* genes and transcripts for both WCA (panel A) and WCB (panel B) phylotypes. Gene copy normalized transcript abundances for WCA (panel C) and WCB (panel D). Dotted line indicates a 1:1 *amoA* gene to transcript ratio.
5. Discussion

5.1. Vertical distributions of Thaumarchaea at Station ALOHA

The present study examined vertical and temporal distributions of MGI Thaumarchaea at Station ALOHA in the NPSG. Sampling for this study relied on near-monthly HOT program cruises to Station ALOHA; as such, the present study benefitted from the robust biogeochemical and physical context available from HOT sampling of this site. By utilizing qPCR amplification of archaeal amoA genes, I was able to assess prominent patterns in the vertical distributions and abundances of these nitrifying microorganisms. Using two sets of phylotype specific amoA oligonucleotide primers, I quantified the abundances and transcriptional activities of two phylogenetically distinct groups of ammonia oxidizing Thaumarchaea from the near surface ocean into the deep sea. The primary objectives of this project were to identify the vertical distributions of two distinct amoA phylotypes, termed WCA and WCB (Beman et al., 2008; Mosier and Francis, 2011), and to examine how the distributions of these phylotypes varied in time. The resulting measurements revealed several robust patterns regarding the distributions of these microorganisms, and gave insight into oceanographic processes shaping these distributions. In particular, both WCA and WCB phylotypes demonstrate strong vertical gradients in abundances through the euphotic zone, with amoA gene abundances increasing by three orders of magnitude between the near-surface waters and the base of the euphotic zone. As a result of this strong gradient, changes in hydrographic forcing,
specifically wintertime deepening of the mixed layer, appear to impart a seasonal
dynamic on amoA gene abundances in the upper ocean.

One of the most prominent features observed in the current study was the sharp
increase in the abundances of amoA genes between the well-lit near surface waters and
the epi-mesopelagic boundary. The large (nearly three order of magnitude) increase in
amoA genes at the base of the euphotic zone increase was most dramatic for the WCA
phytopotypes, which increased from \( \sim 10^4 \) gene copies L\(^{-1} \) in the well-lit upper ocean (<100
m) to peak abundances of \( >10^6 \) gene copies L\(^{-1} \) near the top of the mesopelagic waters,
where concentrations of \( \text{NO}_2^- \) peak (Dore and Karl, 1996a) and surface PAR declines to
<0.1%. Below \( \sim 200 \) m WCA abundances decreased with depth through the meso- and
bathypelagic waters, with abundances in the bathypelagic waters \( \sim 10^4 \) gene copies L\(^{-1} \). In
the meso- and bathypelagic waters, WCB *Thaumarchaeae* were nearly two orders of
magnitude more abundant than WCA phylotype. WCB reached their maximum
abundance (\( \sim 10^6 \) gene L\(^{-1} \)) near 300 m, and abundances remained relatively constant with
depth into the meso- and bathypelagic waters. These observed differences in the
distributions of WCA and WCB phylotypes presumably reflect partitioning of different
niches by these related, but distinct groups of nitrifying *Thaumarchaeae*. In particular, the
differential distributions of these organisms could reflect differences in the kinetic
capacities for substrate utilization by these two clades of ammonia oxidizing
*Thaumarchaeae*. The peak in WCA near the base of the euphotic zone and subsequent
decrease in abundances through the meso- and bathypelagic waters suggests an active
role for these organisms in remineralization of nutrients, with biomass of these organisms
tracking the vertical attenuation of organic matter fluxes (Karl et al., 1984; Martin et al.,
suggesting the organisms comprising the WCA phylotype may rely on the bi-products of ammonification (production of NH$_4^+$) of sinking organic matter. In contrast, the observed distributions of the WCB phylotype suggests these organisms may be better adapted to growth in the energy-deprived region of the deep sea, where flux of reduced nitrogen substrates is substantially reduced. The relatively high abundances of WCB observed in the meso- and bathypelagic waters suggests these populations turnover very slowly, allowing these oligophiles to maintain relatively high abundances despite low fluxes of reduced energy substrates supporting their growth. Alternatively, the high population sizes of the WCB phylotypes could be maintained if these organisms rely on other sources of reduced nitrogen substrates to support their metabolism, rather than surviving exclusively on ammonia. This latter hypothesis is consistent with studies that indicate active assimilation of organic nitrogen (specifically amino acids and urea) by MGI Thaumarchaea (Herndl et al., 2005; Teira et al., 2006; Agogué et al., 2008; Alonso-Sáez et al., 2012).

Recent studies on thaumarchaeal ammonia oxidation indicate these organisms are highly adapted to growth at very low ammonia concentrations (Martens-Habbena et al., 2009; Horak et al., 2013; Nakagawa and Stahl, 2013; Santoro and Casciotti 2011). Laboratory experiments using the ammonia oxidizing MGI Thaumarchaea Nitrosopumilus maritimus SCM1 revealed that this organism has a very high affinity for low concentrations of ammonia, with a half saturation constant (K$_m$) for ammonia oxidation of ~100-130 nM (Martens-Habbena et al., 2009; Horak et al., 2013). Concentrations of ammonium in the open ocean are temporally and spatially variable, with low concentrations (<10 – 30 nM) typical for the euphotic zone, often increasing
slightly (<100 nM) near the base of the euphotic zone (Rees et al., 2006; Beman et al., 2012). The high substrate affinity of *N. maritimus* suggests this organism may actively compete for ammonium. However, based on *amoA* gene phylogenies, *N. maritimus* appears distantly related (<85% and 76% identity, respectively) to WCA and WCB (Konneke et al., 2005; Mincer et al., 2007; Santoro et al., 2011), so the extent to which the physiological adaptations of this organism can be generalized for natural populations of MGI *Thaumarchaeota* remains unclear. A recent study by Santoro and Casciotti (2011) reported growth and ammonia oxidation rates for an enrichment culture of WCA *Thaumarchaeota* (97% of the cells were *Thaumarchaeota*). The growth rates of the WCA enrichments averaged ~0.17 d\(^{-1}\), compared to *N. maritimus* growth rates of ~0.65 d\(^{-1}\); moreover, the WCA enrichments demonstrated lower per cell rates of ammonia oxidation (~2 fmol NO\(_2^-\) cell\(^{-1}\) d\(^{-1}\)) compared to *N. maritimus* (~13 fmol NO\(_2^-\) cell\(^{-1}\) d\(^{-1}\)). To date, there are no isolates or enrichment cultures of WCB representatives so information on possible differences between the physiologies of WCB and WCA phylotypes is lacking.

The environmental factors that establish the apparent vertical segregation of the WCA and WCB phylotypes remain unknown, but have been hypothesized to include specific adaptations to light (Hooper and Terry, 1974; Olson, 1981b; Ward, 1987; Horrigan and Springer, 1990; Hyman and Arp, 1992; Guerrero and Jones, 1996; Merbt et al., 2011), temperature (Tourna et al., 2008), or availability of substrates (Treusch et al., 2005; Herfort et al., 2007). Results from the present study may provide some insights into possible controls on the distributions of these organisms. In addition to the clear depth-dependent distributions of the two phylotypes, my results indicated WCA were persistently more abundant than the WCB *Thaumarchaeota* in the near-surface waters.
Light has been shown to inhibit nitrification in ocean surface waters (Hooper and Terry, 1974; Olson, 1981b; Ward, 1987; Horrigan and Springer, 1990; Hyman and Arp, 1992; Guerrero and Jones, 1996; Merbt et al., 2011). Merbt et al. (2011) reported the growth of nitrifying Thaumarchaea and Bacteria (N. maritimus and Nitrosotalea devanaterra) were reduced by 91% and 81%, respectively, at light intensities above 15 µmol quanta m$^{-2}$ s$^{-1}$. Thaumarchaeal ammonia oxidizers appeared to be more sensitive to cycles of 8-hour light/16-hour dark, and showed no signs of recovery during dark phases (Merbt et al., 2011). The elevated abundances of WCA phylotypes in near-surface waters may suggest that this clade of Thaumarchaea are more tolerant of sunlight compared to WCB.

Prior studies on the abundances and vertical distributions of MGI Archaea described similar vertical patterns to those observed in our study (Karner et al., 2001; Mincer et al., 2007; Church et al., 2010). Using polyribonucleotide probes and FISH to enumerate MGI Archaea, Karner et al. (2001) reported similar distributions of these organisms at Station ALOHA, with Thaumarchaea typically comprising a low fraction (<5%) of picoplankton in the epipelagic waters, increasing sharply below the euphotic zone to account for nearly half of the total picoplankton cells in the meso- and bathypelagic waters. Similarly, consistent with this study, numerous qPCR based studies have described vertical patterns similar to those observed in the present study, with low amoA gene abundances in the upper ocean and increasing with depth (Lam et al., 2007; Beman et al., 2008; De Corte et al., 2009; Church et al., 2010; Santoro et al., 2010; Newell et al., 2011; Alonso-Saez et al., 2012).
5.2. Temporal variability of thaumarchaeal nitrifiers at Station ALOHA

Seven years (2006 – 2012) of near-monthly nucleic acid sample collections at Station ALOHA provided information on both seasonal and interannual variability in ammonia oxidizing *Thaumarchaea*. Hence, I sought to identify whether temporal fluctuations in nitrifying *Thaumarchaea* were potentially related to variability in other physical or biogeochemical properties measured at Station ALOHA. The information gained from this exercise provided information regarding environmental controls on ammonia-oxidizing *Thaumarchaea* at this oligotrophic ocean site.

One of the most striking aspects of this study were the temporal changes in WCA thaumarchaeal *amoA* genes observed in the well-lit regions of the euphotic zone. The upper ocean waters at Station ALOHA are subject to moderate seasonality in physical and biogeochemical conditions, and this seasonality appeared to significantly influence the vertical distributions and abundances of ammonia oxidizing *Thaumarchaea*. Wintertime decreases in sea surface temperatures leads to a destratification of the upper ~100 m of the water column. Coincident with these periods of deeper winter mixing, I observed a significant (~10-fold) increase in the abundances of WCA *amoA* genes. Previous studies have found thaumarchaeal abundances in near-surface waters were highest during winter (Massana *et al.*, 1997; Murray *et al.*, 1998; Church *et al.*, 2003; Hallam *et al.*, 2006a; Wüchter *et al.*, 2006; Mincer *et al.*, 2007; Herfort *et al.*, 2007; Galand *et al.*, 2010; Christman *et al.*, 2011). Based on results from the current study, I hypothesize that wintertime deepening of the surface mixed layer entrains *Thaumarchaea* from lower euphotic zone, where their abundances increase sharply, into the well-lit regions of the euphotic zone. During this study, the mixed layer penetrated to 40 m – 110
m each winter, where WCA amoA gene abundances began to increase dramatically. With
the onset of warming in the spring and summer, the near-surface ocean warms, leading to
an abrupt shoaling of the mixed layer. During these well-stratified periods WCA
abundances decreased and remained low throughout the summer and fall. The same
seasonality was not observed with the WCB phylotype, presumably because this group of
*Thaumarchaeota* undergoes the largest increases in abundance between 150 and 300 m,
below the deepest depths of winter mixing at Station ALOHA. In addition to entrainment
of WCA during mixing, growth of *Thaumarchaeota* appears sensitive to photoinhibition
(Merbt *et al.*, 2011). Hence, decreased light flux during the winter months at Station
ALOHA could also provide conditions more favorable to the growth of ammonia
oxidizing *Thaumarchaeota*.

5.3. Vertical variations in thaumarchaeal amoA transcripts at Station ALOHA

RT-qPCR amplification of amoA mRNA transcripts at Station ALOHA provided
information on the transcriptional activities of both WCA and WCB phylotypes in the
NPSG. Quantifying the expression of amoA genes of the two groups of ammonia
oxidizing *Thaumarchaeota* helped identify vertical patterns in the transcriptional activities
of these phylotypes. On average, WCA amoA transcripts were an order of magnitude or
more than WCB amoA transcripts throughout the upper 1,000 m of the water column.
Hence, despite being nearly three orders of magnitude more abundant in the meso- and
bathypelagic waters, the WCA phylotype transcriptional activity remained greater than
the more abundant WCB phylotype. These results suggest the WCA phylotype might be
an important driver of nitrification in the deep sea despite their low contribution to total *Thaumarchaea* abundance.

The *amoA* transcript abundances derived from this study corroborate previous studies describing the depth-structure of ammonia oxidation rates. In particular, numerous studies indicate peak rates of ammonia oxidation occur near the epi-mesopelagic boundary. In the relatively eutrophic waters of the Gulf of California, rates of nitrification were maximum (348 nmol L\(^{-1}\) day\(^{-1}\)) in the upper ocean (45 m), coinciding with the NH\(_4^+\) and NO\(_2^-\) maxima (Beman *et al*., 2012). At a far offshore station in the central California Current, rates of nitrification were lower (~50 nmol L\(^{-1}\) day\(^{-1}\)) and deeper (150 m) perhaps reflecting deepening of the euphotic zone in these less productive waters (Santoro *et al*., 2013). In the NPSG, rates of ammonia oxidation ranged between 2.2 and 7.3 nmol N L\(^{-1}\) day\(^{-1}\), with peak rates occurring near the primary nitrite maxima (PNM; 100 – 175 m) (Olson 1981a). At Station ALOHA, rates of ammonia oxidation have been reported to range between 1 to 137 nmol L\(^{-1}\) day\(^{-1}\) in the epipelagic, with peak rates occurring near the low light regions of the PNM (150 – 175 m) (Dore and Karl, 1996a). In the present study, thaumarchaeal *amoA* genes and transcripts were greatest in the lower half of the epipelagic (125 – 175 m), coincident with the region of the upper ocean where rates of nitrification appear to peak (Dore and Karl, 1996a; Beman *et al*., 2010; Santoro *et al*., 2013). The high abundance of *amoA* genes and transcripts that we observed between 125 – 175 m, together with the previous reports of on elevated rates of nitrification in this depth region (Dore and Karl, 1996a), may explain the persistence of the NO\(_2^-\) maxima in the lower euphotic zone (Brandhorst, 1959; Olson, 1981b; Dore and Karl, 1996a).
Environmental factors regulating the physiological activities of ammonia oxidizing prokaryotes in the ocean continues to be an active area of research. Cultivation-based laboratory studies using ammonia oxidizing bacteria and natural populations of soil-dwelling *Thaumarchaea* revealed that concentrations of ammonia in the environment appear to regulate the expression of the *amo* operon (Treusch *et al.*, 2005; Berube *et al.*, 2007; El Sheikh and Klotz, 2008; Nakagawa and Stahl, 2013). In the open ocean, ammonia concentrations are typically <100 nmol L$^{-1}$ (Lipschultz, 2001; Woodward and Rees, 2001; Rees *et al.*, 2006), with peak concentrations often detected in the mid to lower euphotic zone (Gruber, 2008). The peak *amoA* transcripts that I observed in the lower half of the epipelagic may reflect a metabolic response in the *Thaumarchaea* to higher ammonia concentrations.

Light has also been demonstrated to have inhibitory effects on ammonia oxidation and the growth of nitrifying microorganisms (Olson, 1981b; Ward, 1987; Horrigan and Springer, 1990; Guerrero and Jones, 1996; Merbt *et al.*, 2011). Previous studies have demonstrated that short wavelength (< 410 nm) may have reversible, but detrimental photooxidative effects on the membrane-bound ammonia monooxygenase protein (Hooper and Terry, 1974; Hyman and Arp, 1992). The inhibitory effects of sunlight on thaumarchaeal *amoA* expression are not fully understood. When normalizing *amoA* transcripts to genes, observations from my study indicate that both clades of ammonia oxidizing *Thaumarchaea* are transcriptionally active in near-surface waters. These patterns are driven by low *amoA* abundances rather than elevated transcript abundances, and hence are highly dependent on accurate *amoA* gene quantifications. Nonetheless, my data indicate WCA and WCB phylotypes are actively expressing *amoA* genes in near-
surface waters of the ocean, possibly as a mechanism to compensate for photooxidative damage to the ammonia monooxygenase protein.

5.4. DNA extraction procedures and quantification of amoA genes

Throughout this study, two different procedures were utilized to extract planktonic DNA. For samples collected between March 2006 and January 2012 DNA was extracted using a slightly modified version of the Qiagen DNeasy Blood and Tissue kit; between March 2012 to February 2013 DNA was extracted using a modified version of the Qiagen DNeasy Plant kit. In general, the concentrations of DNA based on the Blood and Tissue kit were significantly lower than for the Plant kit. Similarly, qPCR analyses revealed large differences in amoA gene abundances of WCA and WCB phylotypes depending on which extraction procedure was employed, with the DNA from the Plant kit yielding significantly greater amoA gene copy abundances than the Blood and Tissue kit. However, the results were not systematic throughout the water column. WCA and WCB amoA gene abundances derived from the upper ocean (0 – 100 m) were similar between the two extraction procedures; however, below the upper ocean, where WCA and WCB abundances increase sharply, the DNA derived from the Plant kit yielded estimates of amoA gene abundances that were more than 50- and 16-fold greater for the WCA and WCB phylotypes, respectively, compared to the Blood and Tissue kit.

By comparing the amoA gene abundances (sum of WCA and WCB phylotypes) measured as part of this study (based on two different extraction kit procedures) to polyFISH-based direct Thaumarchaeota cell counts measured at Station ALOHA between
September 1997 to December 1998 (Karner et al., 2001), several striking patterns emerge. Genomic and metagenomic studies based on *Thaumarchaea* isolates or enrichments indicate these organisms contain a single *amoA* gene (Hallam et al., 2006a; Walker et al., 2010), suggesting direct cell counts and *amoA* gene quantifications should yield equivalent results. The qPCR *amoA* gene abundances (sum of WCA + WCB) derived from the upper ocean (<100 m) varied between $8 \times 10^4$ gene copies L$^{-1}$ and $1 \times 10^5$ gene copies L$^{-1}$, for the Blood and Tissue extraction kit and Plant extraction kit, respectively. In contrast, the polyFISH based direct counts of *Thaumarchaea* averaged ~2 × 10$^7$ cells L$^{-1}$. The reasons for the discrepancy among these methods are unclear; however, the *amoA* gene abundances observed in the upper ocean in the present study are similar to those reported from studies conducted in other regions of the world’s oceans (Beman et al., 2008; Beman et al., 2012; Santoro et al. 2013). For example, at a relatively eutrophic station in the California Current, Santoro *et al.* (2013) report WCA gene abundances in the near surface ocean of ~2 × 10$^5$ *amoA* genes L$^{-1}$, increasing to ~10$^7$ *amoA* genes L$^{-1}$ at 55 m. Similarly, at a more oligotrophic station in the California Current, Santoro *et al.* (2013) reported that *amoA* gene abundances were undetectable (<10$^3$ *amoA* genes L$^{-1}$) in the near-surface ocean, increasing to ~10$^7$ amoA genes L$^{-1}$ at 128 m. QPCR derived abundances of *amoA* and 16S *Thaumarchaea* genes from Monterey Bay and Station ALOHA, together with polynucleotide FISH-based cell counts, indicated upper ocean abundances were <10$^7$ genes L$^{-1}$ (Mincer *et al.*, 2007). Mincer *et al.* (2007) noted that qPCR derived abundances were frequently lower (by 25 – 60%) than polynucleotidie FISH based direct cell counts; these authors suggest that this discrepancy might result from inefficient recovery of plankton DNA during extraction.
Another possible explanation for the large discrepancy between polynucleotide FISH based cell counts and qPCR derived amoA gene abundances might lie with the relatively poor detection limits afforded by direct cell counts based on polynucleotide FISH. The qPCR based detection limit for the current study was ~10^2 genes L^-1 of seawater; the detection limit for a polynucleotide FISH assay depends on the volume of water filtered, but for the upper ocean where total cell abundances are elevated but the *Thaumarchaeota* are scarce, the FISH detection limit would presumably be considerably greater than the qPCR based analyses. If so, then the polynucleotide FISH assays might not be suitable for quantifying organisms in relatively low abundance in near-surface waters.

In addition to the large difference in the upper ocean between the polynucleotide FISH counts of *Thaumarchaea* and the amoA gene-based determinations, the comparison between the polynucleotide FISH determinations and the qPCR derived gene abundances suggested the qPCR-derived amoA gene abundances from the Blood and Tissue kit DNA extracts were likely underestimates below 100 m. In particular, below the euphotic zone, gene abundances from the Plant kit DNA extracts were very similar to the FISH-based cell count determinations. Gene abundances based on the Plant kit DNA extractions converged on the polynucleotide FISH determinations below ~125 m, suggesting the Blood and Tissue kit extraction procedure we utilized was inefficient in recovering *Thaumarchaeota* in the meso- and bathypelagic waters. However, although the gene abundances derived from the two extraction procedures were significantly related, the relatively poor correspondence (based on least squares linear regression analyses) between WCA and WCB amoA gene abundances derived from the two extraction procedures suggested correcting the data using a simple linear relationship was not
appropriate. However, because both extraction procedures yielded similar amoA gene abundances (for both WCA and WCB) in the upper ocean, I examined time-variability associated with amoA genes in the upper ocean in an effort to identify how changes in habitat structure might influence the distributions and abundances of these organisms in the open sea.

Ammonia oxidizing *Thaumarchaea* are ubiquitous in the world ocean and appear to be important regulators of the marine nitrogen cycle. We conducted an eight year time-series study to examine the vertical and temporal dynamics of *Thaumarchaea*. Information gained through our efforts helped me to gain a better understanding of environmental controls on these oligophile nitrifiers. QPCR amplification of clade-specific (WCA and WCB) thaumarchaeal amoA genes revealed prominent vertical and temporal patterns in the abundances of these organisms, while RT-qPCR amplification of amoA gene transcripts provided physiological information on ammonia oxidizing *Thaumarchaea*. WCA *Thaumarchaea* appeared most abundant near the dimly lit epi-mesopelagic boundary, while WCB *Thaumarchaea* were most abundant in meso- and bathypelagic waters. The differences in the distributions of both phylotypes may reflect alternative strategies for substrate utilization. I also observed a temporal dynamic associated with the upper ocean abundances of WCA: amoA gene abundances of this phylotype increased ~10-fold in near-surface waters during the winter coincident with increased mixing. The presence of ammonia-oxidizing *Thaumarchaea* in near-surface waters during winter months may impact nitrification in this region of the water column. Based on patterns of amoA gene expression at Station ALOHA, I found that transcript abundances were less variable with depth when compared to amoA gene abundances,
suggesting *Thaumarchaea* are physiologically active throughout the water column.

Future studies directed towards the factors that control thaumarchaeal physiology (*e.g.* ammonia concentrations, light intensity, temperature, dissolved oxygen concentrations) and abundance (*e.g.* grazers and viral lysis), will help provide a better understanding for factors that control ammonia oxidation in the sea. With this information, I would gain additional insight into the dynamics associated with microbes mediating a crucial process in the marine nitrogen cycle.
6. References


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