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

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ABSTRACT

We sought to investigate environmental controls on two phylogenetically distinct Marine Group I (MGI) ammonia-oxidizing *Thaumarchaea* 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.

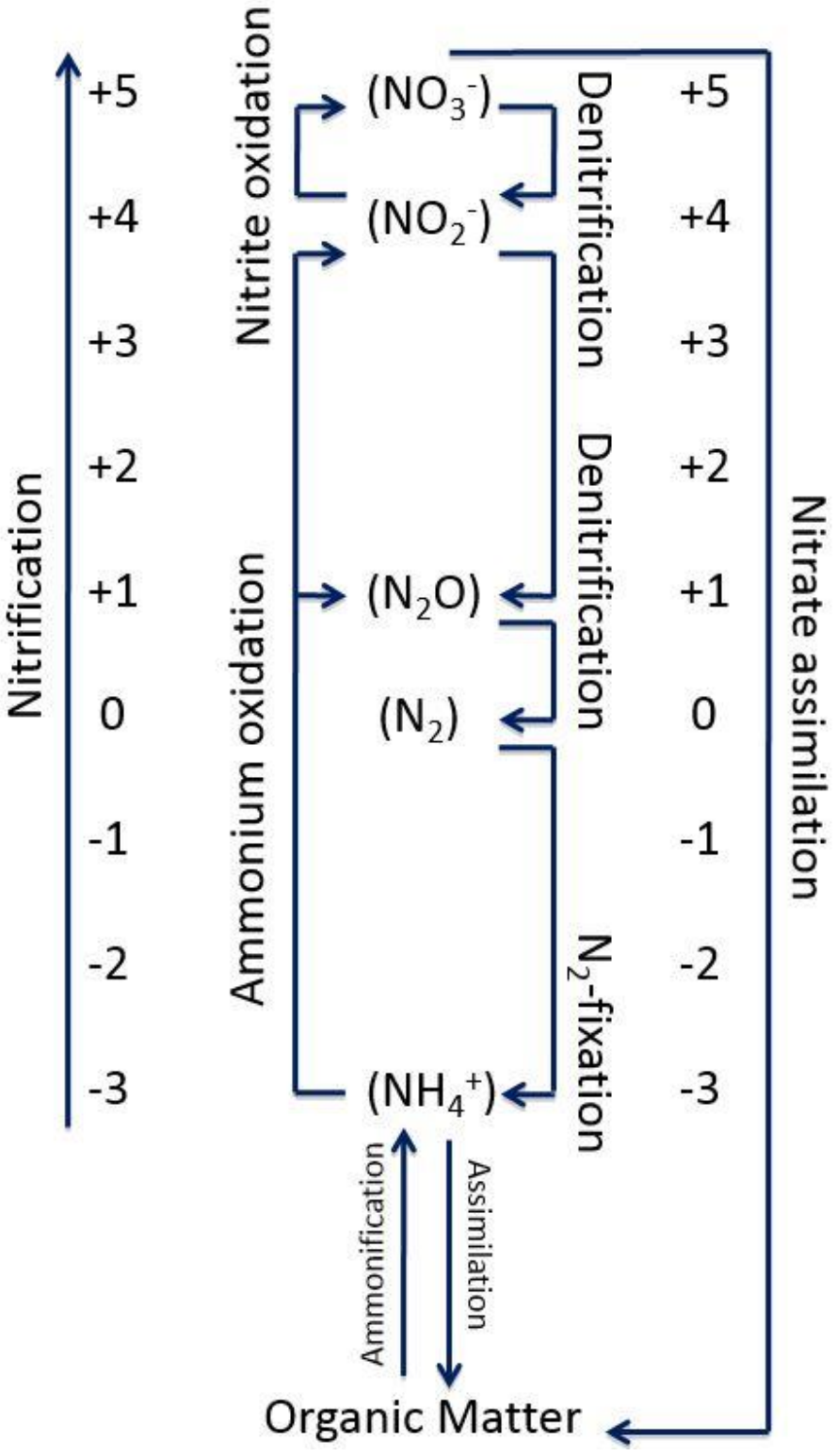

Figure 1. Diagram showing various oxidation states of nitrogen and processes governing transformation of N among these redox states (modifed from Gruber, 2008).

In the ocean, N is found in both organic and inorganic forms. Dinitrogen (N_2) 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 N_2-fixers (called diazotrophs). The largest inventory of fixed N in the ocean is NO_3^- (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 (NH_3) oxidation and anaerobic NH_3 oxidation (Ward, 2008). Similarly, nitrite (NO_2^-) 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 NO_2^- oxidation and anaerobic NH_3 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).

Species	Oceanic Inventory (Tmol N)	Upper Euphotic Station ALOHA (25 m) (μmol l^{-1})	Upper Mesopelagic Station ALOHA (200 m) (μmol l^{-1})
Nitrate (NO_3^-)	4.1×10^4	0.0003 – 0.04	0.9 – 7
Nitrite (NO_2^-)	11	0.0002 – 0.009	0.004 – 0.08
Ammonia (NH_4^+)	24	<0.03	<0.03
Nitrogen gas (N_2)	7.1×10^5	390*	450*
DON	5.5×10^3	3.8 – 7.7	3.6 – 5.7

* Emerson *et al.*, 1995

1.2. Nitrification in the sea

Nitrification describes the aerobic utilization of NH_3 as an energy source by microorganisms, resulting in the production of NO_2^- and subsequently NO_3^- (Ward, 2008). The first step of nitrification occurs when NH_3 is oxidized to NO_2^-, which is then independently followed by NO_2^- oxidation to NO_3^-:

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^- \text{ (ammonia oxidation)}$$

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \text{ (nitrite oxidation)}$$

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 NH_3 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 CO_2 fixation (Forrest and Walker, 1971; Kelly, 1978). To fix one mole of CO_2, approximately 35 mol of NH_3 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_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_3^- as the major allochthonous nutrient substrate, while NH_4^+ 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_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_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_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_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).

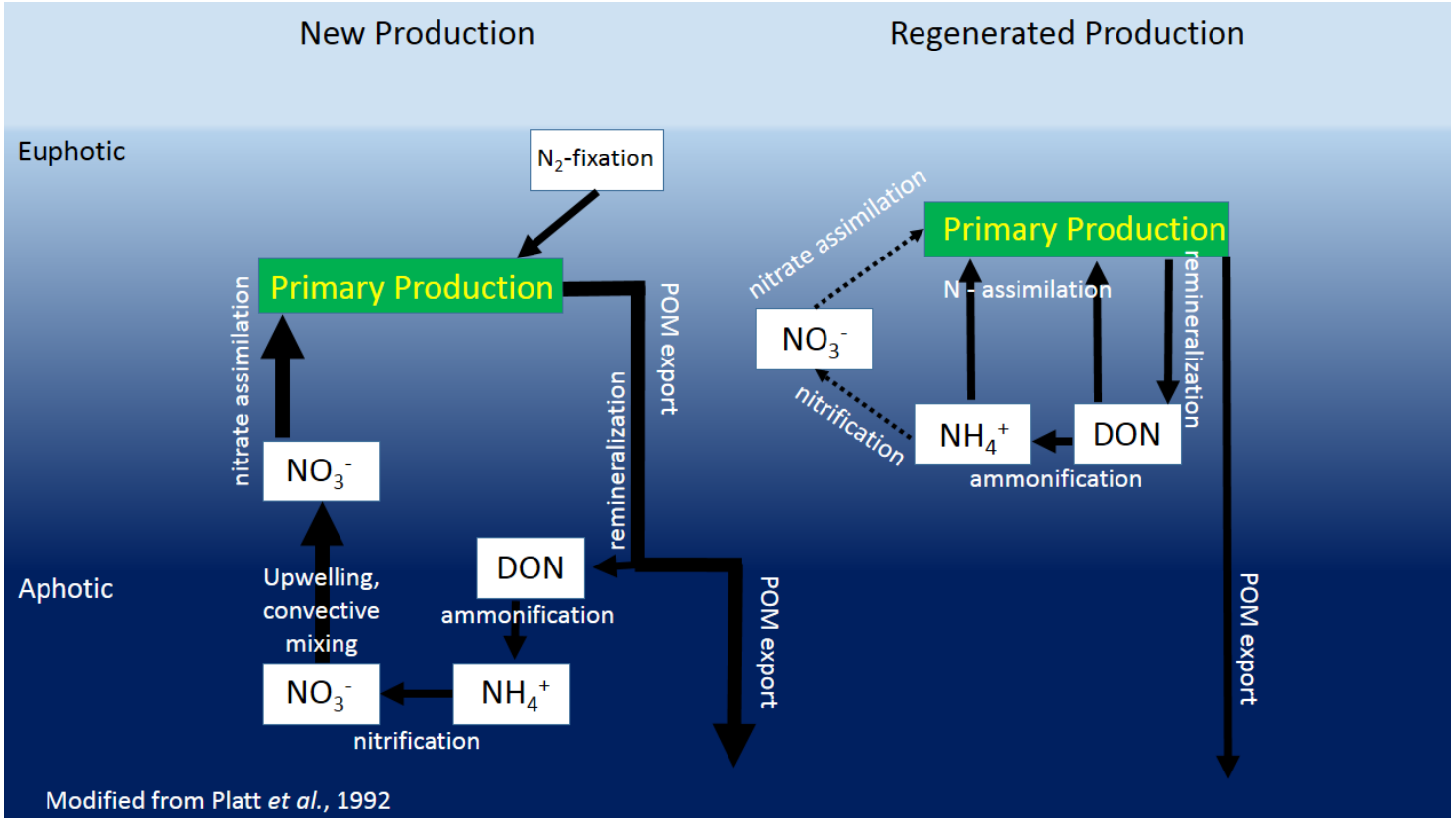

Figure 2. Schematic depiction of new and regerated production. Boxes represent different pools of N. Arrows represent transformations of N, mediated by phylogenetically diverse groups of microorganisms. Dotted arrows depict unresolved fluxes of nitrogen in the euphotic zone.

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 ~10^7 cells L^{-1}, typically increasing through the lower euphotic zone, and ranging from ~10^5 to ~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 (~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 CO_2-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 chemoautotrophy 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 NH_3 to NO_2^-. 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 NH_3 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 NH_3 to nitroxyl (HNO) as an intermediate to the production of NO_2^- (Walker *et al.*, 2010; Pester *et al.*, 2011). One hypothesized implication of this finding is that the nitroxyl pathway uses 0.5 mol O_2 per mol NH_3 oxidized (Schleper and Nicol, 2010), potentially allowing *Thaumarchaea* to oxidize NH_3 in low oxygen environments (Erguder *et al.*, 2009; Pester *et al.*, 2011). Although historically various groups of *Bacteria* were considered to be the predominant NH_3 oxidizing microorganisms in the ocean (Ward, 1990); these discoveries provided the first glimpses that *Thaumarchaea* might play a previously unrecognized role in N cycling.

Our understanding of the physiologies and biogeochemical influence of MGI *Thaumarchaea* 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 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 nM N and oxidation rates of ~13 fmol NH_3 $cell^{-1}$ 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 *Thaumarchaea* enrichment cultures (Santoro and Cascotti, 2011) whose *amoA* gene phylogenies cluster among MGI *Thaumarchaea* 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 (~0.15 d^{-1}) and lower per cell rates of NH_3 oxidation (~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 *Thaumarchaea* 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 *Thaumarchaea* (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; 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 NH_3 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 NH_3 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_2O), a potent heat-trapping gas. In large regions of the open sea, nitrification appears to be a major control on air-sea N_2O 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).

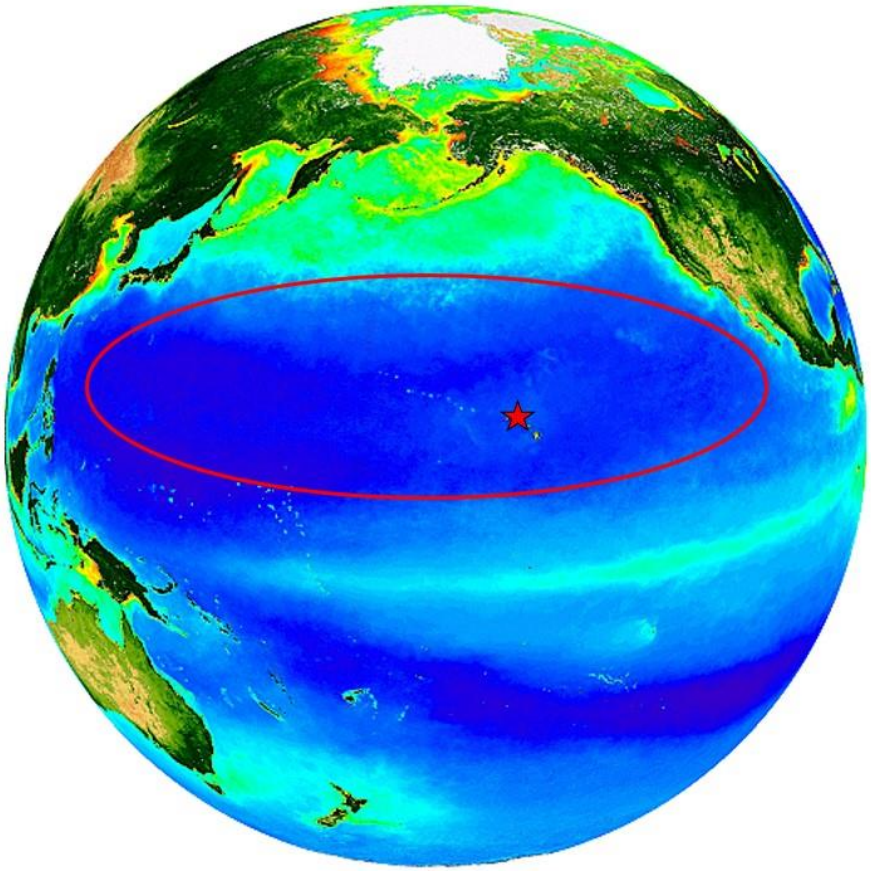

Figure 3. Satellite (SeaWIFS) ocean color view of the North Pacific Subtropical Gyre (in red circle). Location of Station ALOHA (22˚45'N, 158˚W) depicted by the red star. Image obtained from the NASA Goddard Space Flight Center's SeaWIFS Project.

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 *Crenarchaea* (later reclassified as Thaumarchaea; Brochier-Armanet et al. 2008) and *Euryarchaea* (Murray *et al.*, 1998; Massana *et al.*, 1998; Murray *et al.*, 1999; Massana *et al.*, 2000). These studies indicated that the *Thaumarchaea* 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, Euryarchaea,* and *Thaumarchaea* 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. *Euryarchaea* were occasionally present in near surface waters, but typically represented a few percent of the total picoplankton cell abundance, while *Thaumarchaea* 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 *Thaumarchaea* 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 NH_3 to NO_2^- (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 NH_3 to NO_2^- (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 *thaumarchaea* 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*.

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 (Biospec 101, 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.

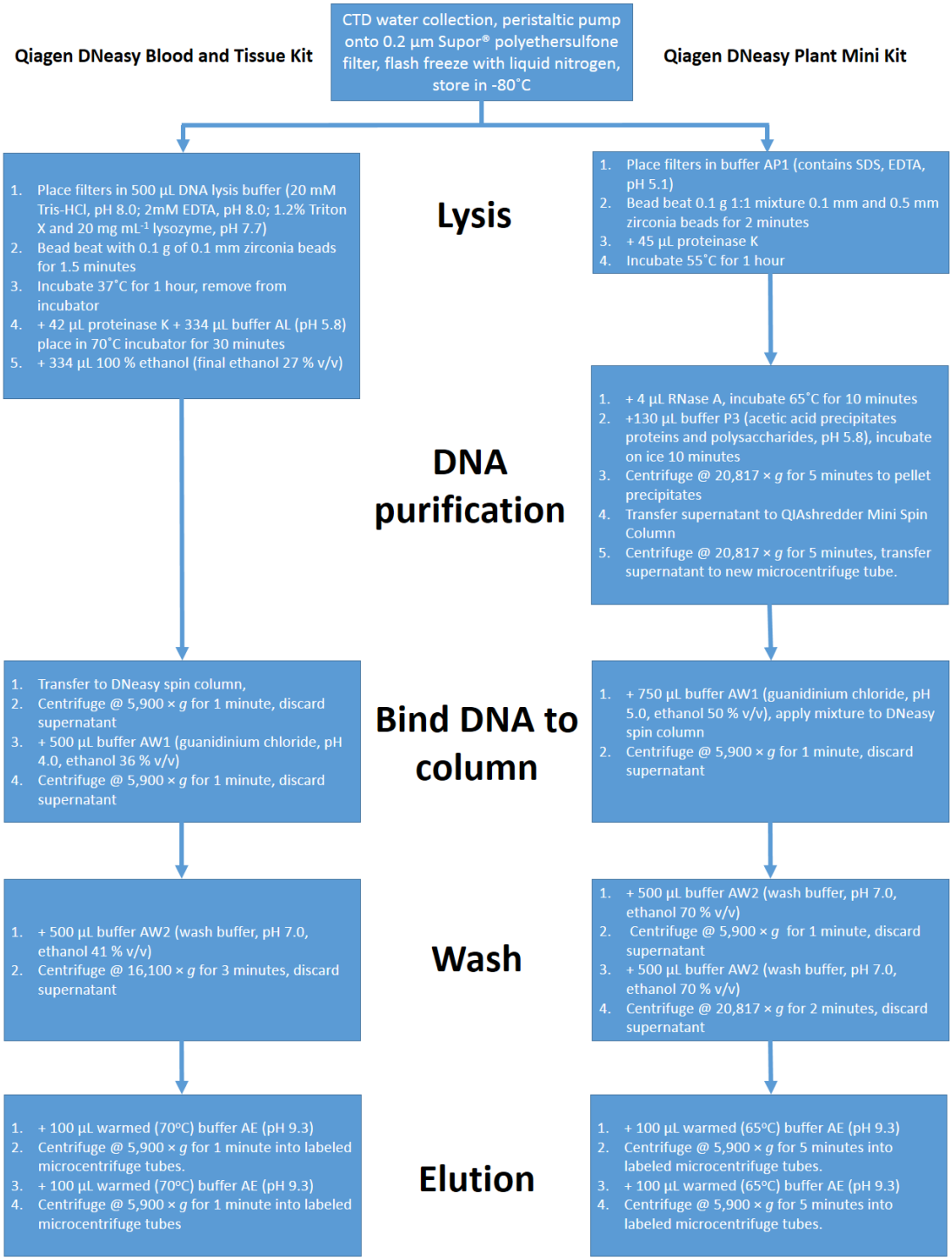

Figure 4. DNA samples collected from HOT cruises 180 – 239 (March 2006 – January 2012) were extracted following slight modifications to the manufacturers protocols for the Qiagen DNeasy Blood and Tissue Kit protocol (left). DNA samples collected from HOT cruises 240 – 254 (March 2012 – February 2013) 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 $MgSO_4$; 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.

Oligonucleotide name	Sequence (5' – 3')	Annealing temp. (˚C)	Expected amplicon (bp)	source
CrenAmoAModR	AAGCGGCCATCCATCTGTA	-	-	Mincer *et al.*, 2007
Cren_amoAF	ATGGTCTGGCTAAGACGMTGTA	53˚C	635	Hallam *et al.*, 2006b
Arch-amoAFA	ACACCAGTTTGGYTACCWTCDGC	52˚C	125	Beman *et al.*, 2008
Arch-amoAFB	ACACCAGTTTGGYTACCWTCDGC	52˚C	125	Beman *et al.*, 2008

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:

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

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 (O_2) 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 (NO_2^- + NO_3^-) 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, NO_3^- + NO_2^- 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).

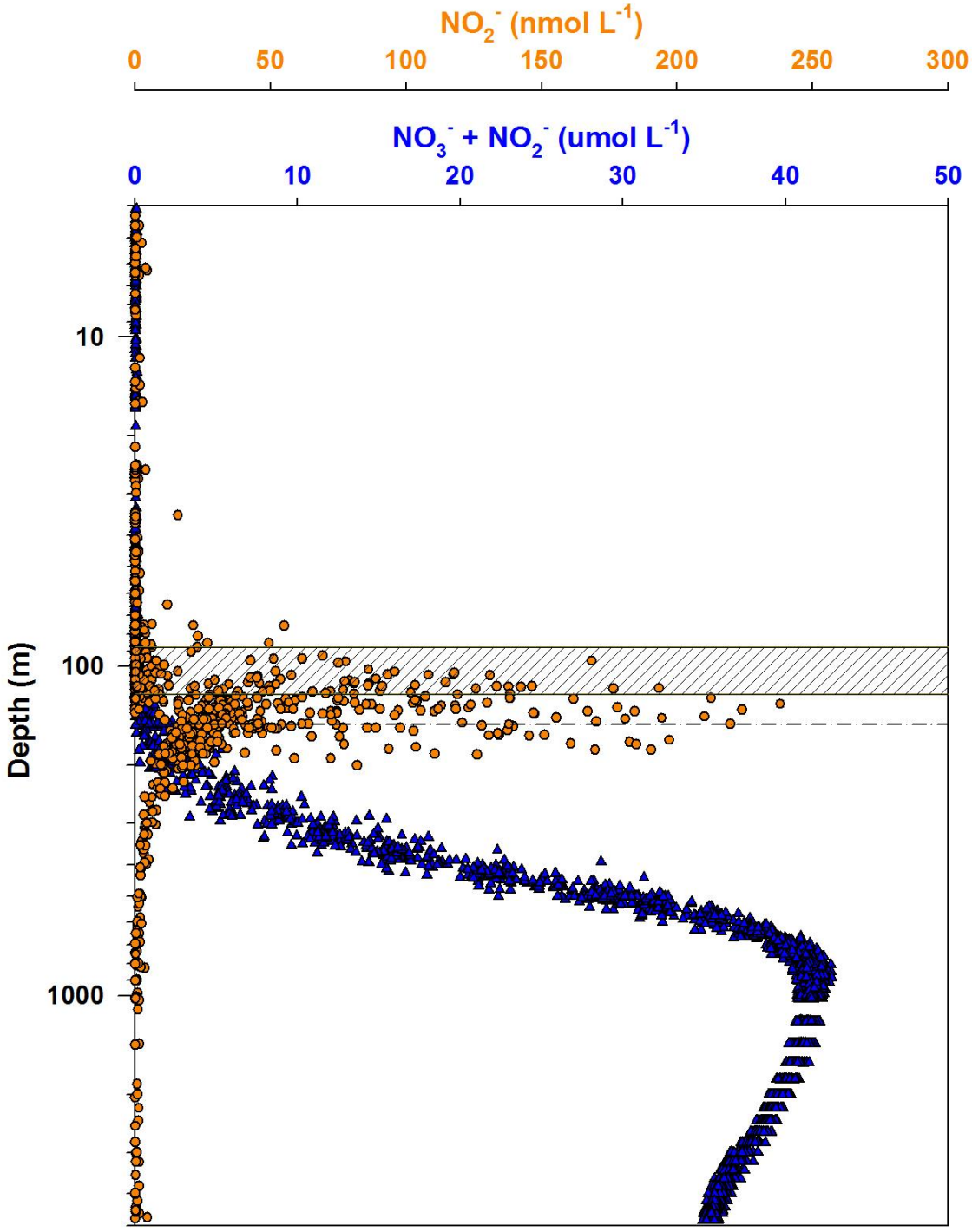

Figure 5. Vertical profiles of NO_3^- + NO_2^- (blue symbols) and NO_2^- (orange symbols), seasonal range of 1% light level (hashed bar), and depth horizon of sediment traps (dash-dot line). Note: Y-axis is plotted on a logarithmic scale.

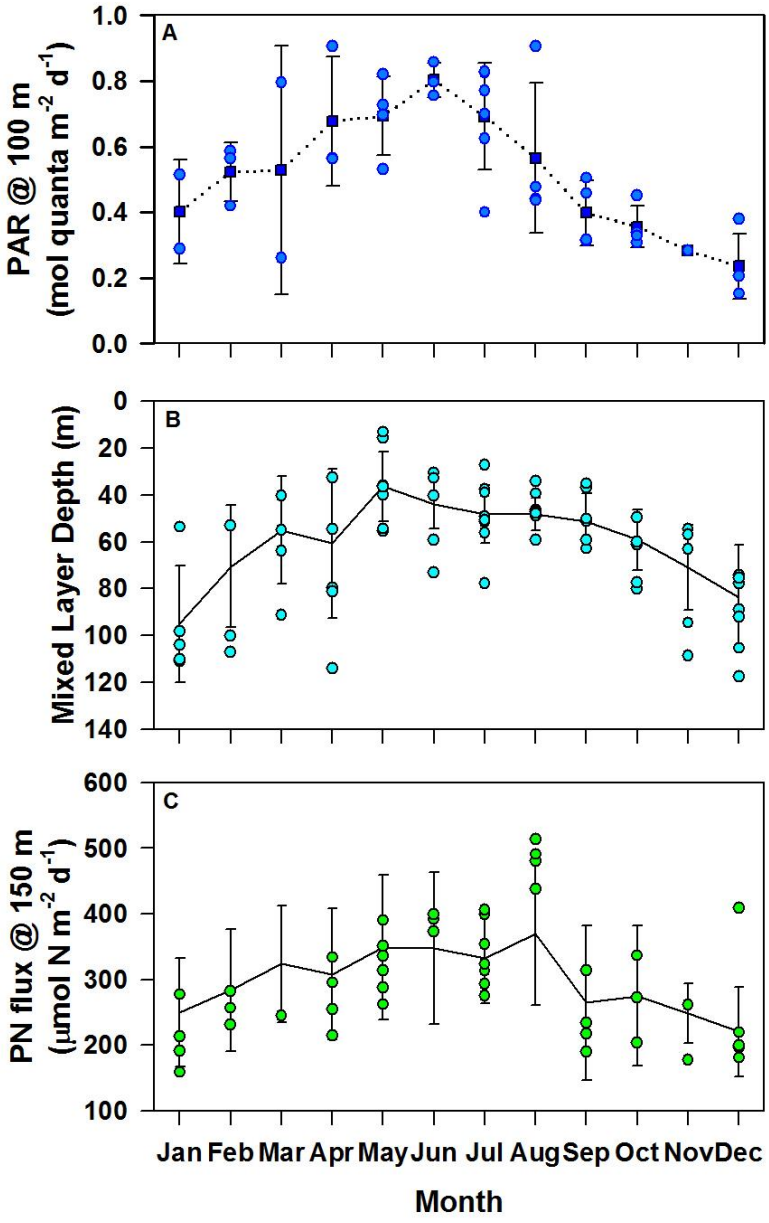

Figure 6. Monthly-binned PAR at 100 m (panel A), mixed layer depth (panel B), and PN flux at 150 m (panel C) at Station ALOHA from April 2006 to February 2013.

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 *Thaumarchaea*. Using clade-specific oligonucleotide primers, I was able to selectively amplify DNA and reverse-transcribed mRNA from two phylogenetically distinct groups of ammonia-oxidizing *Thaumarchaea* (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 *Thaumarchaea* 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 3×10^6 gene copies L^{-1} (ranging $5 \times 10^4 – 3 \times 10^7$ gene copies L^{-1}) at 200 m and decreased to ~1×10^4 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 ~4×10^4 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 2×10^3 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 2×10^6 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 4×10^5 gene copies L^{-1} at 4000 m (ranging $7 \times 10^4 – 1 \times 10^6$ gene copies L^{-1}; Figure 7).

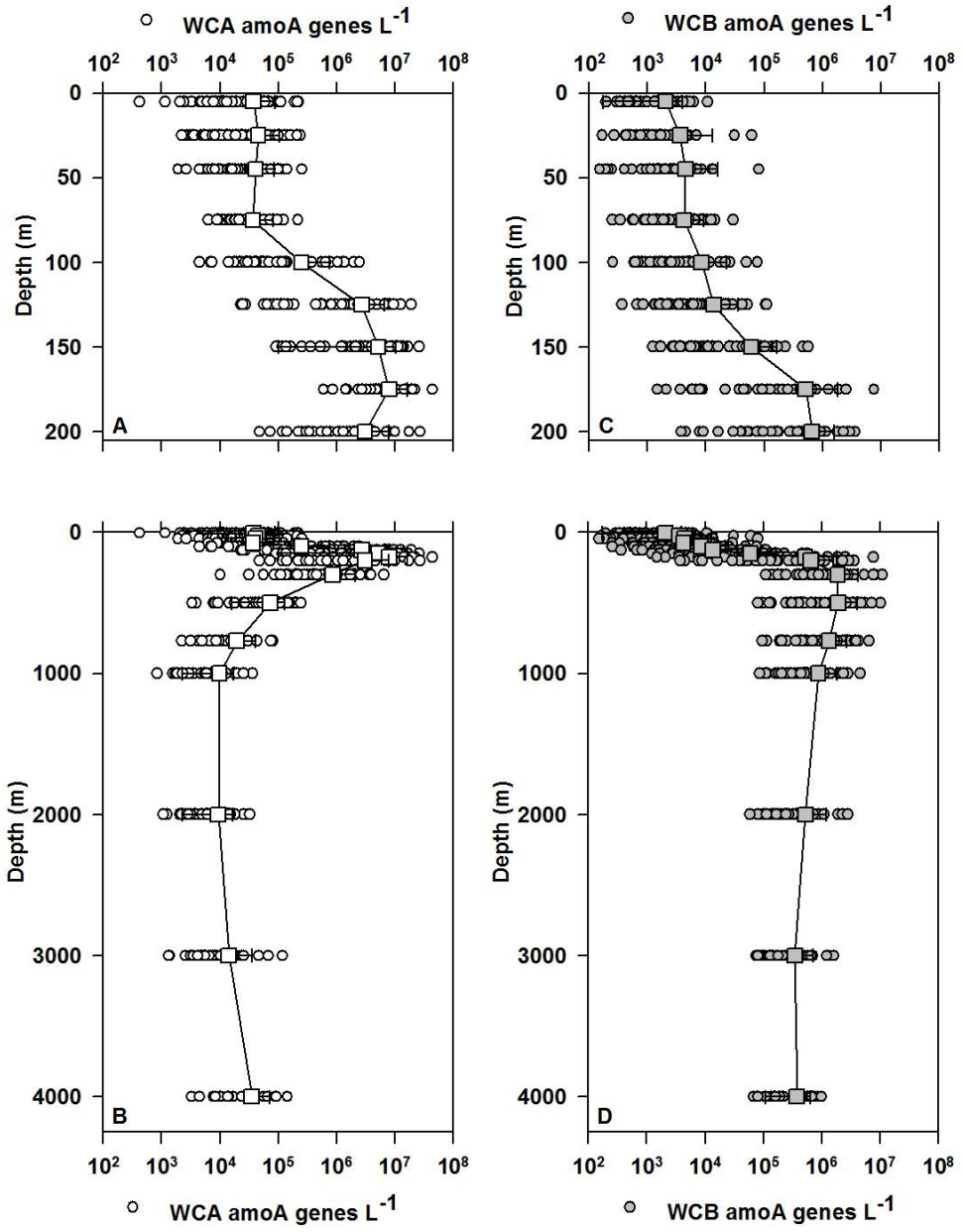

Figure 7. Vertical distributions and abundances of WCA and WCB phylotypes of ammonia-oxidizing *Thaumarchaea* in both the upper ocean (Panels A and C) and through the meso- and bathypelagic waters (panels B and D) at Station ALOHA. Depicted are *amoA* gene abundances analyzed from samples collected from 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; $p = 0.891$).

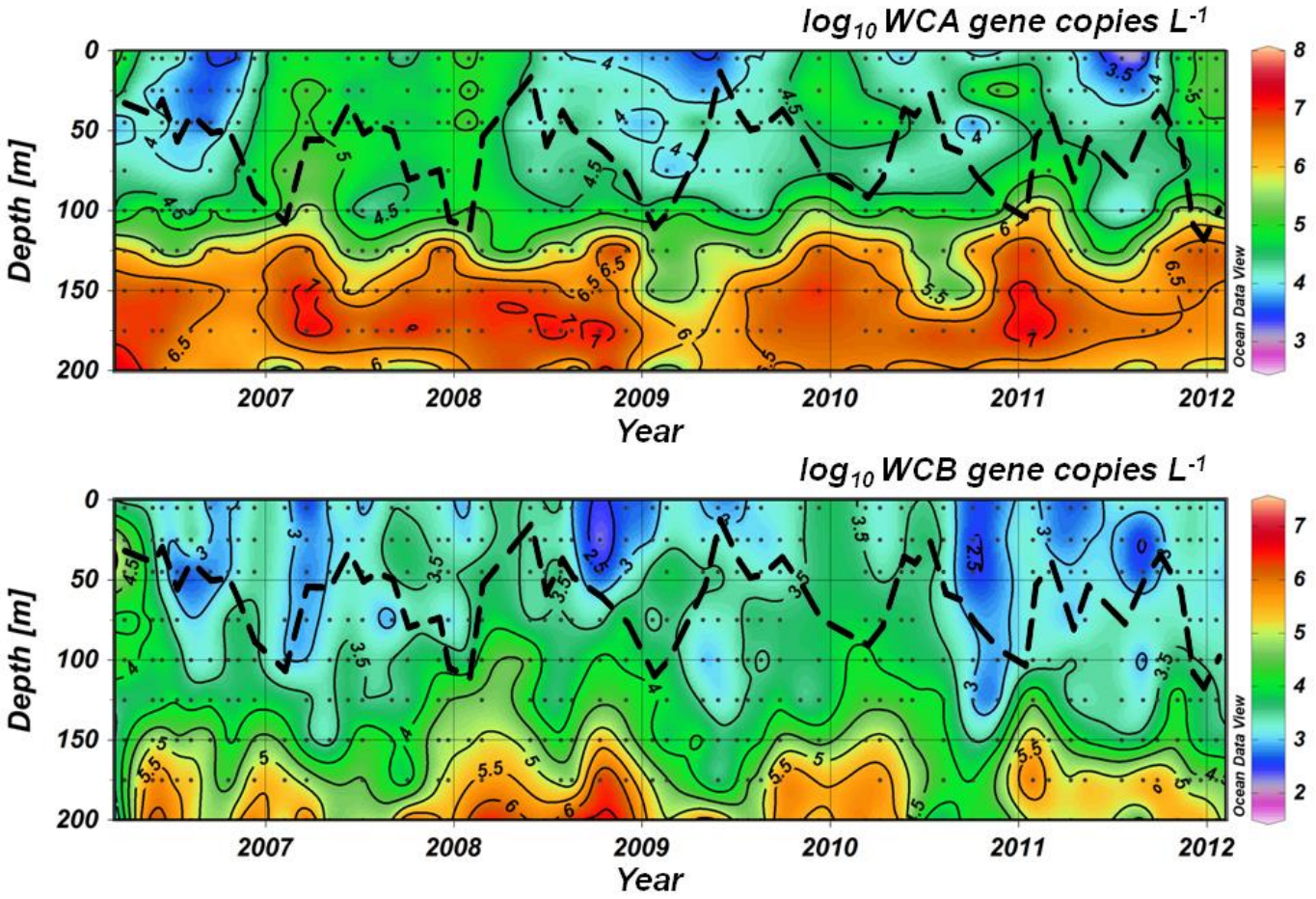

Figure 8. Time-series of upper ocean (0 – 200 m) WCA (top) and WCB (bottom) gene abundances at Station ALOHA between 2006 and 2012. Depicted are log *amoA* gene copies L^{-1}; pink dashed line indicates mixed layer depth (based on 0.125 kg m^{-3} change in potential density).

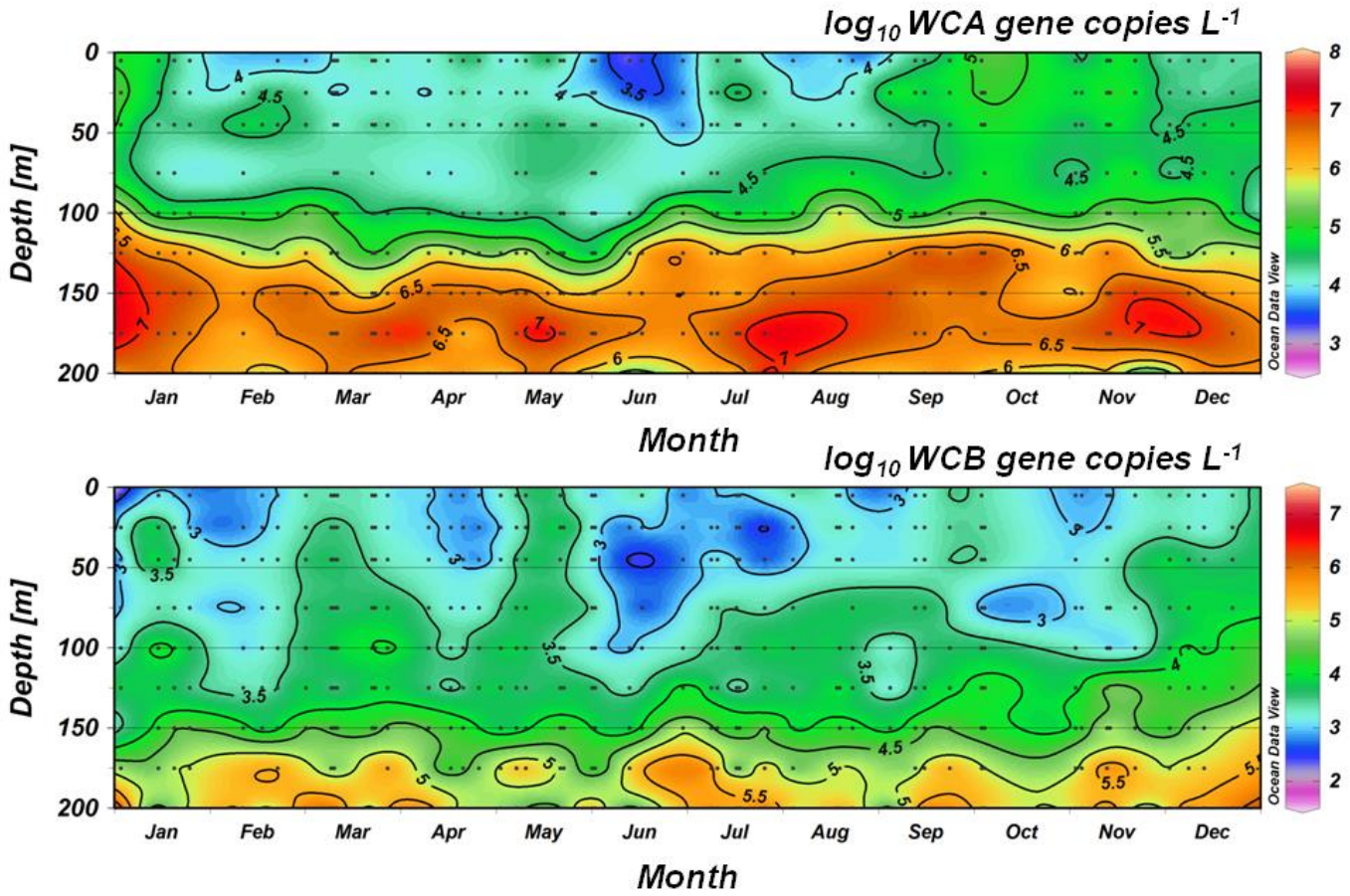

Figure 9. Monthly-binned WCA (upper panel) and WCB (lower panel) *amoA* phylotype abundances in the upper ocean (0 – 200 m) at Station ALOHA between 2006 and 2012. Depicted are $\log_{10}$ *amoA* gene copies L^{-1}. Note months arranged to highlight seasonally in WCA abundances.

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 *Thaumarchaea* gene inventories are provided.

Season	PAR (mol quanta m^{-2} d^{-1}) 100 m	N flux (µmol m^2 d^{-1}) 150 m	Mixed layer depth (m)	NO_3^- + NO_2^- (mmol m^{-2}) 0 – 100 m	NO_3^- + NO_2^- (mmol m^{-2}) 100 – 200 m	WCA (*amoA* gene copies m^{-2})		WCB (*amoA* gene copies m^{-2})	
						0 – 100 m	100 – 200 m	0 – 100 m	100 – 200 m
Winter Dec. 22 – Mar. 19	0.43 ± 0.13	248 ± 75.4	87± 27	10.0 ± 6.74	1740 ± 388	1×10^{10} $1 \times 10^{9} – 3 \times 10^{10}$	5×10^{11} $6 \times 10^{9} – 1 \times 10^{12}$	4×10^{8} $3 \times 10^{7} – 1 \times 10^{9}$	2×10^{10} $2 \times 10^{9} – 5 \times 10^{10}$
Spring Mar. 20 – June 21	0.72 ± 0.13	317 ± 57.0	44 ± 20	6.27 ± 5.62	125 ± 87.6	5×10^{9} $1 \times 10^{9} – 2 \times 10^{10}$	4×10^{11} $6 \times 10^{10} – 1 \times 10^{12}$	6×10^{8} $6 \times 10^{7} – 4 \times 10^{9}$	2×10^{10} $7 \times 10^{8} – 6 \times 10^{10}$
Summer June 22 – Sept. 21	0.59 ± 0.20	358 ± 98.5	49 ± 11	4.77 ± 1.87	1233 ± 655	3×10^{9} $2 \times 10^{8} – 1 \times 10^{10}$	4×10^{11} $5 \times 10^{10} – 1 \times 10^{12}$	3×10^{8} $1 \times 10^{7} – 8 \times 10^{8}$	1×10^{10} $8 \times 10^{8} – 6 \times 10^{10}$
Fall Sept. 22 – Dec. 21	0.28 ± 0.09	225 ± 50.3	77 ± 20	6.94 ± 5.24	135 ± 68.3	9×10^{9} $1 \times 10^{9} – 7 \times 10^{10}$	5×10^{11} $4 \times 10^{10} – 1 \times 10^{12}$	4×10^{8} $4 \times 10^{7} – 9 \times 10^{8}$	3×10^{10} $3 \times 10^{8} – 2 \times 10^{11}$

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 *Thaumarchaea* 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 *Thaumarchaea amoA* genes reached abundances up to 6×10^7 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 5×10^4 gene copies L^{-1} (ranging $7 \times 10^2 – 2 \times 10^5$ gene copies L^{-1}) in near-surface waters, increasing to 8×10^7 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 5×10^7 gene copies L^{-1} (ranging $2 \times 10^7 – 8 \times 10^7$ gene copies L^{-1}) at 200 m and decreased to ~2×10^5 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 2×10^5 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 5×10^3 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 2×10^7 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 6×10^6 gene copies L^{-1} at 4000 m (ranging $2 \times 10^6 – 1 \times 10^7$ gene copies L^{-1}; Figure 10).

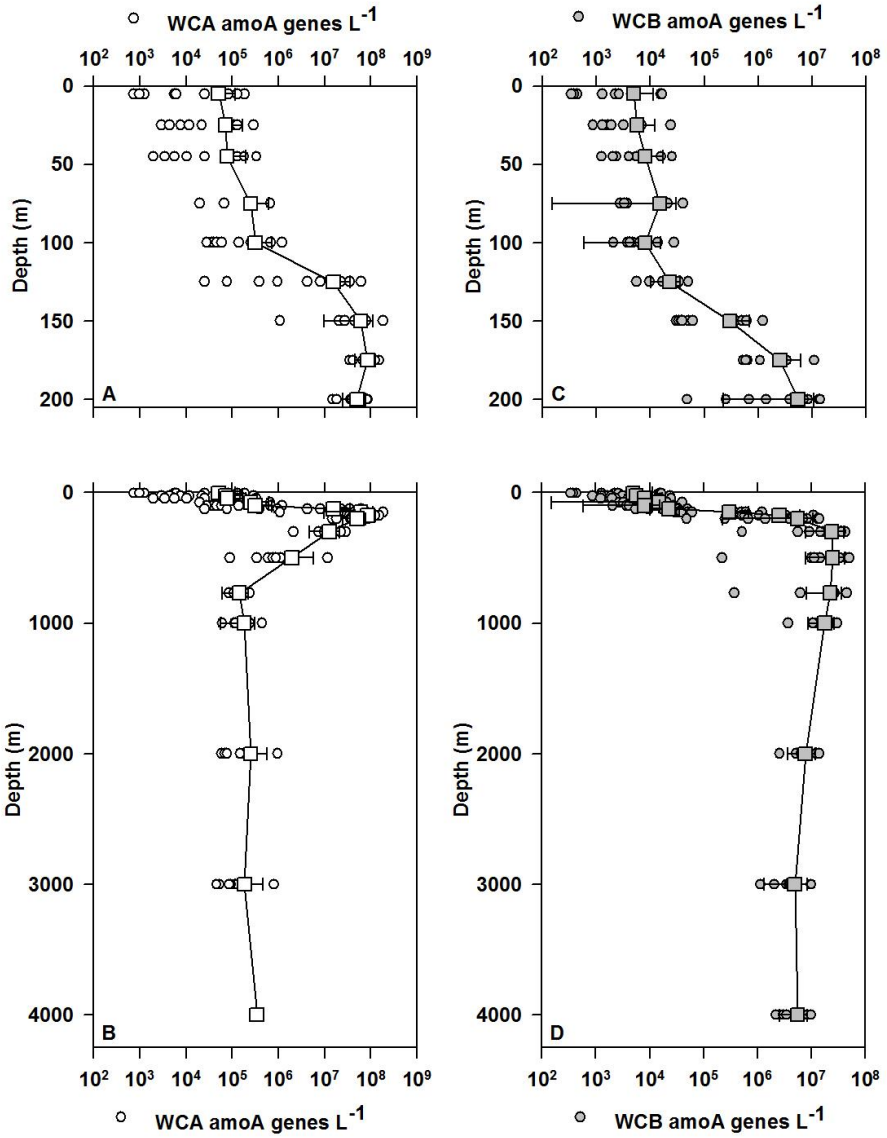

Figure 10. Vertical distributions and abundances of WCA and WCB phylotypes of ammonia-oxidizing *Thaumarchaea* in both the upper ocean (Panels A and C) and through the meso- and bathypelagic waters (panels B and D) at Station ALOHA. Depicted are *amoA* gene abundances analyzed from samples collected from March 2012 to February 2013.

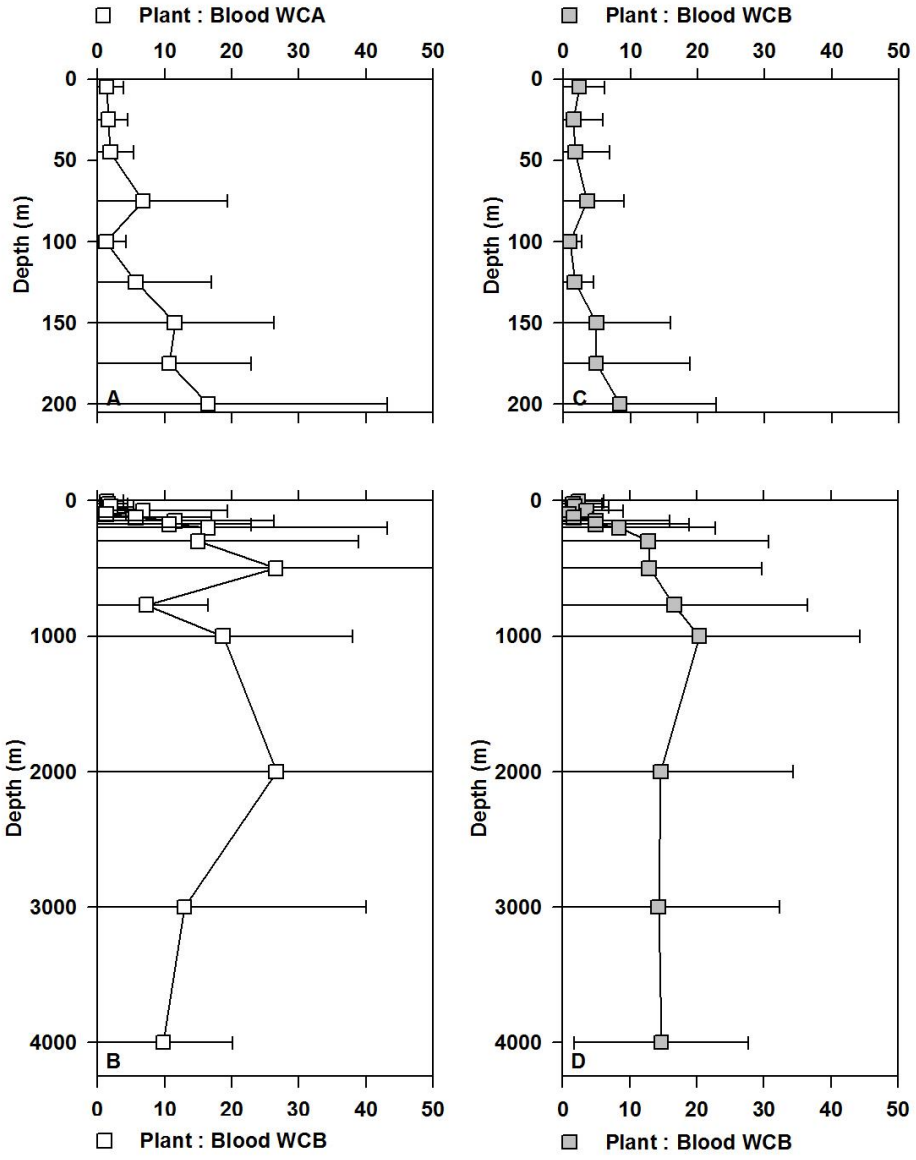

Figure 11. Ratio of time-averaged WCA and WCB phylotypes extracted using the Qiagen Blood and Tissue kit (Blood) and the Plant kit (Plant) in both the upper ocean (Panels A and C) and through the meso- and bathypelagic waters (panels B and D). Plant kit samples were extracted for cruises between March 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.

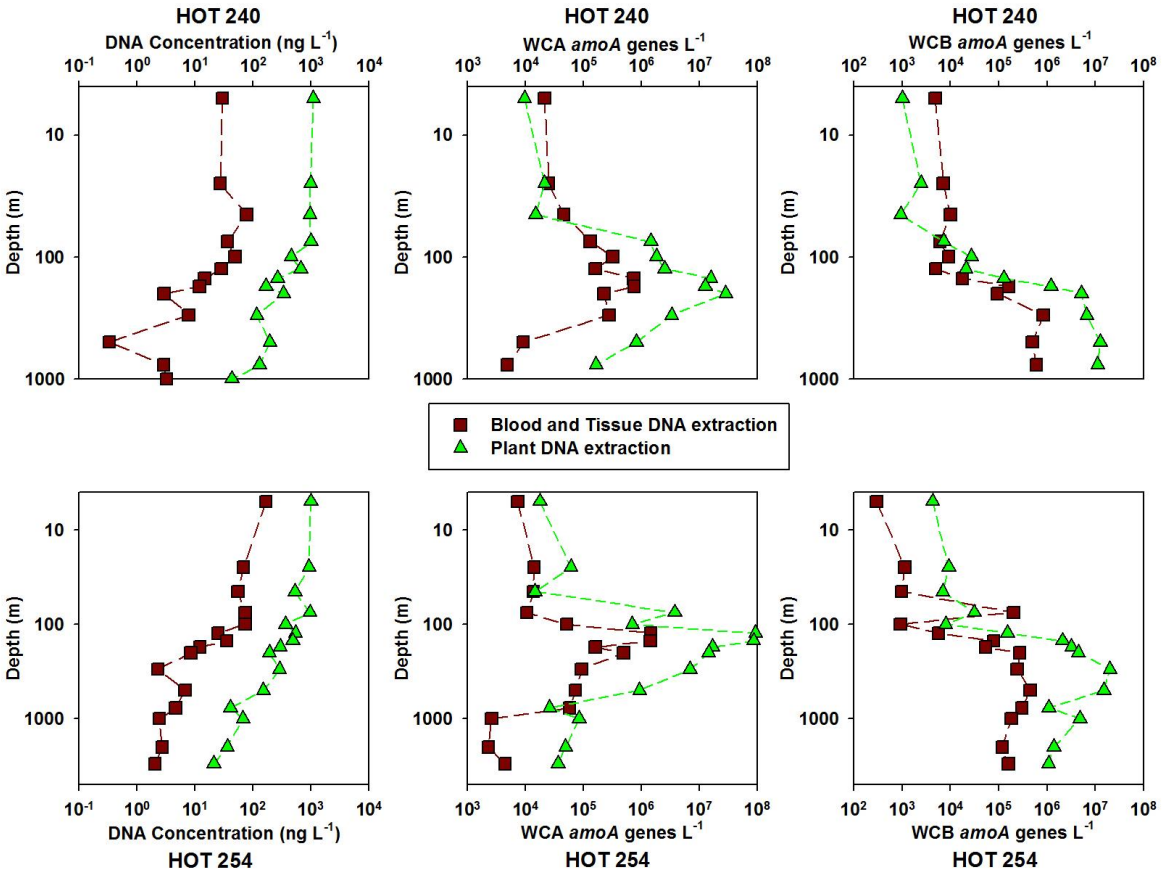

Figure 12. Comparison of two DNA extraction methods used during this study. Duplicate samples were collected on HOT 240 and HOT 254 and DNA was extracted using the Qiagen DNeasy Plant kit and the Qiagen DNeasy Blood and Tissue kit. Depicted are resulting DNA concentrations (panel A and B, respectively), WCA *amoA* gene abundances (panel C and D, respectively), and WCB *amoA* gene abundances (panel E and F, respectively) for the two cruises.

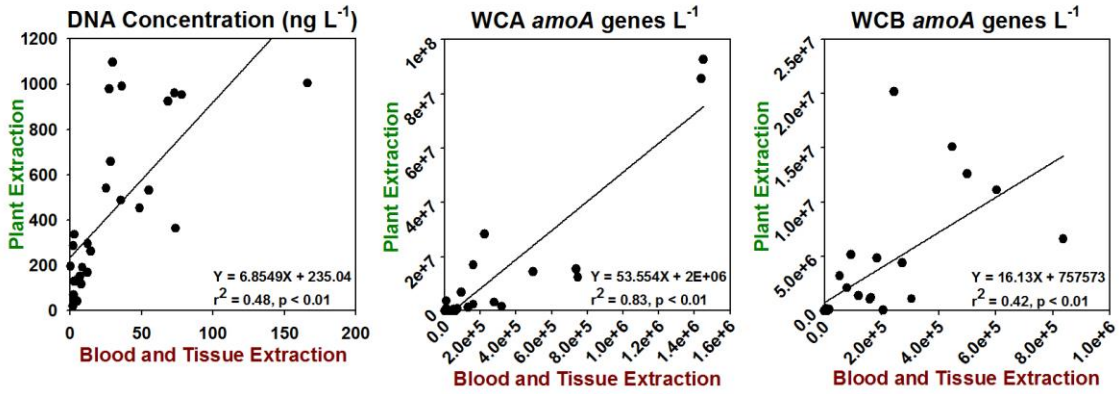

Figure 13. Least squares linear regression analyses describing relationships between seawater DNA concentrations extracted using the Qiagen DNeasy Plant kit and the Qiagen DNeasy Blood and Tissue kit (panel A). Comparison of WCA *amoA* gene abundances (panel B), and WCB *amoA* gene abundances (panel C) derived from DNA samples extracted using two different extraction procedures from samples collected on HOT 240 and HOT 254.

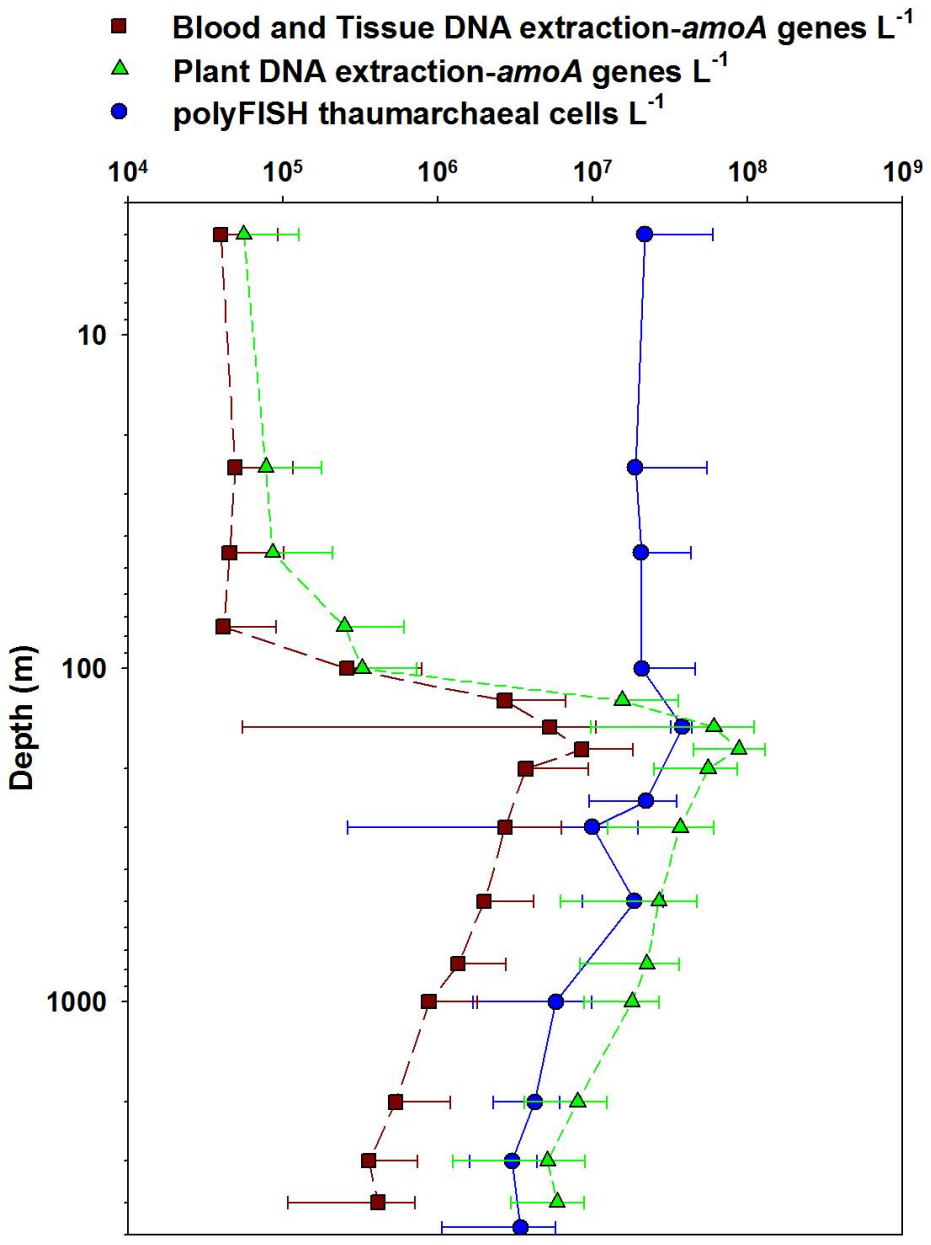

Figure 14. Various estimates of thaumarchaeal abundance at Station ALOHA. Blue circles represent polyFISH-derived MGI cell abundances (Karner *et al.*, 2001), red squares represent the sum of WCA + WCB *amoA* genes L^{-1} using the Blood and Tissue DNA extraction kit (April 2006 to January 2012 in the 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 $8 \times 10^4 – 4 \times 10^6$) transcripts L^{-1} in the top half of the epipelagic (0 – 100 m), increasing to ~3×10^6 (ranging $9 \times 10^4 – 3 \times 10^7$) transcripts L^{-1} in the lower epipelagic (125 – 175 m), before decreasing to ~1×10^5 (ranging $1 \times 10^4 – 5 \times 10^5$) transcripts L^{-1} at the bottom of the mesopelagic zone (Figure 15). WCB phylotype transcripts remained relatively constant around 5×10^4 (ranging $3 \times 10^3 – 5 \times 10^5$) transcripts L^{-1} throughout the epipelagic (Figure 15), reaching a maximum of ~1×10^5 (ranging $1 \times 10^4 – 4 \times 10^5$) transcripts L^{-1} at 500 m, decreasing to ~2×10^4 (ranging $3 \times 10^3 – 3 \times 10^4$) 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).

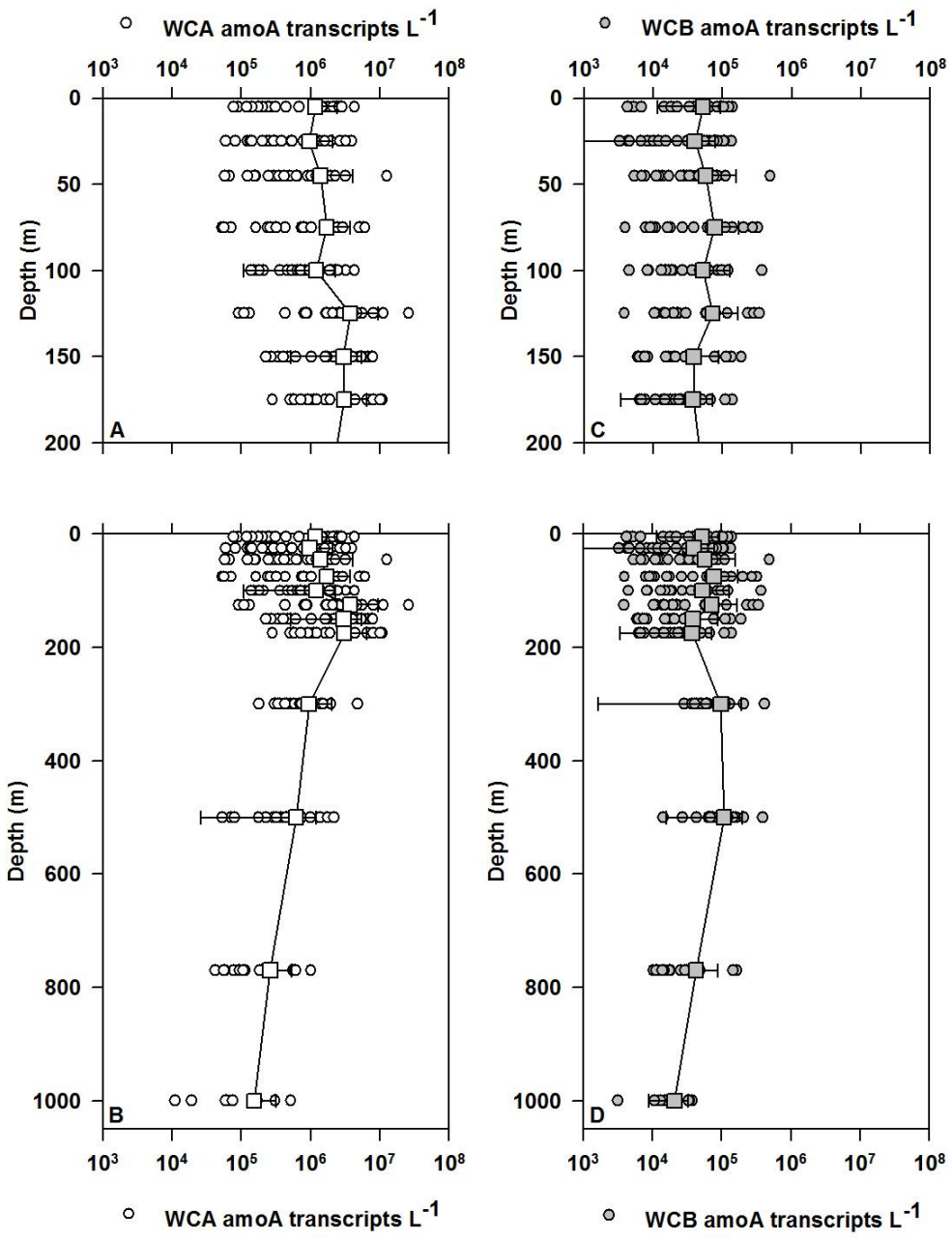

Figure 15. Vertical profiles of *amoA* gene transcripts at Station ALOHA. Upper ocean WCA and WCB (panels A and C, respectively) and WCA and WCB through the mesopelagic waters (panels B and D, respectively).

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 *Thaumarchaea* transcript inventories are provided.

Season	N flux (µmol m^2 d^{-1}) 150 m	WCA (*amoA* transcripts m^{-2})		WCB (*amoA* transcripts m^{-2})	
		0-100 m	100-200 m	0-100 m	100-200 m
Winter Dec. 22 – Mar. 19	248 ± 75.4	5×10^{11} $1 \times 10^{11} – 1 \times 10^{12}$	3×10^{12} $3 \times 10^{11} – 1 \times 10^{13}$	2×10^{10} $3 \times 10^{9} – 6 \times 10^{10}$	5×10^{10} $3 \times 10^{10} – 8 \times 10^{10}$
Spring Mar. 20 – June 21	317 ± 57.0	5×10^{11} $1 \times 10^{11} – 1 \times 10^{12}$	2×10^{12} $1 \times 10^{12} – 4 \times 10^{12}$	1×10^{10} $4 \times 10^{9} – 2 \times 10^{10}$	1×10^{10} $6 \times 10^{9} – 2 \times 10^{10}$
Summer June 22 – Sept. 21	358 ± 98.5	7×10^{11} $5 \times 10^{10} – 2 \times 10^{12}$	2×10^{12} $2 \times 10^{11} – 5 \times 10^{12}$	3×10^{10} $3 \times 10^{9} – 9 \times 10^{10}$	5×10^{10} $3 \times 10^{9} – 1 \times 10^{11}$
Fall Sept. 22 – Dec. 21	225 ± 50.3	5×10^{11} $6 \times 10^{10} – 9 \times 10^{11}$	2×10^{12} $1 \times 10^{12} – 3 \times 10^{12}$	2×10^{10} $5 \times 10^{9} – 3 \times 10^{10}$	5×10^{10} $2 \times 10^{10} – 7 \times 10^{10}$

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).

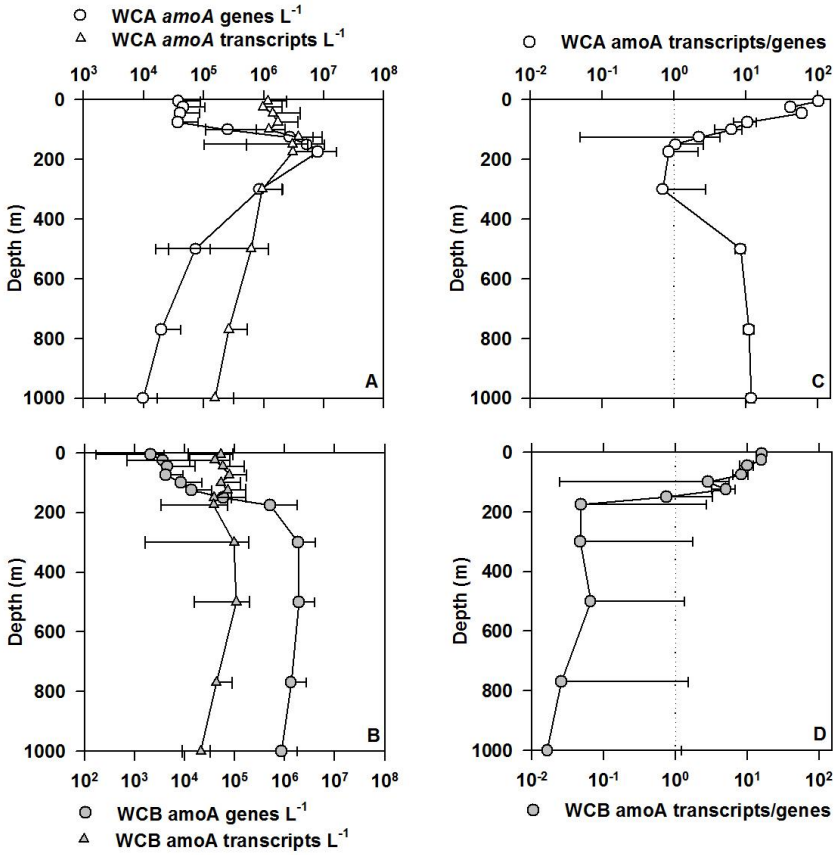

Figure 16. Vertical profiles of thaumarchaeal *amoA* genes and transcripts for both WCA (panel A) and WCB (panel B) phylotypes. Gene copy normalized transcript abundances for WCA (panel C) and WCB (panel D). Dotted line indicates a 1:1 *amoA* gene to transcript ratio.

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 phylotypes, which increased from ~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 NO_2^- peak (Dore and Karl, 1996a) and surface PAR declines to <0.1%. Below ~200 m WCA abundances decreased with depth through the meso- and bathypelagic waters, with abundances in the bathypelagic waters ~10^4 gene copies L^{-1}. In the meso- and bathypelagic waters, WCB *Thaumarchaea* were nearly two orders of magnitude more abundant than WCA phylotype. WCB reached their maximum abundance (~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 *Thaumarchaea*. 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 *Thaumarchaea.* 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.*,

1987; Buessler *et al.*, 2007), 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 *Thaumarchaea* remains unclear. A recent study by Santoro and Casciotti (2011) reported growth and ammonia oxidation rates for an enrichment culture of WCA *Thaumarchaea* (97% of the cells were *Thaumarchaea*). 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 *Thaumarchaea* 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 *Thaumarchaea* 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 *Thaumarchaea* 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 *Thaumarchaea.*

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 *Thaumarchaea* 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 *Thaumarchaea* 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 8×10^4 gene copies L^{-1} and 1×10^5 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 $\sim 2 \times 10^7$ 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 $\sim 2 \times 10^5$ *amoA* genes L^{-1}, increasing to $\sim 10^7$ *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 $\sim 10^7$ 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 polynucleotide 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 *Thaumarchaea* 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 *Thaumarchaea* 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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