UNIVERSITY OF CALIFORNIA, SAN DIEGO

Microbial Metabolism in the Deep Ocean

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Oceanography

by

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2008

The Dissertation of Roberta Lynn Hansman is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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DEDICATION

This dissertation is dedicated to my family:

Mom, Dad, Michael, Stephen (& Ernie)

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Chapter 3, in part, will be submitted for publication with S. Griffin, J.T. Watson, E.R.M. Druffel, A.E. Ingalls, A. Pearson, and L.I. Aluwihare to PNAS. Chapter 4, in part, is being prepared for submission for publication in Environmental Microbiology with L.I. Aluwihare. Chapter 5, in part, is being prepared for submission for publication with L.I. Aluwihare. The dissertation author was the primary investigator and author of these papers.

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FIELDS OF STUDY

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Professor Lihini I. Aluwihare

ABSTRACT OF THE DISSERTATION

Microbial Metabolism in the Deep Ocean

by

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To address the major role microorganisms play in the biogeochemical cycling of carbon and nutrients in the marine environment, the work presented in this dissertation uses a combination of geochemical and molecular biological techniques to investigate carbon and nitrogen metabolism in the planktonic microbial community of the deep ocean. A method was developed to isolate microbial DNA from the marine water column suitable for radiocarbon analysis, which was then applied to determine

the sources of carbon fueling microbial production in the mesopelagic. Fresh organic matter delivered from sinking particles was confirmed as an important carbon source for free-living microbes, but the extent of autotrophic carbon fixation was also significant and variable with depth, highlighting the requirement for particle-delivered reduced nitrogen by the total microbial community in the deep ocean. Both findings stressed the importance of constraining particle-derived carbon and nitrogen flux to the deep ocean. The importance of methane as a carbon source in unique environments above cold methane seeps was examined using both stable carbon isotope measurements of microbial DNA and quantitative PCR of the gene encoding for particulate methane monooxygenase. The carbon isotope measurements showed that methane-carbon played an insignificant role in fueling planktonic microbial production in the deep water column just above methane seeps. The PCR measurements substantiated this result by showing that the methane oxidizing community represented only a small percentage of the microbial community in this environment. And finally, bacterial heterotrophic nitrate assimilation was studied and determined to increase with depth and/or nitrate concentration, but appeared to only be a possible nitrogen acquisition method for a small (> 1%) fraction of the bacterial community. Sequencing of the heterotrophic nitrate assimilation gene isolated from the marine environment further demonstrated that these genes were depth stratified. In addition both isotopic analyses and molecular biological techniques such as quantitative PCR indicated distinct differences between free-living and particle-attached microbial communities. Overall, future research efforts should be focused on

expanding the current data set, constraining particle flux, and gaining a better understanding of microbial physiology, including *in situ* growth rates, in the subsurface marine environment.

I. Introduction

Microorganisms play critical roles in both the marine carbon and nitrogen cycles. While the general role of microorganisms has been known for some time the individual players, and their abundance and "rates of participation" still remain poorly characterized and quantified.

For example, in the ocean, heterotrophic bacteria are thought to derive their substrates primarily from the dissolved phase (defined here as dissolved organic carbon or DOC) and this forms the basis of the microbial loop (Hagström *et al*., 1979; Fuhrman and Azam, 1982; Azam et al., 1983; Figure 1.1). This DOC is produced in surface waters primarily by phytoplankton exudation, zooplankton sloppy feeding, and viral lysis (Carlson and Ducklow, 1995). In the deep ocean, many investigators have indicated that DOC produced from the sinking particle flux is the main carbon source supporting heterotrophic production; however, microbial respiration rates in the deep ocean (Arístegui *et al*., 2003) and bacterial carbon demand calculations (e.g., Steinberg *et al*., 2008) show that measured sinking particle flux is inadequate for supporting microbial production in dark ocean. In addition, contrary to the hypothesis that marine prokaryotes are primarily heterotrophic and consume only the recently produced fraction of DOC, uptake studies and the carbon isotopic signature of particular cellular biochemicals suggest that planktonic archaea are chemoautotrophs (Pearson *et al*., 2001; Wuchter *et al*., 2003; Herndl *et al*., 2005; Ingalls *et al*., 2006). In further support of this, the recently cultured crenarchaeote *Nitrosopumilus maritimus* is an ammonia-oxidizing chemoautotroph (Könneke *et al*., 2005). In fact, the gene coding for a subunit of archaeal ammonia monooxygenase (*amoA*) has been

found to be widely distributed throughout the marine environment (Francis *et al*., 2005), and Beman *et al*. (2008) and Wuchter *et al*. (2006) have demonstrated archaeal ammonia oxidation in the marine environment through labeled $^{15}N\text{-}NH_4^+$ studies and enrichment cultures. Although early marine studies highlighted the importance of ammonia oxidizing bacteria, they were typically found to represent only a small percentage of the total community (e.g., Ward *et al*., 1989; Mincer *et al*., 2007). In contrast, marine planktonic archaea are widespread and can be comparable to bacterial abundance in the deep ocean (Karner *et al*.. 2001; Herndl *et al*., 2005). In fact, it is unclear whether known fluxes of organic nitrogen to the deep ocean can provide enough reduced nitrogen to support ammonia oxidation coupled to chemautotrophy in the deep ocean. What does seem to be clear however, is that archaea are the major nitrifiers in the marine environment (Wuchter *et al*., 2006; Ingalls *et al*, 2006).

These recent studies have highlighted that much of the carbon and nitrogen cycle in the deep ocean remains poorly understood. With this in mind, the goal of this dissertation was to gain a better understanding of the carbon and nitrogen metabolisms of the prokaryotes in the deep ocean through a combination of geochemical analyses and molecular biological techniques.

The deep ocean carbon cycle

Delivery and sequestration of both organic and inorganic carbon in the bathypelagic ocean, either in the particulate or dissolved form, is a critical link in the carbon cycle. The distribution of DIC in the deep ocean, the Inorganic Carbon Pump,

is governed primarily by the slow advection of water masses. Some inorganic carbon is also injected into the deep ocean as a result of organic carbon remineralization and dissolution of calcite – as part of the Biological Carbon Pump. Organic carbon may enter the deep ocean by a variety of means including sinking particles, suspended particles, slowly sinking aggregates, and advected DOC – the remaining components of the Biological Carbon Pump. There is also mounting evidence that DOC may be released from particles as they sink through the water column - potentially a significant flux of carbon to the deep ocean (Aluwihare, 1999; Nagata *et al*., 2000; Azam and Long, 2001 and references therein; Figure 1.1).

Bacterial oxidation of DOC may be significant. Growing interest in the biological carbon pump has prompted numerous studies of the microbial loop in surface waters. In contrast, the microbial ecology of the ocean interior has received relatively little attention. Many factors have kept this region a black box, including the relatively low bacterial abundances, apparently slow growth rates and enzymatic activities, low concentration of organic substrates, and difficulties in isolating viable microorganisms from the deep ocean. However, a growing number of studies are beginning to shed light on the meso- and bathypelagic bacterial community (*e.g.*, Hoppe and Ullrich, 1999; Wirsen and Mollyneaux, 1999; Nagata *et al*., 2000). This environment appears to harbor complex relationships between DIC, DOC, and dead and living POC, and therefore it plays a unique role in the Global Carbon Cycle. In addition, advances in microbial biology have illuminated the great degree of biodiversity among pelagic microbes. The stratified niches of the SAR clusters

(Giovannoni *et al*., 1996) and the abundance of Group I Crenarchaeaota (Karner *et al*., 2001) are just two examples that hint at the degree of undiscovered complexity in the deep ocean.

Organic substrate availability

The cycling of organic matter has been studied extensively in the surface ocean, yet, carbon cycling in the interior of the ocean, which contains the largest and oldest reservoir of reduced organic matter in the water column: DOC, remains understudied. While DOC is less abundant than DIC, the deep ocean reservoir of DOC is 550 GT (Hedges, 1992), close to the atmospheric inventory of carbon dioxide and far greater than the standing stock of live biomass or particulate organic carbon (POC).

Organic carbon enters the deep ocean through a variety of means. The ^{14}C age of DOC (> 5000 years) is greater than the ocean's ventilation time (~ 1500 years), indicating that the large reservoir of DOC in the deep ocean is delivered primarily by ocean re-circulation, and is well-mixed (Druffel *et al.*, 1992; Figure 1.2). The age of DOC also is used to suggest that this material is refractory and resists biological degradation. However, some studies have observed deep ocean gradients in DOC concentration (Hansell and Carlson, 1998; Druffel and Bauer, 2000), which have been interpreted as the slow degradation of DOC along the path of deep ocean circulation.

Sinking POC also provides a major source of carbon to the deep ocean, and it is estimated that about 10% (Martin *et al.*, 1987) of marine primary productivity,

between 2.3 and 5.5 Pg C yr^{-1} (Buesseler *et al*., 2007), sinks below 500 m and enters the meso- and bathypelagic ocean. Sinking POC is not only remineralized to inorganic carbon in the deep ocean - it also dissolves to become new DOC (Cho and Azam, 1988; Karl *et al*., 1988; Azam and Long, 2001; Kiørboe and Jackson, 2001). The enzymatic activity of particle-attached bacteria indicates polymer hydrolysis rates greatly exceed the carbon demand of these bacteria (Karner and Herndl, 1992; Smith *et al*., 1992) leading to the particle decomposition paradox. The release of a substantial amount of carbon into the dissolved organic reservoir - a result of the hyperproduction of hydrolytic enzymes (Cho and Azam, 1988) - would provide a satisfactory solution to this paradox (Azam and Long, 2001).

In support of this hypothesis, studies show elevated growth rates of free bacteria in the presence of colonized marine snow (Herndl, 1988). In addition, deep sea bacterial production is coupled, spatially and temporally, with sinking POC fluxes (Nagata *et al.*, 2000; Hansell and Ducklow, 2003). Based on leucine incorporation rates, Nagata *et al*. (2000) estimate that the flux of carbon into free-living bacteria could be easily satisfied by the particle flux if up to 60% of the sinking POC were converted to DOC. Therefore, recently produced labile DOC delivered to the interior of the ocean by rapidly sinking particles and marine aggregates may be the major substrate for bacteria living in an otherwise substrate poor environment. The essential question is the extent to which the free-living organisms discriminate between the aged, refractory DOC and the labile, injected material. If free-living and particle-attached bacteria were 100% heterotrophic and consumed only the new fraction of

DOC, their biomass would have a modern ^{14}C concentration. *Modern* carbon is defined here as being in isotopic equilibrium with the surface water DIC at the sampling location. This is demonstrably false, as Cherrier *et al*. (1999) measured the $\Delta^{14}C$ of marine nucleic acids and showed that living marine microbes contain a minor, but significant aged carbon component.

Inorganic substrate availability

Radiocarbon analyses of sinking and suspended POC show that this material is not composed exclusively of modern carbon (*e.g.*, Druffel *et al*., 1992; Hwang and Druffel, 2003). For example, a small ^{14}C-depletion is generally observed with depth in suspended POC. The $\Delta^{14}C$ values of organic fractions isolated from suspended POC in the deep ocean at Station M in the northeast Pacific ranged from +22 to -187‰ (Hwang and Druffel, 2006) and were all lower than surface DIC (60 ± 20‰; Masiello *et al*., 1998). Several explanations have been offered for this ^{14}C-depletion, including the physical or biological (attached bacteria) uptake of old DOC (Druffel *et al*., 1992; Hwang and Druffel, 2006) by suspended POC in the deep ocean. Another mechanism that would contribute to this depth-dependent "aging" of suspended POC is the uptake of ^{14}C-depleted DIC by the particle-attached bacteria via anaplerotic β-carboxylation reactions (Rau *et al*., 1986; Rau, 1991) could also (Druffel *et al*., 1992). The carboxylation of pyruvate to form oxaloacetate in the citric acid cycle is one such example; it can provide up to 10% of total biomass-C in heterotrophic consumers (Sorokin, 1978).

This brings us to another important aspect of the deep ocean carbon cycle – what role does ambient DIC play in microbial production? The contribution of autotrophic prokaryotic production to the deep water column remains largely unknown. Recent evidence suggests that some prokaryotic species in the marine water column, specifically the Group I Crenarchaeaota, seem to function as obligate chemoautotrophs (Pearson *et al*., 2001; Wuchter *et al*., 2003; Ingalls *et al.,* 2006). Radiocarbon dating of the lipids of marine planktonic archaea shows that these organisms assimilate >80% of their biomass from DIC (Pearson *et al*., 2001; Ingalls *et al.,* 2006). Recently, Könneke *et al*. (2005) have isolated a marine crenarchaeote in culture, *Nitrosopumilus maritimus*, that grows chemolithoautotrophically through the aerobic oxidation of ammonia to nitrite. Numerous studies have documented the widespread distribution of planktonic archaea in the world's ocean (*e.g.*, DeLong, 1992; Fuhrman, 1992; Fuhrman and Davis, 1997; Murray *et al*., 1998). Karner *et al*., (2001) quantified these organisms in waters off Hawaii to show the archaeal and bacterial abundances are comparable at depths near 1000 m. The abundance and distribution of these archaea indicate that autotrophic carbon production is likely to be a significant component of the carbon cycle at meso- and bathypelagic depths. The extent to which obligate autotrophy is distributed among bacterial groups (as distinct from archaea) is largely unknown; however, the total pelagic chemoautotrophic production is limited by the available free energy.

The marine nitrogen cycle

Nitrogen cycling in the ocean consists primarily of microbially-mediated processes that control the availability of nitrogen necessary for biological productivity (Zehr and Ward, 2002). These processes include nitrogen fixation, organic nitrogen remineralization, nitrification, and denitrification. Nitrogen fixation is the biological reduction of atmospheric N_2 to ammonia and is performed by microorganisms known as diazotrophs, which include the filamentous cyanobacterium *Trichodesmium* (Zehr *et al*., 1999). Biological denitrification is the reduction of oxidized nitrogen forms through the pathway of nitrate --> nitrite --> nitric oxide --> nitrous oxide --> dinitrogen gas, and these reactions generally occur under low oxygen conditions (Knowles, 1982).

While nitrogen fixation and processes such as denitrification that occur under anoxic conditions are steps catalyzed by microbes that complete the nitrogen cycle, remineralization of organic nitrogen and nitrification appear to be the more relevant processes for most of the microbial community in the deep ocean (Figure 1.3). Heterotrophic bacteria are primarily responsible for the release of inorganic nitrogen through the decomposition of organic matter, though they recently have been shown to compete with phytoplankton for inorganic nitrogen in surface waters (Kirchman, 2000). The importance of heterotrophic nitrate assimilation in the deep ocean has not yet been determined. Nitrification is a two-step process involving the oxidation of ammonium to nitrite and then the conversion of nitrite to nitrate (Ward, 2000). Neither ammonium nor nitrite accumulates in oxygenated seawater, though the flux of nitrogen through these pools is significant (Ward *et al*., 1989). Nitrifying bacteria,

including both nitrite- and ammonia-oxidizers, are Proteobacteria (Teske *et al*., 1994), but are believed to be < 1% of the total microbial community (Ward and Carlucci, 1985). Recently, the marine *Crenarchaeota* have been identified as the major marine nitrifiers (Könneke *et al*., 2005; Francis *et al*., 2005; Wuchter *et al*., 2006), but the degree to which this group of microbes contributes to the overall marine nitrogen cycle is still being explored.

Molecular biogeochemistry

Rapid advances in molecular biological techniques have enabled a more detailed study of microorganisms and microbial communities in their environments. DNA isolation and genomic sequencing from environmental samples have revolutionized what is known about microbial diversity and abundance in a way that could never have been previously determined from culture dependent studies. With particular reference to the marine environment, previously unknown phylogenetic groups and domains of life that were believed to be unique to extreme environments appear to be ubiquitous in the water column (DeLong, 1992; Fuhrman, 1992; Giovannoni *et al*., 1996; Fuhrman and Davis, 1997; Massana *et al*., 2000; Venter *et al*., 2004). This newly exposed phylogenetic diversity highlights the vast physiological capabilities of the microbial community. However, genetic studies alone cannot elucidate the microbial ecology of the oceans or the biogeochemical role microorganisms play in the environment.

Recently, 'molecular biogeochemical' studies (the combination of molecular

biology and molecular-level geochemistry) have been very successful at identifying the ecological niches occupied by particular phylogenetic groups (Hinrichs *et al*., 1999; Valentine and Reeburgh, 2000; Orphan *et al*., 2001). These studies used a combination of fluorescence *in situ* hybridization (FISH) using specific 16S rRNA-targeted oligonucleotide probes, molecular-level isotopic studies of specific lipid biomarkers, and secondary ion mass spectrometry (SIMS)-assisted carbon isotopic studies to identify the biogeochemical role of archaea and bacteria in sediments. Based on the success of these studies it is clear that molecular-level organic geochemistry, when combined with existing and ongoing molecular biological studies, has the potential to provide valuable insight into the particular ecological and biogeochemical niches occupied by diverse members of the marine microbial community.

Radiocarbon as a geochemical tracer

Figure 1.4 shows radiocarbon depth profiles for various carbon pools in the North Pacific Ocean (DOC - Druffel *et al*., 1992; DIC - Kumamoto *et al*., 2002; sinking POC - Hwang *et al*., 2004). The profile for inorganic carbon is controlled by water mass advection and shows modern or post-bomb radiocarbon signatures in the surface ocean (+70‰). Radiocarbon values decrease up to about 1000 m and remain constant at deeper depths (-236‰). These data indicate that, relative to a pre-bomb surface DIC value of -50‰, the DIC in deep Pacific waters is 2000 years older – approximately equal the transit time for the advection of water to the deep Pacific

Ocean. The total DOC profile shows a shape similar to DIC, but is much more depleted in radiocarbon; this reduced carbon pool is therefore, much *older* than DIC at all depths in the ocean. Surface values are -179‰ and deep water values below 1000 m are -525‰. The constant offset throughout the water column indicates that the same processes distribute both DIC and DOC to the deep ocean, namely, the circulation of water masses. The older age of DOC (6000 years in the deep Pacific) therefore, probably implies that a major fraction of DOC is recycled within the water column several times before exiting the ocean (Druffel *et al*., 1992). Surface water radiocarbon values for DOC can be explained by conservative mixing of a modern (+70‰) component with the background / deep concentration of old (-525‰) DOC.

Figure 1.4 clearly demonstrates the unique $\Delta^{14}C$ values of different carbon reservoirs in the deep Pacific Ocean. Three distinct sources of carbon are available to free-living prokaryotes, DIC ($\Delta^{14}C$ as low as -239‰), old DOC (with an average $\Delta^{14}C$ value of -525‰), and particle injected DOC ($\Delta^{14}C \sim +60‰$). The radiocarbon content of bulk organic matter isolated from microorganisms will be some combination of the above three values and will be controlled by the relative contribution of autotrophic versus heterotrophic prokaryotic production in these deep waters. For example, if microorganisms at this site are primarily heterotrophic and consume only fresh DOC injected from dissolving particles, the $\Delta^{14}C$ value of organic matter isolated from these organisms will be close to +60‰. On the other hand, if these microbes are a mixture of both autotrophs and fresh DOM-consuming heterotrophs, we would expect values somewhere between +70‰ and -239‰ (Figure 1.2). By measuring the radiocarbon

value of organic compounds representative of the entire microbial community, we can begin to assess the types of carbon pools assimilated by these organisms.

Functional genes as indicators of metabolic potential in the deep ocean

In addition to detecting the presence or absence of particular groups of microbes in the marine environment, PCR can be used to help evaluate the metabolic potential of a sampled community. Surveying for genes that code for enzymes catalyzing specific metabolic functions ("functional genes") can determine the potential for certain processes in a sample. This technique has been used to look for the metabolic potential of a variety of processes in the marine environment, including nitrogen fixation (*nif*; Zehr and McReynolds, 1989), ammonia oxidation (*amo*; Rotthauwe *et al*., 1997; Alzerreca *et al*., 1999), and methane oxidation (*pmo*; Holmes *et al*., 1995). When analyzing DNA, only metabolic potential as opposed to gene expression can be assessed. However, correlations between the presence of functional genes and occurrence of their specific metabolic process have been demonstrated; for example, *amoA* gene copies were positively correlated with ammonia oxidation rates in the Gulf of California (Beman *et al*., 2008).

Organization of this dissertation

This dissertation focuses on aspects of prokaryotic metabolism in the deep ocean in an effort to better understand the critical roles occupied by marine microbes in the biogeochemical cycling of carbon and nitrogen. A combination of geochemical

and molecular biological techniques is used to trace carbon assimilation and assess metabolic potential in the microbial community throughout the water column. **Chapter 2** describes the development of a method for extracting microbial DNA suitable for radiocarbon analysis. That method is applied in **Chapter 3** to determine the sources of carbon fueling prokaryotic production in the mesopelagic ocean, using the distribution of natural abundance radiocarbon in the marine environment. **Chapter 4** investigates the importance of an alternative carbon source, methane, available to planktonic microbes above cold methane vents, in supporting the total prokaryotic community in these systems. In **Chapter 5**, the focus shifts to nitrogen metabolism where the potential for heterotrophic nitrate assimilation by the microbial community throughout the water column is examined. General conclusions from the thesis work are presented in **Chapter 6**.

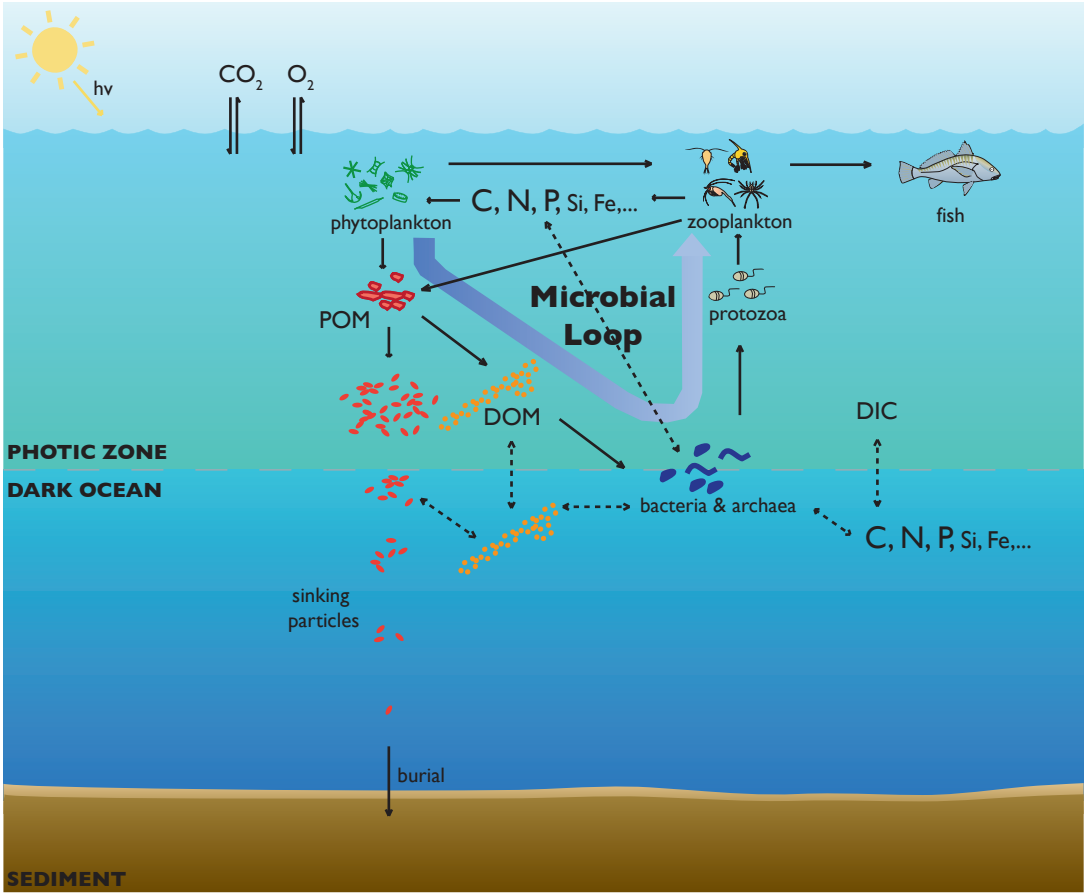

Figure 1.1. Schematic of the microbial loop, adapted from Azam and Malfatti (2007).

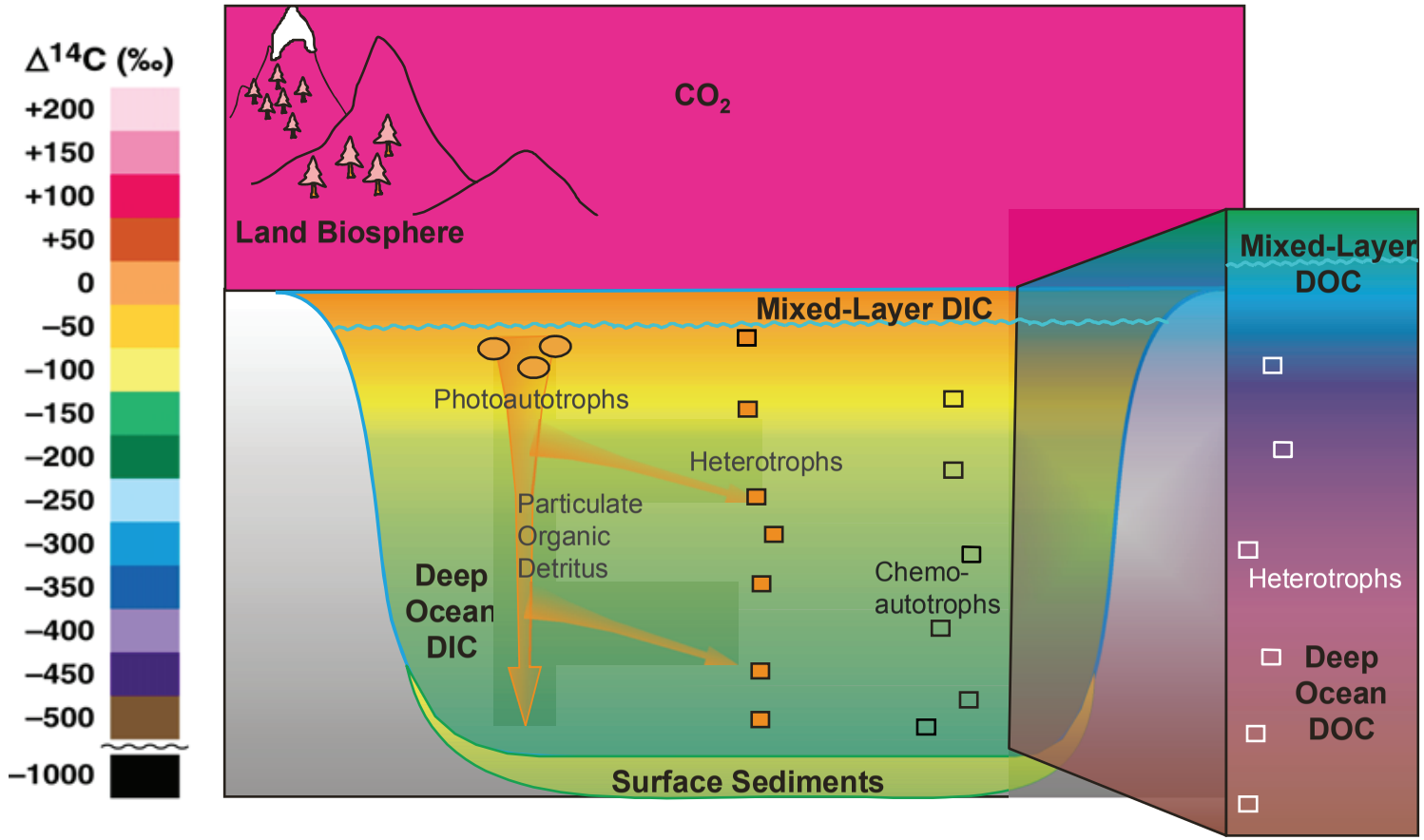

Figure 1.2. Radiocarbon content of the major carbon pools on earth (from McNichol and Aluwihare, 2007). The positive $\Delta^{14}C$ of the atmosphere is a result of the above-ground testing of nuclear weapons that occurred in the 1950s and 1960s. This 'bomb spike' has penetrated the surface ocean and is reflected in recently-produced autotrophic biomass as well as in the heterotrophic consumers of this fresh organic matter. Particles that sink rapidly through the water column retain some of their young component, but also incorporate older sources of DOC and POC. DOC is older than DIC everywhere in the ocean. Like POC, DOC is heterogeneous and also contains young and old carbon.

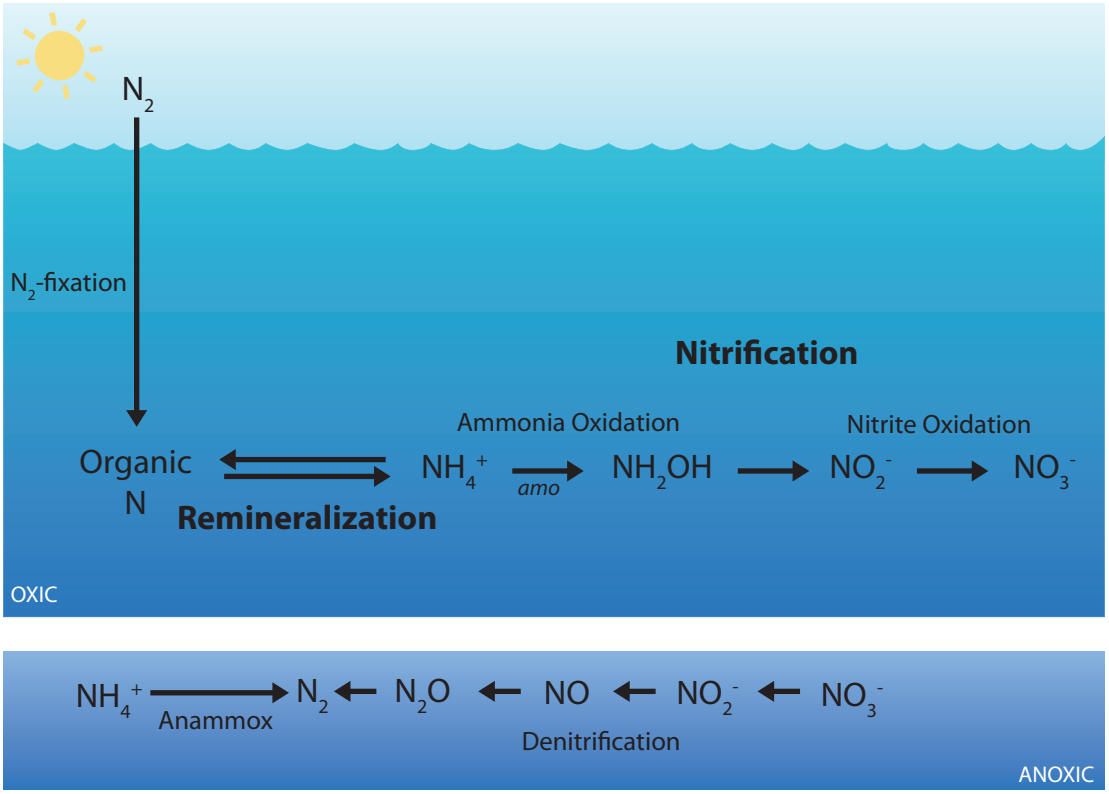

Figure 1.3. Microbially-mediated processes of the marine nitrogen cycle.

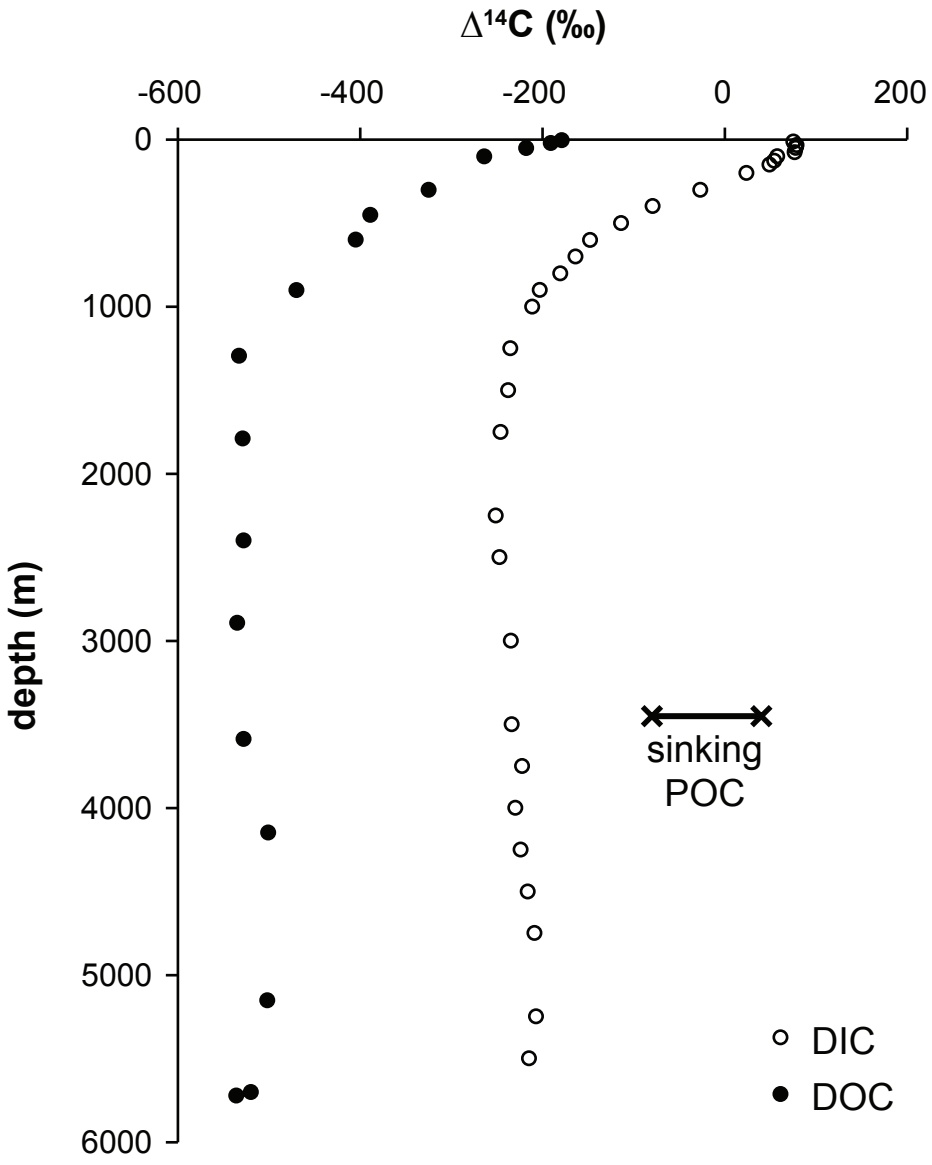

Figure 1.4. Radiocarbon ($\Delta^{14}C$) depth profiles for various carbon pools in the North Pacific Ocean. $\Delta^{14}C$-DOC (Druffel *et al*., 1992) and $\Delta^{14}C$-sinking POC (Hwang *et al*., 2004) data are from Station M (31˚00'N, 159˚00'W). $\Delta^{14}C$-DIC data (Kumamoto *et al*., 2002) are from 35˚00'N, 155˚00'E.

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II. Methods for the extraction of microbial DNA suitable for radiocarbon analysis

This chapter will serve as a comprehensive reference for the extraction of significant amounts of microbial DNA (on the order of 100's of μgs) suitable for radiocarbon analysis from large volumes of seawater. In addition to being largely used for a radiocarbon measurement, the resulting DNA can also be analyzed for stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and amplified by PCR for a number of molecular biology analyses. The primary goal for obtaining a $\Delta^{14}C$ value of extracted microbial DNA is to determine the source(s) of carbon supporting prokaryotic production in the subsurface ocean. The utility of measuring natural abundance carbon isotopes in DNA for tracing carbon assimilation by microbes in aquatic environments has been previously demonstrated with ^{13}C by Coffin *et al*. (1990) for estuarine bacteria and with ^{14}C by Cherrier *et al*. (1999) in the surface ocean. The aim of the subsequent molecular biological analyses is to gain information about the abundance, composition, and genetic potential of the microbial community present at the time of sampling.

Sample collection

Significant volumes of seawater (1,000-50,000 L) were filtered through two acid-cleaned large volume capacity 10-inch cartridge filters plumbed in-line. The pre-filter (either 0.5-μm or 1.2-μm pore size) composed of cellulose esters (Millipore Opticap) serves to trap larger particles, while the bulk of the microbial biomass is collected on the polyethersulfone 0.2-μm pore size filter (Pall Supor or Millipore Opticap). One primary location for sample collection was the Natural Energy

Laboratory of Hawaii Authority (NELHA) in Kona, HI (Chapter 3). Large volumes of seawater from the surface (21 m), 670 m, and 915 m were easily accessible and available through the pumping station here, and filter setups were plumbed directly to a tap with flow regulated to approximately 5 L min^{-1}. Samples were also collected at sea from surface water using tubing extended off the side of the ship and a diaphragm pump, or from depth by filtering water collected in Niskin bottles mounted on a rosette (Chapter 4). More recently, a McLane WTS-LV pump (McLane Labs, Falmouth, MA), modified to house the cartridge filters, was used to pump large volumes of seawater *in situ* at 3000 m depth at a rate of approximately 7 L min^{-1}.

Nucleic acid extraction protocol

Nucleic acids were chemically extracted using a modified Marmur (1961) method adapted from Blair *et al.* (1985). The 10-inch cartridge filters were cut open using a combusted hacksaw, torn into pieces, and extracted in 1.5 L of 1.5 M $NaClO_4$ for 48 h at 4˚C with periodic shaking to lyse the prokaryotic cells and release the cellular contents into solution. Enzymes and other lysis agents such as detergents were avoided in order to minimize exposure to possible organic carbon contaminants. Filter pieces were rinsed with Milli-Q water. The resulting 2 L sample solution was then concentrated down to approximately 20 ml by tangential flow filtration with a Pellicon cartridge (5000 MWCO; Millipore) that had been extensively cleaned with 0.1 N NaOH and thoroughly rinsed with Milli-Q water, and then transferred into two 50-mL centrifuge tubes. An organic extraction was performed on the sample by

adding an equal volume of chloroform:isoamyl alcohol (24:1) to each tube, shaking for 15 min, then centrifuging at 10000 x g for 10 min. The use of phenol was avoided due to potential ^{13}C fractionation effects (Coffin *et al*., 1990). The resulting aqueous layer containing the nucleic acids was transferred to a new centrifuge tube while the organic layer was re-extracted with 2 ml 15 mM NaCl-1.5 mM Na_2HPO_4. The aqueous layers from both extractions were combined. Nucleic acids were precipitated out of the concentrated sample by the addition of 0.1x 3 M NaCl and 2.5x cold 95% EtOH and placed overnight at -20°C. Sodium acetate was originally used in the place of NaCl for this step; however, through ^{1}H-NMR we determined the acetate was persisting in the precipitated nucleic acids and ultimately contaminating the sample for ^{14}C analysis. Therefore, NaCl was used in all subsequent extractions. The precipitated nucleic acids were pelleted by centrifugation at 10000 x g for 30 min at 4°C. The supernatant was discarded and the pellet was then air-dried upside-down for 10 min before being dissolved in 2 ml 15 mM NaCl-1.5 mM Na_2HPO_4 and 200 µl 1.5 M NaCl-0.15 M Na Na_2HPO_4. A second organic extraction was performed by adding 2.2 ml chloroform:isoamyl alcohol (24:1). The aqueous layer was transferred to a clean tube and precipitated by the addition of 0.1x 3 M NaCl and 2.5x cold 95% EtOH overnight at -20°C. The precipitated nucleic acids were once again pelleted by centrifugation at 10000 x g for 30 min at 4°C, the supernatant decanted, and the pellet washed with 3 ml cold 95% EtOH. After an additional centrifugation at 10000 x g for 5 min, the supernatant was once again decanted and the pellet was air-dried upside-down for 10 min. The nucleic acids were then dissolved in a small volume (2 ml) of

nuclease-free water. Aliquots were taken out to check quantity and quality of the nucleic acids, as well as for PCR and other molecular biological analyses. The majority of the remaining sample was then transferred to a combusted 8-inch long quartz tube (9 mm o.d. x 7 mm i.d. x 8" long) and lyophilized to yield the solid nucleic acid sample for radiocarbon analysis.

DNA quantification

DNA concentrations were estimated using absorbance at 260 nm as determined on a NanoDrop 1000, and quantified more accurately using the PicoGreen assay (Molecular Probes) as specified by the manufacturer. There was typically relative agreement between the two methods of quantitation amongst samples, though the DNA concentration calculated from its absorbance at 260 nm was generally higher on a per sample basis when compared to results from the PicoGreen assay. One possible explanation for this discrepancy is the presence of other cellular components in the samples, such as proteins, that also absorbed light at 260 nm, causing an overestimation of DNA concentration.

Nucleoside digestion and separation

In an attempt to improve upon the DNA extraction method and decrease uncertainty of DNA purity, bulk DNA samples in solution were hydrolyzed into individual nucleosides by enzyme digestion and then separated by HPLC. The enzyme digestion solution was composed of acid phosphatase (Sigma), S1 nuclease

buffer (Invitrogen), and S1 nuclease (Invitrogen). DNA was denatured for 5 min at 95˚C, added immediately to the digestion mixture, and rotated in a hybridization oven at 37˚C for 12 h. Any remaining proteins and undigested DNA were pelleted by centrifugation, and the resulting supernatant was injected onto the HPLC for nucleoside separation. Nucleosides were separated and purified by HPLC on a Zorbax SB-CN column using a methanol / water gradient elution method (Figure 2.1).

Collected peaks of individual nucleosides were checked for purity and identity by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy (Figure 2.2), then freeze-dried in quartz tubes for radiocarbon analysis. Nucleoside standards (deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine; Sigma) were also injected onto the HPLC and peaks collected for radiocarbon measurements, in addition to the pure solid taken directly from the bottle. Blanks of 'column bleed' collected at the expected retention times for each individual nucleoside were also submitted for ^{14}C analysis. As indicated from the results listed in Table 2.1, yields for sample individual nucleosides were quite low, especially when compared to the amount of carbon in the 'column bleed.' Comparisons of ^{1}H-NMR spectra for pure nucleosides and sample material collected off of the HPLC showed the presence of additional peaks in what was believed to be the sample nucleosides (Figure 2.2), suggesting contamination from either the column or the digestion protocol. Additionally, discrepancies between the Δ^{14}C values of the pure, unprocessed nucleoside standards from the bottle and those collected off the HPLC raised questions about the utility and accuracy of this method so it was ultimately abandoned in favor of measuring ^{14}C in

the bulk DNA samples.

Process blanks

GF/F-filtered seawater collected off of the Scripps Pier was amended with nitrate, phosphate, and either glucose or acetate as a carbon source. The natural assemblages of marine bacteria were grown up to 10^9 cells ml^{-1} (approximately 3 days) and then harvested by filtration onto a large volume capacity 0.2-μm polyethersulfone cartridge filter (Pall Supor). These filters were subjected to the exact same DNA extraction procedure as environmental samples, and the resulting DNA was analyzed for $\Delta^{14}C$ and $\delta^{13}C$.

Radiocarbon analysis

Samples for radiocarbon analysis were sealed in pre-combusted, evacuated quartz tubes with added silver and cupric oxide, and then combusted to CO_2 at 850˚C for 2 h. The resulting CO_2 was quantified manometrically and converted to graphite according to the methods of Vogel *et al*. (1987) for analysis by accelerator mass spectrometry (AMS) at the University of California-Irvine Keck Carbon Cycle AMS facility. Carbon yields were comparable to those calculated from DNA concentrations determined from sample absorbance at 260 nm. $\Delta^{14}C = [f_m e^{(1950-x)\lambda} - 1] * 1000$ as indicated by Stuiver and Polach (1977) for geochemical samples, in which $\lambda = 1/8267$ (yr^{-1}), f_m = fraction modern ^{14}C corrected for isotopic fractionation using $\delta^{13}C$, and x = year of collection.

Lipid extraction

In addition to extracting DNA from the microbial cells captured on the cartridge filters, we attempted to also isolate lipids to be used as biomarkers for the organisms present in the samples. The permeate (< 5,000 MWCO) from the ultrafiltration step of the DNA extraction was extracted in the method of Bligh and Dyer (1959). For every 100 ml aqueous sample in a 1-L separatory funnel, 375 ml 1:2 $CHCl_3$:MeOH was added and mixed, followed by 125 ml $CHCl_3$, and finally 125 ml solvent-extracted Milli-Q water. The lower organic fraction was collected and this was repeated for the approximate 1.5 L of sample permeate. All of the organic fractions from this extraction were evaporated to dryness on a rotary evaporator and combined with the organic layer from the DNA extraction performed with the ultrafiltered retentate to form the total lipid extract (TLE).

In the laboratory of Kai Hinrichs at Universität Bremen (Bremen, Germany) numerous techniques were utilized to isolate and analyze lipids. TLEs in 1:1 MeOH:DCM were analyzed for intact polar lipids (IPLs) by LC-MS. Portions of the TLEs were derivatized with 10 µl pyridine and 10 µl BSTFA at 70˚C for 1 h, dried down completely under N_2, and taken up in 20 µl hexane before being analyzed by GC-MS. Fatty acid fractions of the TLEs were isolated as follows: 1 ml of 6% KOH in MeOH was added to the dried-down TLE and placed at 80˚C for 3 h to saponify. After cooling, it was extracted 4x with 1 ml hexane. The combined aqueous layers were acidified to pH 1 with approximately 3 drops of 4 N HCl and extracted 4x with 1

ml hexane. The resulting organic layers were combined, dried-down, derivatized as above, and analyzed with GC-MS. In general, little to no lipid biomarkers were identified in these samples. We concluded that our extraction method was not sufficient for isolating lipids from the polyethersulfone cartridge filters, as the microbial lipids surely present in the cells captured were either destroyed or 'trapped' at some stage during the extraction procedure. To investigate the possibility that lipid yields were affected by ultrafiltration, we also tried obtaining TLEs directly from the cellular lysis solution prior to concentration by ultrafiltration but saw no measurable increase in yields. For future work in analyzing lipids isolated from microbial biomass in the water column, we have switched to collecting samples on GF/F filters for improved and cleaner lipid yields.

Polymerase chain reaction (PCR) and clone libraries

PCR was carried out using a variety of published primer sets (Tables 2.2 and 2.3) to amplify domain and group specific 16S rRNA genes as well as a number of functional genes from extracted microbial DNA. Reaction conditions and cycling parameters were typically as described in the literature for each primer set. ExTaq polymerase (Takara) was commonly used for amplifying environmental DNA. For clone libraries, products of 3 to 6 reactions were pooled together, cleaned using a Qiagen PCR purification kit, and eluted in 30 µl nuclease-free water. 4 µl of cleaned product were used in a cloning reaction with 1 µl provided salt solution and 1 µl vector pCR2.1-TOPO (Invitrogen). The reaction vial was incubated for 5 min at room

temperature and then 2 µl were used to transform a vial of One Shot® TOP10 competent cells (Invitrogen) by incubating for 5 min on ice, heat-shocking the cells for 30 s in a 42˚C water bath, and then transferring back to ice for 2 min. 250 µl of provided S.O.C. media was added to the vial of cells, which was then shaken horizontally at 37˚C for 1 h. Transformed cells were then plated onto LB agar plates containing kanamycin and incubated overnight at 37˚C for growth. White colonies were typically checked for inserts by colony PCR, then submitted for sequencing as either extracted plasmid DNA (to SeqXcel; San Diego, CA) or as glycerol stocks (to Agencourt Bioscience; Beverly, MA).

Quantitative PCR (qPCR)

For qPCR assays, each 25 µl reaction consisted of 12.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.4 µM each primer, 2 ng BSA, and 1-8 ng DNA (as determined by PicroGreen). Primer sets (Tables 2.2 and 2.3) and cycling parameters used were as published (Allen *et al*., 2005; Mincer *et al.*, 2007; Beman *et al*., 2008), and all assays were run on an MP3000X Real-time PCR Thermal Cycler (Stratagene). PCR products for each gene of interest cleaned using Qiagen PCR purification kit and quantified by PicoGreen were used as standards and diluted 10-fold to range from 10^2 to 10^7 gene copies. Results are typically presented as relative abundances amongst samples of gene copies per ng total DNA.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was used to compare community composition between samples of extracted microbial DNA. Portions of bacterial and archaeal 16S rRNA genes were amplified by PCR using primer sets with 40-bp GC clamps added to the 5' ends of the forward primers (Tables 2.2 and 2.3; Muyzer *et al*., 1993) and a touchdown thermocycling program (Don *et al*., 1991). PCR products were run on 30-70% gradient polyacrylamide gels in 0.5x TAE at a constant voltage of 85 V and temperature of 60˚C for 16 h. The gels were subsequently stained with SYBR Green (Molecular Probes) and visualized under UV light. Figure 2.3 shows the resulting banding patterns for both bacteria (a) and archaea (b) from samples collected at NELHA from 21 m (2 left lanes) and 670 m (4 right lanes). As expected, the microbial community appears to be quite different in the mesopelagic than in surface waters, and the archaea seem to dominate at 670 m compared to the surface.

Fluorescent in situ hybridization (FISH)

FISH was tested as a method of determining the abundances of bacteria versus archaea present at the time of sampling. Samples were collected separately from the large volume filtrations, and generally 10 ml of seawater was fixed in 3.7% formaldehyde and placed at 4˚C for 1 to 24 h. The fixed sample was then filtered onto a 25 mm white polycarbonate membrane filter (Millipore), rinsed with a 2% NaCl-50% EtOH solution, air-dried, and stored at -20˚C until further processing. Back at the lab, cells were embedded onto the filter surface by dipping the filter in 0.2% low-melt agarose.

Because detection was very low using standard oligonucleotide probes, catalyzed reporter deposition FISH (CARD-FISH) with horseradish peroxidase (HRP)-labeled oligonucleotide probes was used to try to increase detections of bacteria and archaea. The method of Teira *et al*. (2004) that optimizes the permeabilization of marine archaea with proteinase K was generally followed. Briefly, filter pieces were incubated in either lysozyme (for bacteria detection using probe Eub338-HRP; Amann *et al*., 1990) or proteinase K (for archaea detection using probe Arch915-HRP; Stahl and Amann, 1991) for 1 h at 37˚C to permeabilize cell membranes. The filters were then washed 3 times in Milli-Q water and incubated in 0.01 M HCl at room temperature for 20 min to inactivate the permeabilization enzymes. Filters were washed twice in Milli-Q water, dehydrated in 95% EtOH, and air-dried. For hybridization, filters were incubated overnight (8-12 h) at 35˚C in 500 µl hybridization buffer + 20 µl HRP-labeled probe. Buffer stringency was probe-dependent (Table 2.4). Following hybridization, filters were washed for 15 min in pre-warmed wash buffer at 37˚C, rinsed in PBS-T for 15 min at room temperature, and incubated for 30 min at 37˚C in 493 µl amplification buffer + 5 µl tyramide-Alexa488. After a final 15 min room temperature wash in PBS-T, filters were then rinsed with Milli-Q water and 95% EtOH before being mounted onto microscope slides using Vectashield® with DAPI (Vector Labs). Table 2.5 shows results of the abundance of bacteria and archaea (as % of DAPI-stained cells) in samples collected from 21 m and 670 m at NELHA, as well as from the SIO Pier. The total combined % of hybridized cells was low compared to the DAPI counts for the NELHA samples. Because the

archaea abundances were comparable to those found at nearby Station ALOHA for these depths (Karner *et al*., 2001), we hypothesized that the hybridization efficiency of the Eub338 probe was probably low. Though it did not appear to be the case for our experiments, the Arch915 probe has been shown to unspecifically hybridize to bacteria populations (Pernthaler *et al*., 2002); so for future work we planned to use the archaeal group-specific probes Cren537 (targeting *Crenarchaeaota*) and Eury806 (targeting *Euryarchaeota*) (Teira *et al*., 2004). Ultimately, difficulties in achieving high efficiencies led us to abandon CARD-FISH as a method for determining abundances in future samples in favor of other techniques such as qPCR, which could be applied to the same DNA extracted for isotopic analyses rather than separately collected samples.

Table 2.1. Sizes and $\Delta^{14}C$ values of nucleoside standards (pure solids and as collected from the HPLC), hydrolysis enzymes, HPLC blanks, and sample HPLC peaks. n.a., not available; samples too small for accurate radiocarbon measurement.

Sample	Size (µgC)	$\Delta^{14}C$ (‰)	UCIAMS# / UCIG#
S1 nuclease	94	-976 ± 4	24612 / 10169
acid phosphatase	861	-670 ± 1	24606 / 10172
dA from bottle	130	+31 ± 2	24611 / 10165
dC from bottle	172	+33 ± 3	24604 / 10174
dG from bottle	33	+51 ± 4	24614 / 10167
dT from bottle	70	-454 ± 3	24613 / 10171
dA HPLC	137	+38 ± 2	24594 / 10146
dC HPLC	169	+33 ± 2	24593 / 10145
dG HPLC	31	+125 ± 4	24554 / 10117
dT HPLC	56	-384 ± 3	24595 / 10147
9’ column bleed	37	-870 ± 6	24553 / 10118
12’ column bleed	2	-840 ± 96	24577 / 10112
18’ column bleed	1	n.a.	n.a.
24’ column bleed	7	-416 ± 18	24564 / 10106
9’ nucleoside	1	n.a.	n.a.
12’ nucleoside	1	n.a.	n.a.
18’ nucleoside	10	-13 ± 17	24562 / 10105
24’ nucleoside	11	+251 ± 20	24561 / 10119

Table 2.2. Primer sets, probes, and targeted genes for PCR amplification and fluorescent *in situ* hybridization (FISH).

Primer set	Gene target	Reference
27f, 1492r	bacteria 16S rRNA	Lane, 1991
21f, 958r	archaea 16S rRNA	DeLong, 1992
Type IF, Type IR	type I methanotrophs 16S rRNA	Chen *et al*., 2007
Arch-amoAF, Arch-amoAR	archaeal amoA	Francis *et al*., 2005
A189f, mb661r	pmoA	Costello and Lidstrom, 1999
wcpmoA189f, wcpmoA661r	water column pmoA	Tavormina *et al*., 2008
nas22, nas1933	nasA nested PCR	Allen *et al*., 2001
nas964, nasA1735	nasA nested PCR	Allen et al., 2001
341f-GC, 534r	bacteria 16S rRNA (DGGE)	Muyzer *et al*., 1993
ARC344f-GC, 517r	archaea 16S rRNA (DGGE)	Bano *et al*., 2004
932f, 1062r	bacteria 16S rRNA (qPCR)	Allen et al., 2005
GI_741F, GI_956R	GI Crenarchaeaota 16S rRNA (qPCR)	Mincer *et al*., 2007
Arch-amoAFA, Arch-amoAFB	archaeal amoA (qPCR)	Beman *et al*., 2008
Eub338	bacteria 16S rRNA (FISH)	Amann *et al*., 1990
Arch915	archaea 16S rRNA (FISH)	Stahl and Amann, 1991
Cren537	Crenarchaeota 16S rRNA (FISH)	Teira *et al*., 2004
Eury806	Euryarchaeota 16S rRNA (FISH)	Teira *et al*., 2004

Table 2.3. Sequences of primers and probes listed in Table 2.2.

Primer	Sequence (5' – 3')
27f	AGA GTT TGA TCM TGG CTC AG
1492r	GGY TAC CTT GTT ACG ACT T
21f	TTC CGG TTG ATC CTG CCG GA
958r	YCC GGC GTT GAM TCC AAT T
Type IF	ATG CTT AAC ACA TGC AAG TCG AAC G
Type IR	CCA CTG GTG TTC CTT CMG AT
Arch-amoAF	STA ATG GTC TGG CTT AGA CG
Arch-amoAR	GCG GCC ATC CAT CTG TAT GT
A189f	GGN GAC TGG GAC TTC TGG
mb661r	CCG GMG CAA CGT CYT TAC C
wcpmoA189f	GGN GAC YGG GAT TTC TGG
wcpmoA661r	CAG GMG CAA CGT CYT TAC C
nas22	TGY CCN TAY TGY GGN GT
nas1933	CAR TGC ATN GGN AYR AA
nas964	CAR CCN AAY GCN ATG GG
nasA1735	ATN GTR TGC CAY TGR TC
341f-GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG
534r	ATT ACC GCG GCT GCT GG
ARC344f-GC	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC CAC GGG GCG CAG CAG GCG CGA
517r	ATT ACC GCG GCT GCT GG
932f	CGC ACA AGC RGY GGA GYA TGT G
1062r	CAC RRC ACG AGC TGA CGA
GI_741F	GTC TAC CAG AAC AYG TTC
GI_956R	HGG CGT TGA CTC CAA TTG
Arch-amoAFA	ACA CCA GTT TGG YTA CCW TCD GC
Arch-amoAFB	CAT CCR ATG TGG ATT CCA TCD TG
Eub338	GCT GCC TCC CGT AGG AGT
Arch915	GTG CTC CCC CGC CAA TTC CT
Cren537	TGA CCA CTT GAG GTG CTG
Eury806	CAC AGC GTT TAC ACC TAG

Table 2.4. CARD-FISH buffer compositions.

Buffer	Eub338-HRP	Arch915-HRP
hybridization buffer	0.9M NaCl 20mM Tris-HCl (pH 7.5) 10% dextran sulfate 0.02% SDS 1% blocking reagent 55% formamide	0.9M NaCl 20mM Tris-HCl (pH 7.5) 10% dextran sulfate 0.02% SDS 1% blocking reagent 20% formamide
wash buffer	5mM EDTA (pH 8) 20mM Tris-HCl (pH 7.5) 0.01% SDS 13mM NaCl	5mM EDTA (pH 8) 20mM Tris-HCl (pH 7.5) 0.01% SDS 145mM NaCl
PBS-T	145mM NaCl 1.4mM NaH_2PO_4 8mM Na_2HPO_4 (pH 7.6) 0.05% Triton X-100	
amplification buffer (in PBS)	10% dextran sulfate 2M NaCl 0.1% blocking reagent 0.0015% H_2O_2	

Table 2.5. Prokaryotic cell abundances as determined by CARD-FISH.

Sample	Eub338-HRP	Arch915-HRP	Total % DAPI
NELHA 21 m	36.4%	6.6%	43.0%
NELHA 670 m	28.4%	21.3%	49.7%
SIO Pier	68.3%	5.1%	73.4%

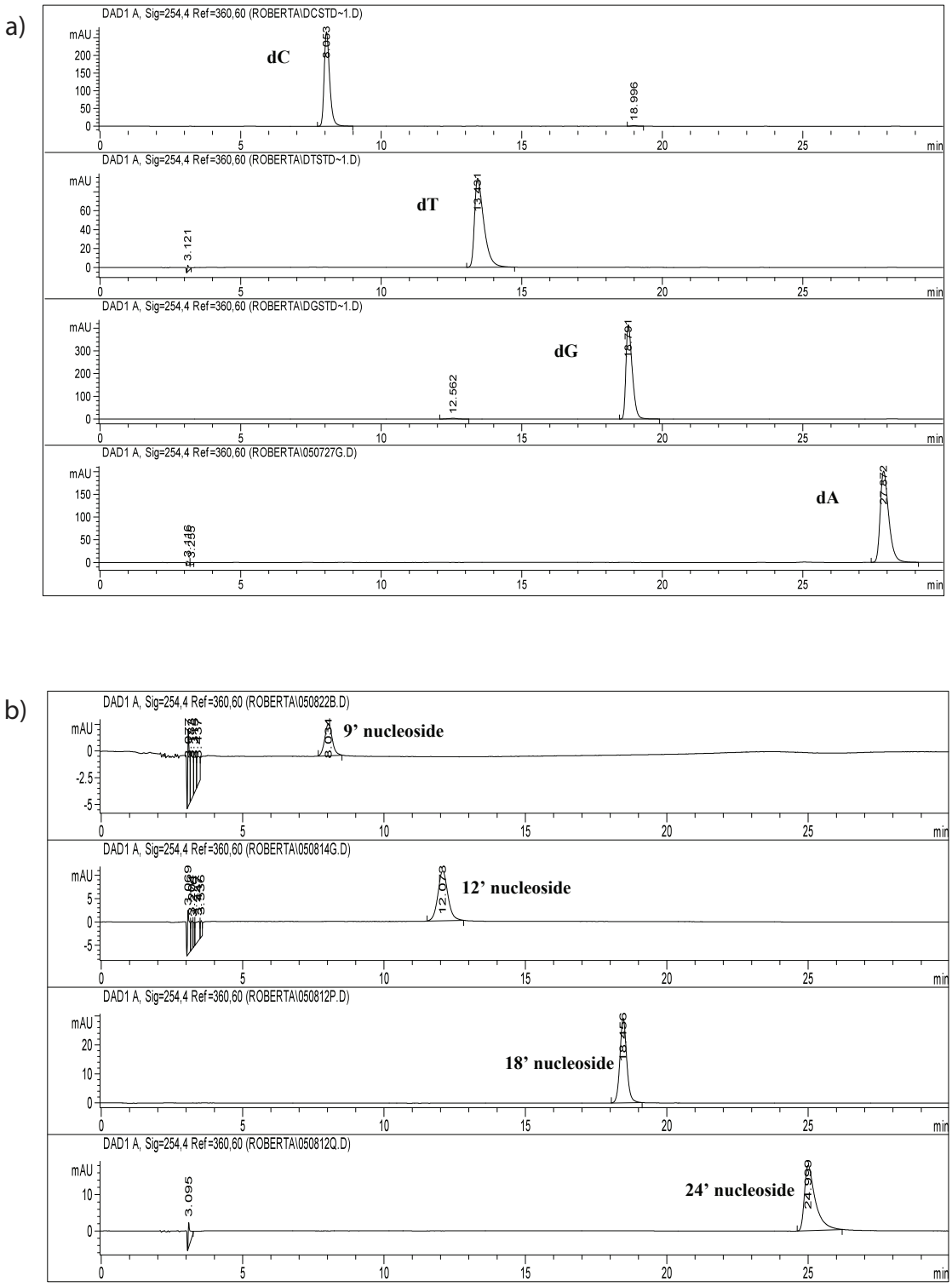

Figure 2.1. HPLC chromatograms showing retention times of a) nucleoside standards, and b) collected fractions of hydrolyzed sample DNA.

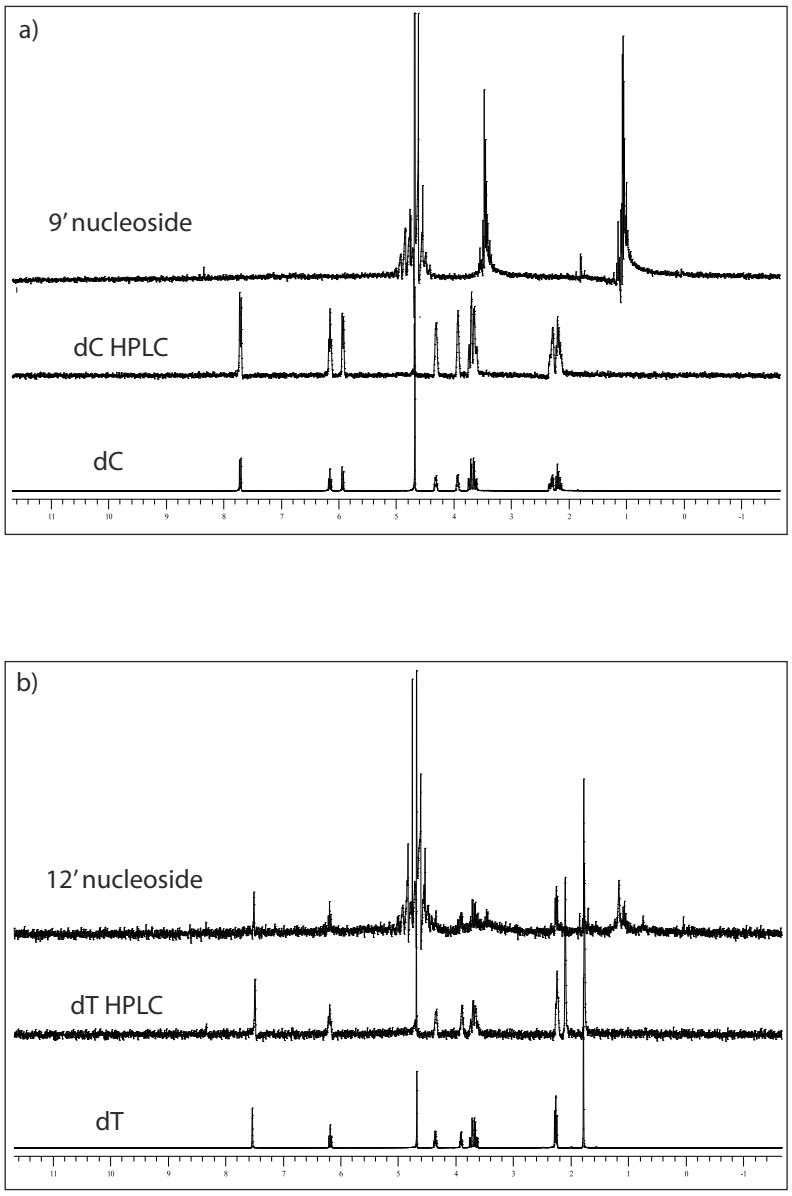

Figure 2.2. ^{1}H-NMR spectra of pure nucleoside standards, standards collected off the HPLC, and hydrolyzed sample DNA fractions for a) deoxycytidine (dC), and b) thymidine (dT)

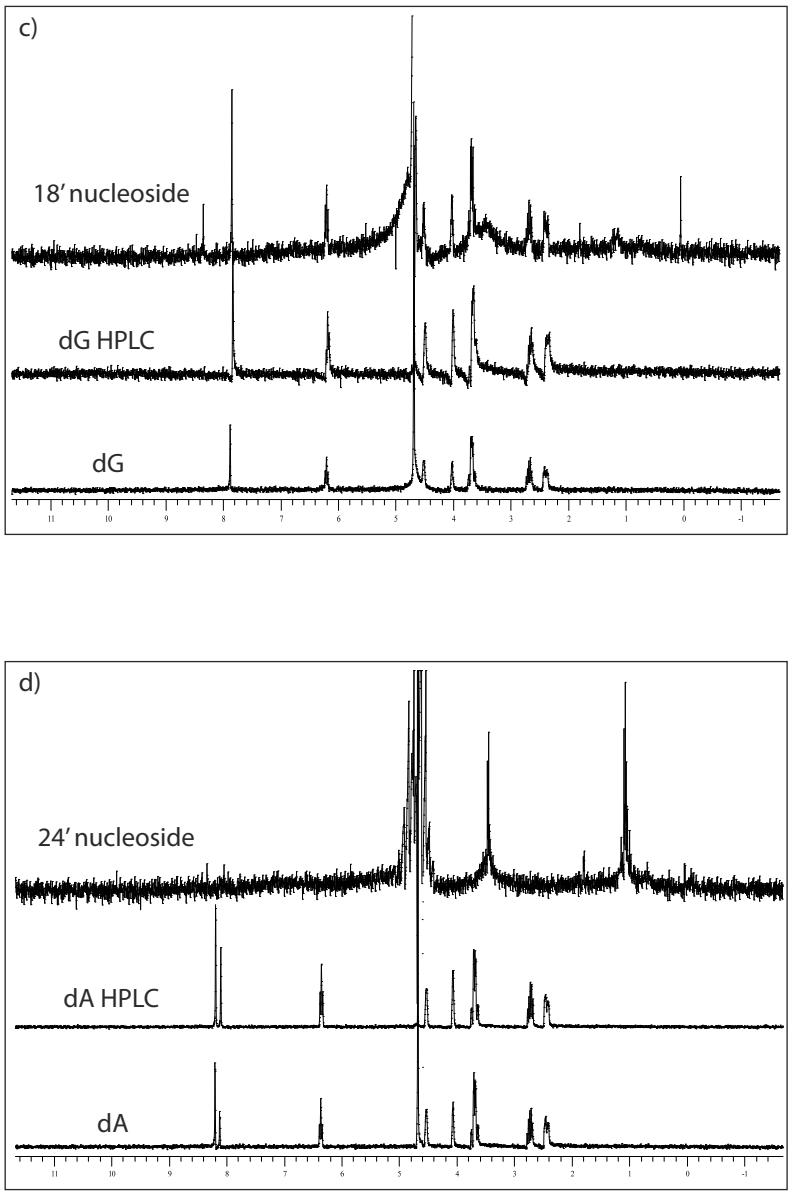

Figure 2.2, continued. ^{1}H-NMR spectra of pure nucleoside standards, standards collected off the HPLC, and hydrolyzed sample DNA fractions for c) deoxyguanosine (dG), and d) deoxyadenosine (dA).

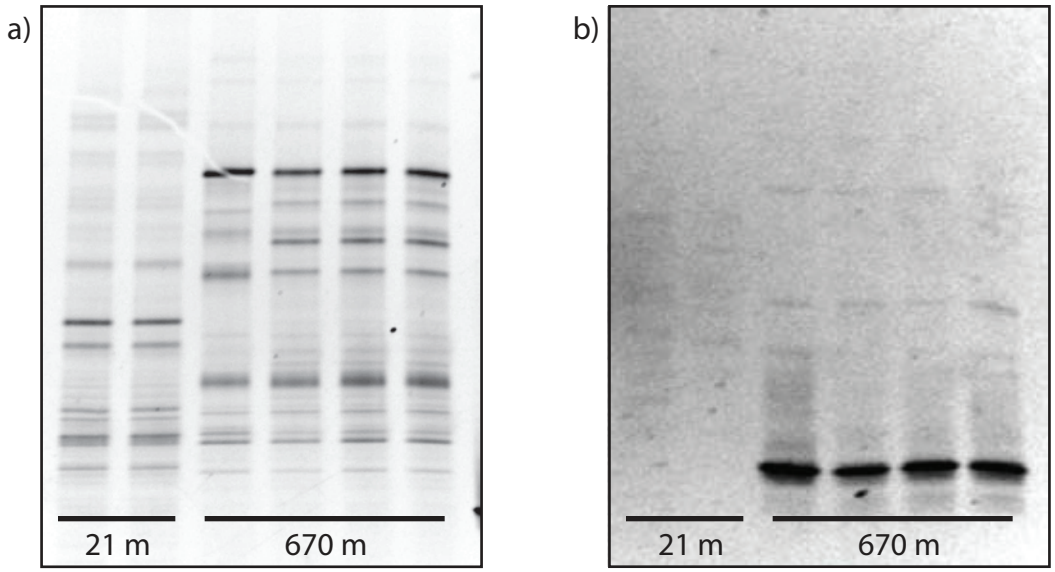

Figure 2.3. DGGE banding patterns for a) bacterial 16S rRNA and b) archaeal 16S rRNA from extracted DNA samples collected from 21 m (2 left lanes) and 670 m (4 right lanes) at NELHA.

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III. The radiocarbon signature of microbial organisms in the mesopelagic ocean

Abstract

Dramatic attenuation of the sinking particle flux in the top 1000 m of the oceanic water column, the short residence time of suspended particulate organic carbon in the meso- and bathypelagic ocean, and the detection of rRNA in a significant fraction of microorganisms in the deep ocean indicate that microbes are metabolically active and respiring carbon in this environment. In addition, growing evidence also suggests that archaea are fixing inorganic carbon in the dark ocean. Carbon flow through the microbial community at two depths in the mesopelagic zone of the North Pacific Subtropical Gyre is examined here by exploiting the unique radiocarbon signatures ($\Delta^{14}C$) of the three major carbon sources in this environment. The radiocarbon content of nucleic acids, a biomarker for active cells, isolated from size-fractionated particles (0.2-0.5 μm and > 0.5 μm) showed the direct incorporation of carbon delivered by rapidly sinking particles. However, at the two mesopelagic depths examined (670 m and 915 m), autotrophic carbon fixation also appeared to support a significant fraction (36-100%) of the free-living microbial community (0.2-0.5 μm size fraction). The results further showed that utilization of the ocean's largest reduced carbon reservoir, dissolved organic carbon, was negligible in this environment. This isotopic portrait of carbon assimilation by the *in-situ,* free-living microbial community, integrated over > 50,000 L of seawater, implies that recent, photosynthetic carbon is not the major carbon source supporting this community in the mesopelagic ocean.

Introduction

Marine microbes play a critical role in transforming inorganic and organic carbon in the ocean (Azam and Malfatti, 2007), and their interactions with the carbon reservoir, in its variety of phases, have important implications for the sequestration of CO_2 in either the deep ocean or sediments. However, the role that microorganisms play in the carbon cycle of the deep ocean, the mechanisms they employ, and the extent to which they employ them, are poorly understood. Most of the ocean's reduced carbon accumulates in the meso- and bathypelagic as dissolved organic carbon (DOC; approximately 700 Pg,(Hedges, 1992)), and here is where the fate of a significant fraction of organic carbon leaving the euphotic zone is decided (Buesseler *et al*., 2007). Therefore, microbial transformations of carbon in the "twilight zone" (defined here according to Buesseler *et al.* (2007) as depths between the base of the euphotic zone and 1000 m) play an essential role in determining the ocean's ability to sequester atmospheric CO_2.

Below the euphotic zone, light and then labile organic matter become increasingly scarce. Although DOC is present in this environment, it has long been assumed that this reservoir is unavailable to support heterotrophic production. For example, depth profiles of DOC (Williams and Druffel, 1987), dissolved combined amino acids (Lee and Bada, 1977; Williams, 1986), and dissolved carbohydrates (Mopper, 1977; Williams, 1986) appear to be static below 600 m, consistent with findings that DOC from the deep ocean resists microbial degradation (Barber, 1968). The most convincing evidence of the refractory nature of deep ocean DOC is its

average radiocarbon age, which lies between 4000 and 6500 radiocarbon years in the deep ocean (Williams and Druffel, 1987; Druffel *et al*., 1992; Druffel and Bauer, 2000).

Despite the apparently static nature of the largest carbon reservoir available to support heterotrophic production in the meso- and bathypelagic, the abundance and activity of microorganisms are still measurable (Azam *et al*., 1992; Massana *et al*., 1998; Perez *et al*., 2003; Teira *et al*., 2006). One study suggests that 20-33 Pg C yr^{-1} are respired to CO_2 in the dark ocean (Arístegui *et al*., 2003), and the majority of this carbon is most likely derived from particulate organic carbon (POC) that sinks out of the euphotic zone. Measurements using particle traps have shown that POC flux is strongly attenuated in the mesopelagic ocean (Martin *et al*., 1987; Buesseler *et al*., 2007); and a parameterization of this flux attenuation – the Martin Curve (Martin *et al*., 1987) - demonstrated that 90% of the organic carbon leaving the euphotic zone was lost from the particle phase within the upper 1500 m. This loss may occur as a result of respiration by particle-attached bacteria or through DOC release. Several geochemical observations suggest that there is a "fresh" or new component within the mesopelagic DOC pool that is masked by the much larger refractory fraction identified above (Mortazavi and Chanton, 2004; Repeta and Aluwihare, 2006), and sinking POC is a likely conduit for the rapid delivery of this new DOC to the deep ocean.

Bacteria attached to particles and marine snow sinking out of surface ocean waters produce hydrolytic enzymes that appear to transform POC into DOC at a rate faster than that which it is assimilated, and it is hypothesized that this decoupling of

hydrolysis and uptake rates produces a "plume" of DOC and other nutrients that trail these particles as they sink through the water column (Karner and Herndl, 1992; Smith *et al*., 1992; Azam and Long, 2001; Kiørboe and Jackson, 2001). Other authors have noted strong correlations between deep ocean free-bacterial abundance and both episodic and long-term particle flux out of the overlying water column (Cho and Azam, 1988; Nagata *et al*., 2000; Hansell and Ducklow, 2003), again suggesting carbon transfer from sinking POC to DOC to heterotrophic microbes. In support of this hypothesis, high-resolution depth profiles demonstrating that suspended POC contains bomb ^{14}C at abyssal depth (Druffel *et al*., 1996) indicate that rapidly sinking particles disaggregate to form suspended POC - either an intermediate or a by-product of particle dissolution. Therefore, at least a fraction of free-living heterotrophic microbes in the deep ocean are likely supported by inputs of recently-fixed carbon provided by the dissolution of sinking POC to DOC.

Archaea have been shown to contribute up to 50% of prokaryotic abundance in the deep ocean (Karner *et al*., 2001), and, as a domain, demonstrate the ability to take up amino acids as well as fix inorganic carbon (Ouverney and Fuhrman, 2000; Pearson *et al*., 2001; Wuchter *et al*., 2003; Herndl *et al*., 2005; Ingalls *et al*., 2006). For example, the recently cultured crenarchaeote *Nitrosopumilus maritimus* is an ammonia-oxidizing chemoautotroph (Könneke *et al*., 2005), and the gene coding for subunit A of an archaeal ammonia monooxygenase (*amoA*) has been found to be ubiquitous throughout the marine environment (Francis *et al*., 2005). Crenarchaeaota are now believed to be important players in the marine nitrogen cycle through

chemoautotrophic nitrification (Wuchter *et al*., 2006). Studies have shown that planktonic archaea distributions and apparent metabolisms vary with depth and across water masses (Herndl *et al*., 2005; Varela *et al*., 2008). However, the contribution of chemosynthesis (by these and other organisms) to the total production of the microbial community and how that might change throughout the water column have not been fully determined (Herndl *et al*., 2005; Ingalls *et al*., 2006*)*.

In this study, natural variations in the abundance of the radioactive isotope of carbon (^{14}C) in different reservoirs were exploited to identify carbon sources fueling production of the total microbial community and explicitly test the hypothesis that the microbes in the mesopelagic ocean are supported primarily by particle flux from overlying waters. The three pools of carbon discussed above, dissolved inorganic carbon (DIC), refractory DOC, and fresh DOC injected from particles, have unique ^{14}C signatures ($\Delta^{14}C$) in the deep ocean that can be traced into the intracellular biochemicals of organisms that utilize these various carbon sources. Fresh DOC delivered from the surface ocean has a modern $\Delta^{14}C$ value > +50‰, while DIC in the mesopelagic zone has pre-bomb $\Delta^{14}C$ values and is depleted in ^{14}C due to radioactive decay (≈ -240 to -100‰). The bulk DOC pool is the "oldest," with an average $\Delta^{14}C$ value of -525‰ in the deep North Pacific Ocean (Druffel *et al*., 1992). Microbial DNA is used as a biomarker for the total microbial community as it is synthesized *de novo* in cells using carbon, nitrogen, and phosphorus acquired from the surrounding environment. DNA is expected to be primarily present in viable cells, and so, the $^{14}C/^{12}C$ ratio of DNA will provide a snapshot of the active community at the time of

sampling. This technique represents a direct method by which to evaluate carbon metabolism *in situ*, and was first used to trace assimilated carbon through aquatic microbial communities by Coffin *et al.* (1990) and Cherrier *et al.* (1999).

Results of the study directly supported the hypothesis that organic carbon delivered from rapidly sinking particles represents an important energy source for free-living microorganisms in the subsurface ocean. However, the study also revealed the importance of chemoautotrophy in driving total community production in the mesopelagic ocean. Together, these data provide a geochemical glimpse of the diverse roles microorganisms play in the carbon cycle of the ocean's "twilight zone."

Results

To demonstrate the incorporation of source carbon into microbial DNA and to quantify any carbon contamination introduced from the DNA extraction and isolation procedure, a set of ^{14}C process blanks was obtained. GF/F-filtered seawater collected off the Scripps Institution of Oceanography (SIO) pier was amended with 883 μM nitrate and 36.3 μM phosphate, in addition to a carbon source with a known $\Delta^{14}C$ value (glucose, $\Delta^{14}C = +87 \pm 2‰$; or acetate, $\Delta^{14}C = -987 \pm 4‰$). These carbon sources were chosen to provide a range of $\Delta^{14}C$ values from modern ($\Delta^{14}C > 0‰$) to radiocarbon dead ($\Delta^{14}C = -1000‰$) in order to constrain possible carbon contamination from either end of the $\Delta^{14}C$ spectrum. The natural, marine microbial community was then incubated in the dark for three days until biomass was approximately 10^9 cells ml^{-1} and then subjected to the same filtration, lysis, and

extraction method as all environmental samples. Isolated DNA was processed for $\Delta^{14}C$ and $\delta^{13}C$ analyses with the results shown in Table 3.1. ^{14}C sample sizes were comparable to the amount of carbon analyzed for environmental $\Delta^{14}C$-DNA (Table 3.2). $\Delta^{14}C$-DNA values, +63 ± 2‰ (average ± S.D.; n = 3) for the glucose culture and -821‰ for the acetate culture, deviate to different extents from the $\Delta^{14}C$ signature of the respective carbon source (glucose = +87 ± 2‰; acetate = -987 ± 4‰). The observed deviation from the added carbon source could be due to a combination of several possible factors: utilization of labile ambient DOC present in the collected seawater by co-metabolism with the added glucose or acetate (Carlson *et al*., 2002), selective utilization of the individual carbon atoms of an isotopically inhomogeneous carbon source (e.g., glucose), and contamination from either process chemicals or passive adsorption of ambient bulk DOC to filters during cell isolation. It is clear that no consistent carbon contamination from either a modern or ^{14}C-dead source is introduced through our extraction procedure, but an exogenous component with an intermediate $\Delta^{14}C$ value (between modern and ^{14}C dead) could cause the observed deviation.

A mass balance calculation using $\Delta^{14}C$-DNA results from the two identical experiments conducted in 2007 can be solved by a 24 µgC contaminant with a $\Delta^{14}C$ value of -235‰. (Values used for mass balance calculations are provided in the supporting information section). Cleaned, blank filters (n = 2) subjected to the entire method yielded a negligible 0.7 ± 0.4 µgC with an average $\Delta^{14}C$ value of -379 ± 300‰ (UCIG# 11408-11409). The small yield of carbon indicates that blanks, due to

either reagents or filter material, are not solely responsible for the deviation. All sample DNA $\Delta^{14}C$ values have been corrected for this blank. The other source of possible contamination, bulk DOC adsorption onto filters, would then be responsible for much of the observed deviation. However, source carbon and bacterial DNA had very similar $\delta^{13}C$ values following the incubation ($\Delta\delta^{13}C < \pm 1‰$; Table 3.1), which is inconsistent with 24 μg of ambient DOC co-isolation during DNA extraction. Furthermore, experimental glucose cultures processed in 2003 and 2007, which were amended with the same batch of chemicals but conducted in different volumes of seawater, yielded identical $\Delta^{14}C$-DNA values (Table 3.1), again suggesting that differences between source C and DNA could not be easily attributed to the adsorption of the bulk DOC present in ambient seawater. Therefore, a combination of other factors discussed above is likely to have contributed to the observed differences.

At the surface (21 m), the measured $\Delta^{14}C$ value of DNA isolated from the 0.2-0.5 μm microbial community is modern, as expected, with a value of +36 ± 2‰ (Figure 3.1). The measured $\Delta^{14}C$-DNA value of the 0.2-0.5 μm size fraction at 670 m was -187 ± 14‰, significantly lower than the value for the > 0.5 μm fraction at this same depth (-106 ± 4‰; Table 3.2; Figure 3.1). At 915 m, measured $\Delta^{14}C$ values for both size fractions were higher than those at 670 m, -87 ± 10‰ for the 0.2-0.5 μm size fraction and -27 ± 4‰ for the > 0.5 μm fraction (Figure 3.1). Reported $\Delta^{14}C$ errors are propagated AMS errors and blank corrections. In two cases replicate samples were run (670 m 0.2-0.5μm and 915 m > 0.5 μm samples), and differences in measured $\Delta^{14}C$ values ranged from 5 to 20‰ between replicates. $\delta^{13}C$ values of all

DNA samples at all depths are between -21 and -19‰, within the expected range for samples of marine origin (Table 3.2).

A variety of different techniques were used to assess the quantity and quality of extracted sample DNA including UV-vis absorbance at 260 nm, agarose gel electrophoresis, and amplification by PCR. Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy was also used to insure that there was no contamination of DNA samples from process chemicals, which would appear as sharp, well-defined resonances. In addition, the presence of significant ^{1}H-NMR resonances in the region between 7 to 8.5 ppm indicative of aromatic groups was used to assess the purity of DNA. These functional groups are not typically abundant in marine detrital organic matter.

In order to estimate the possible contamination introduced through DOC adsorption, we examined the C:N ratios of each sample. Sample C:N values ranged from 4.05 to 5.32 (Table 3.2), and pure DNA is assumed to have a C:N of approximately 3 (confirmed by measured ratios in a pure *E. coli* standard). Variations in the sample C:N ratio from pure DNA could represent co-isolation of carbon-rich intracellular biochemicals, such as carbohydrates and lipids, that should provide the same radiocarbon information as pure DNA and does not represent a contamination of the radiocarbon sample. However, it is possible that elevated C:N ratios result primarily from adsorption of bulk DOC to filters followed by co-precipitation with DNA in the extraction method. If DOC is co-precipitated with our DNA, the extremely ^{14}C-depleted DOC at these depths in the ocean would contaminate our

measured Δ^{14}C-DNA values. Correction for this possible source of contamination (see Supporting Information) shifts our Δ^{14}C-DNA values toward modern (indicated as dashed lines on Figure 3.1) and brings them into better agreement with the Δ^{14}C value of DIC in the surface ocean and at 670 m depth. Corrected Δ^{14}C-DNA values for the 0.2-0.5 μm size fraction are +60‰ at the surface, and -140‰ and -15‰ at 670 m and 915 m, respectively. The > 0.5μm size fractions had corrected Δ^{14}C values of -73‰ at 670 m and +69‰ at 915 m (Table 3.2)

Due to the nature of the seawater supply system, we were motivated to analyze the microbial community sampled in order to confirm that we were not isolating DNA from organisms that were unique to the seawater intake pipe. 16S rRNA bacterial and archaeal clone libraries establish that the microbes sampled are representative of those found in other mesopelagic marine environments (including nearby station ALOHA; Tables 3.3 and 3.4), and no absolute trends based on depth or size fraction are apparent. The majority of archaeal clones from all depths and size fractions (> 85%) are Group I *Crenarchaeota*, with the remainder belonging to the Group II *Euryarchaeota*. Of note are a number of clones with 98-99% identity to the ammonia-oxidizing *N. maritimus* (Könneke *et al*., 2005). Difficulties amplifying the ~1400bp bacterial 16S rRNA fragment from the > 0.5 μm sample from 670 m lead us to believe this sample is partially degraded (confirmed by examination on an agarose gel), so sequences from that sample are absent on the bacterial phylogenetic classification table.

Discussion

The similar $\Delta^{14}C$ signatures of DIC in surface waters (+71 ± 3‰ at the time of sampling; (Ingalls *et al*., 2006)) and freshly produced DOC make it difficult to distinguish between autotrophic inorganic carbon fixation and heterotrophic consumption of modern organic carbon by microorganisms at this depth. A primarily autotrophic microbial community in the surface ocean would have a $\Delta^{14}C$ signature that is identical to surface DIC. Archaeal lipids conform to this expected trend – surface archaeal GDGTs in the 0.2-0.5 μm size class had an average $\Delta^{14}C$ value of +82 ± 12‰ (Ingalls *et al*., 2006). Sterols from autotrophs isolated in the > 0.5 μm fraction at this site had values of +56 ± 9‰ and +69 ± 9‰ (Ingalls *et al*., 2006). Additionally, sugars isolated from the DOC reservoir, a possible substrate for heterotrophic bacteria, had $\Delta^{14}C$ values between 40 and 67‰ at this site in 2001 (Repeta and Aluwihare, 2006). One previous study, Cherrier *et al.* (1999), measured depleted $\Delta^{14}C$ values for bacterial DNA (-34 ± 24‰) isolated from surface waters of the eastern North Pacific Ocean (34°50' N, 123°00' W; Station M) and suggested incorporation of an old DOC component ($\Delta^{14}C$ = -549‰). Our $\Delta^{14}C$ value for DNA is much more enriched and when corrected to +60‰ based on measured C:N ratios it is comparable to potential carbon sources in surface waters.

Utilization of refractory DOC in the mesopelagic zone appears to be negligible, but the relative importance of ambient DIC-fixation varies with depth. The extremely depleted $\Delta^{14}C$-DNA value for the 0.2-0.5 μm size fraction at 670 m suggests that microbes are accessing a carbon reservoir with a $\Delta^{14}C$ value that is

similar to *in situ* DIC (which has a $\Delta^{14}C$ value of -151 ± 3‰; Ingalls *et al*., 2006; Figure 3.1), either directly through autotrophy or through secondary production. The depleted $\Delta^{14}C$-DNA value (Table 3.2) could also result from consumption of a fraction of the ambient DOC pool (Figure 3.2) and the current dataset does not exclude this possibility. However, archaeal lipids isolated from the > 0.2 μm community at 670 m had an average $\Delta^{14}C$ value of -112‰ and showed that archaea derived 83% of their carbon from autotrophy at this depth (Ingalls *et al*., 2006). These latter data, which demonstrate chemoautotrophy at this depth, favor the hypothesis that the depletion of total $\Delta^{14}C$-DNA reflects the importance of DIC as the primary carbon source for microbial production at this site and depth. Surprisingly, $\Delta^{14}C$-DNA from the 670 m 0.2-0.5 μm sample is lower than the same size fraction at 915 m (Figure 3.1). At 915 m, the 0.2-0.5 μm size fraction $\Delta^{14}C$-DNA is greater than $\Delta^{14}C$-DIC (-171 ± 3‰), indicating carbon from both sinking particles and DIC sustain the microbial population. Based on corrected $\Delta^{14}C$ values, 36% of DNA carbon in the 0.2-0.5 μm size fraction at 915 m is derived from ambient DIC fixation as compared to ≥ 95% at 670 m depth.

The > 0.5 μm size fractions at 670 m and 915 m followed a similar trend with depth but had higher $\Delta^{14}C$ values (Figure 3.1) than their 0.2-0.5 μm counterparts. Particles would be isolated into this larger size fraction and the more enriched $\Delta^{14}C$-DNA values are attributable to POC metabolism by particle-attached cells. However, the relatively small size-cutoff of this filter probably led to the isolation of some free-living microorganisms as well. GF/F filters (0.7-μm nominal pore size) have been

shown to collect over 50% of marine bacterioplankton (Lee and Fuhrman, 1987; Hollibaugh *et al*., 1991), and Kirchman *et al*. (2007) observed that archaea are larger than bacteria in some marine environments. Therefore, ^{14}C-DNA values for the > 0.5 μm size fraction likely integrate the ^{14}C signature of DNA from both free-living, mesopelagic microorganisms and those attached to particles. Modern DNA from particle-attached organisms would result in more ^{14}C-replete values than those observed for the 0.2-0.5 μm fraction, but the co-isolation of some free-living organisms would contribute to any observed deviation from the surface Δ^{14}C-DNA value. The similar depth trend, with the more ^{14}C-depleted > 0.5 μm sample at 670 m compared to 915 m, is consistent with the co-isolation of an *in-situ* community that incorporates more DIC at 670 m. Together, these data from the two different size-fractions suggest that free-living microbes isolated by the current method have adapted to utilizing different carbon sources from those attached to particles in their respective environments; however, this distinction is not explicitly reflected in the limited phylogenetic data (Tables 3.3 and 3.4).

The depth-dependent variation in autotrophy implied by the Δ^{14}C-DNA data is consistent with the observed variability in archaeal *amoA* gene copies observed in other marine studies (Wuchter *et al*., 2006; Mincer *et al*., 2007; Beman *et al*., 2008). The latter suggest spatial gradients in the extent of archaeal ammonia oxidation and by extension, autotrophy. In an effort to explore the possible metabolisms that may contribute to the assimilation of carbon measured within the extracted DNA, quantitative PCR (qPCR) was used to determine the number of marine group I

Crenarchaeota 16S rRNA and archaeal *amoA* gene copies present per ng total DNA in the same samples isolated from the mesopelagic zone for radiocarbon analyses. While this is only an indication of community composition and metabolic potential and not gene expression, a correlation has been shown between both marine *Crenarchaeota* 16S rRNA and archaeal *amoA* copy number and ammonia oxidation rates in the Gulf of California (Beman *et al*., 2008). qPCR assays with the 0.2-0.5 µm DNA samples show comparable numbers of bacterial 16S rRNA genes at 670 m and 915 m, but more of both marine group I *Crenarchaeota* 16S rRNA and archaeal *amoA* gene copies per ng total DNA from 670 m than 915 m (Figure 3.3). These trends are consistent with the $\Delta^{14}C$ data and suggest that DIC fixation fuels a greater proportion of microbial community production at 670 m depth. While the focus has been on archaeal autotrophy, carbon fixation by other members of the microbial community cannot be ruled out. For example, so far planktonic archaea appear to be primarily nitrosifyers, able to perform only the first step in the process of nitrification; therefore, the contribution to autotrophy by bacterial nitrifiers must also be considered (Könneke *et al*., 2005; Mincer *et al*., 2007).

Significant differences were not observed for inorganic nitrogen between the two depths as $NO_3^- + NO_2^-$ concentrations were 39.6 µmol kg^{-1} at 670 m and 40.1 µmol kg^{-1} at 915 m. NH_4^+ concentrations were also comparable with values of 0.29 µmol kg^{-1} and 0.33 µmol kg^{-1} at 670 m and 915 m, respectively. There were O_2 variations as the oxygen concentration at 670 m of 60.9 µmol kg^{-1} was depleted relative to the 90.1 µmol kg^{-1} at 915 m (Figure 3.1). Based on these measured *in situ*

geochemical parameters, spatial variations in the strength of the autotrophic contribution are not anticipated. However, our two sampling depths in the mesopelagic zone are separated not only vertically in the water column but also > 1180 m in horizontal space due to the design of the seawater intake pipes down the slope of Hawaii at NELHA (http://www.nelha.org). While delivery of fresh organic material from sinking particles generally decreases rapidly with depth in the mesopelagic ocean (Buesseler *et al*., 2007), spatial heterogeneity in the quantity and quality of the particle flux is not uncommon.

As discussed previously in Ingalls *et al*. (2006), the importance of chemoautotrophy in the deep ocean, if fueled primarily by ammonium oxidation, necessitates a starring role for regenerated, reduced N in the ocean's twilight zone. Therefore, the dominant free-living prokaryotic metabolisms at both depths (autotrophy, if supported primarily through nitrification, at 670 m and heterotrophy of recently produced carbon at 915 m) require fresh organic material delivered presumably from sinking particles. For example, the current study suggests that at least 95% of the microbial carbon accumulating at 670 m could ultimately be derived from chemoautotrophy, and it is not yet known whether enough reduced N is being delivered to the deep sea to sustain the estimated carbon-fixation rate of either the *Crenarchaeota* or other autotrophic organisms. However, there are other factors such as *in situ* growth rates and carbon fixation rates of microorganisms in the deep ocean, that also determine the magnitude of the reduced N requirement, and these parameters remain poorly constrained (Herndl *et al*., 2005). Particle flux to the deep ocean is also

unsatisfactorily defined (Buesseler *et al*., 2007) and believed to be inadequate to support carbon respiration rates in the mesopelagic (e.g., Arístegui *et al*., 2003). In fact, at nearby Station ALOHA bacterial carbon demand has been shown to outweigh POC flux by 3- to 4-fold (Steinberg *et al*., 2008), and investigators have invoked vertically migrating zooplankton as one additional mechanism of carbon transport to the mesopelagic at this site (Al-Mutairi and Landry, 2001; Steinberg *et al*., 2008). Dissolved nitrogen is also a major waste product of vertically migrating zooplankton (e.g., Longhurst *et al*., 1989). Therefore, these organisms may have a major impact on the nitrogen cycle at depth (Steinberg *et al*., 2002) and could mediate the decoupling of reduced N and fresh carbon delivery to various depths in the mesopelagic that is needed to explain the observed difference between 670 m and 915 m at this site.

Neutral sugars isolated from high-molecular-weight DOC at this site were found to have an average $\Delta^{14}C$ value of -123 ± 10‰ at 670 m (Repeta and Aluwihare, 2006) and believed to be a combination of newly synthesized sugars released from sinking particles and older material introduced by advection. However, given the $\Delta^{14}C$-DNA of the free-living microbial community at this depth, these sugars may have been freshly produced by *in situ* autotrophic organisms utilizing DIC. These sugars could additionally provide a ^{14}C-depleted carbon source for heterotrophs that would minimize the amount of chemosynthesis required to balance the sample $\Delta^{14}C$-DNA value at 670 m.

Our approach provides a true *in situ* measurement with no tracer amendments or environmental manipulations and integrates production by the total microbial

community in a significant volume of seawater (~50,000 L) over approximately one week of sampling. This approach differs significantly from the snapshot view captured in other studies via discrete water column sampling, such as those that assess RNA gene expression. The data show that source(s) of carbon utilized by the microbial community in the sub-surface ocean can vary with depth (670 m vs. 915 m) and between free-living and particle-attached organisms (0.2-0.5 μm fraction vs. > 0.5 μm fraction). It has been previously hypothesized that the microbial community at depth is supported by the flux of fresh DOC released from surface-derived sinking particles, and the current results confirm that free-living, heterotrophic microorganisms do benefit from this carbon source, particularly at 915 m. Modern carbon is also being incorporated into the DNA of organisms that are probably attached to particles isolated in the > 0.5 μm size fraction at both 670 m and 915 m. And finally, ^{14}C-depleted carbon, whether from *in situ* DIC or aged DOC, makes up a significant fraction of the DNA carbon in both 0.2-0.5 μm samples (Figure 3.2). The observed spatial variation in *Crenarchaeota* 16S and *amoA* DNA gene sequences together with previous work on archaeal lipids isolated from the same site (Ingalls *et al*., 2006) bolsters the interpretation that ^{14}C-depleted DNA results from chemoautotrophic DIC assimilation. However, the results cannot, at present, distinguish whether the majority of free-living microorganisms, both bacteria and archaea, are autotrophic at 670 m or a tight coupling between chemoautotrophs and heterotrophs exist in the mesopelagic zone. The direct demonstration of the importance of both rapid DOC delivery to the mesopelagic ocean and

chemoautotrophy in maintaining total microbial production was only possible through the Δ^{14}C-DNA method developed here.

The results presented here argue for a concerted focus on delineating both the *in situ* microbial physiology of the deep ocean and constraining carbon and nitrogen fluxes into this environment. Finally, the study developed here may prove useful for examining spatial variations in the flux of particles to the deep-ocean. A relatively high-resolution depth profile of the Δ^{14}C signature of microbial DNA would demonstrate the importance of surface-derived carbon to microbes living in the mesopelagic zone and identify depths where significant respiration of vertical carbon flux occurs, thereby providing a microbial perspective on the shape of the Martin Curve (Martin *et al*., 1987).

Methods

Sampling and DNA Extraction

0.5-µm cellulose ester filters (Millipore Opticap) plumbed inline with 0.2-µm polyethersulfone filters (Pall Supor) were used to collect particulate organic matter from approximately 50,000 L of surface (21 m), 670 m, and 915 m seawater using the large-volume pumping capabilities at the Natural Energy Laboratory in Kona, HI (19.74ºN, 156.05ºW). Filters were stored at -80˚C until DNA was extracted. To isolate the nucleic acids, a method modified from Blair *et al.* (1985) was used to minimize organic carbon contamination for ^{14}C analysis. Filters were cut open using a combusted hacksaw, unfolded, and extracted in 1.5 M $NaClO_4$ at 4˚C for 48 h with

shaking to lyse collected cells. The resulting ~2 L solution was ultrafiltered (Pellicon MWCO 5,000Da; Millipore) down to 20 mls and an organic extraction was performed with an equal volume of chloroform and isoamyl alcohol (24:1). Nucleic acids were precipitated from the resulting aqueous fraction with ethanol, pelleted by centrifugation, and re-dissolved in sterile water. All reagents used in the extraction of environmental DNA were the same and used in the same quantities as for the ^{14}C process blanks. Fractions were set aside for elemental and stable isotope analysis (UC-Davis Stable Isotope Facility; SIO Analytical Facility), ^{1}H-NMR spectroscopy, and molecular biology procedures, and the majority of the sample was lyophilized in 9 mm quartz tubes, combusted to CO_2 at 850˚C with the addition of Ag and CuO, quantified, and converted to graphite according to the methods of Vogel *et al.* (1987) for AMS analysis at the UCI Keck Carbon Cycle AMS facility. $\Delta^{14}C$ values are reported as per geochemical samples according to Stuiver and Polach (1977).

PCR, Cloning, and Sequencing

Portions of the 16S rRNA gene were amplified from environmental DNA samples using Takara ExTaq Polymerase under the conditions described by the manufacturer's protocol. Universal bacterial (27F, 1492R) and archaeal (21F, 958R) primers were used at a final concentration of 0.5 µM. Products from 6 reactions were pooled, cleaned using the Qiagen PCR Purification kit, and eluted in 30 µl water. 4 µl of this cleaned product was used in a TOPO cloning reaction with vector pCR2.1 as described by the manufacturer (Invitrogen). Plasmid DNA was extracted from white

colonies using a Qiagen Miniprep kit and was submitted to SeqXcel (San Diego, CA) for sequencing. The 5' end of both strands of the bacterial 16S rRNA gene was sequenced with primer 536r and M13f/r, while M13f&r were used to sequence entire archaeal inserts. DNA sequences were assembled and edited using VectorNTI (Invitrogen), checked for chimeras using the Bellerophon server (Huber *et al*., 2004), aligned to the Silva database (Pruesse *et al*., 2007), grouped into OTUs using DOTUR (Schloss and Handelsman, 2005), and inserted into ARB for phylogenetic analysis (Ludwig *et al*., 2004). Sequences were deposited into GenBank under accession numbers EU817582-EU817654.

Quantitative PCR

For the qPCR assays, each reaction consisted of 12.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.4 µM each primer, 2 ng BSA, and 1-8 ng 670 m and 915 m 0.2-0.5 µm size fraction DNA as determined by PicoGreen (Molecular Probes) in a final volume of 25 µl. Archaeal *amoA* assays were run in triplicate using primers and cycling parameters from Beman *et al*. (2008) though run for 40 cycles, with the marine group I *Crenarchaeota* assays similarly adapted from Mincer *et al*. (2007. Bacterial 16S rRNA genes were quantified using primers 932F and 1062R (Allen *et al*., 2005). PCR products generated using the qPCR primers were used as standards in tenfold dilution series from 10^7 to 10^2 copies based on PicoGreen quantification. PCR efficiencies and correlation coefficients for standard curves were as follows: 96% and $r^2 = 0.997$ for archaeal *amoA*; 82% and $r^2 = 0.994$ for

GI *Crenarchaeota* 16S rRNA; and 94% and $r^2 = 0.999$ for bacterial 16S rRNA. Results are presented as the relative abundance of gene copies per ng total DNA normalized to the 670 m 0.2-0.5 μm DNA sample.

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Supporting Information

Process Blanks

A mass balance calculation using the two process blanks (experiments conducted in 2007; Table 3.1) and their source carbon can be solved by a 24 μgC contaminant with a $\Delta^{14}C$ value of -235‰. An absolute blank, rather than a fraction of the DNA yield, is favored for the 2007 experiments as seawater volumes used for the incubation, volumes of culture sample filtered, and process chemicals used were all identical for each experiment. This "contaminant" $\Delta^{14}C$ value is moderately enriched in ^{14}C relative to that of bulk DOC in surface waters of the eastern North Pacific Ocean, -302‰ in 2004 at an open ocean site (34˚50'N, 123˚00'W) (Beaupré *et al*., 2007), but it is still possible that adsorption from this pool onto our filters and co-precipitation with DNA during our extraction procedure leads to the observed deviation in sample $\Delta^{14}C$ from the source. However, source carbon and bacterial DNA from these process blanks had very similar $\delta^{13}C$ values, which can constrain adsorption of bulk DOC (with a $\delta^{13}C$ value of -22.5 in surface waters of the eastern North Pacific; Beaupré *et al*., 2007) to 6-8%. Coffin *et al.* (1990) showed that the $\delta^{13}C$ signature of bacterial DNA reflected the isotopic composition of source carbon to within 2.4‰.

Assessing Contamination from DOC Co-Isolation

We cannot use process blank results to constrain the contamination introduced from ambient DOC adsorption during sample isolation (if this is indeed an important

source of contamination) because seawater volumes filtered during sample isolation were much larger than those used for lab cultures and DOC concentrations were much lower for mesopelagic samples (DOC composition is also different at depth; Williams 1986). Instead here we use the C:N of our samples to constrain the potential importance of DOC adsorption. This approach has been used before for riverine systems (McCallister *et al*., 2004). Data shown in Table 3.2 were used for the calculation, and in the case of the 0.2-0.5 μm sample from 670 m where C:N was not measured, the C:N for the same size fraction isolated from 915 m (5.06) was used. In this section we assume that the C:N of pure DNA is 3 (based on the measured elemental composition of *E. coli* DNA (Sigma, cat. # D2001)), and that any deviation from this ideal value results from the adsorption and subsequent precipitation of ambient DOC with our DNA sample. We note that this is the extreme case because the deviation may also be caused by co-precipitation of other intracellular biochemicals, which does not represent contamination. In addition, we use relatively low C:N values for deep DOC (16; surface values are assumed to be 14; Bronk, 2002) and so the calculated contribution from DOC contamination is likely an overestimate. We further assume that the absorbed and co-precipitated DOC has C:N and $\Delta^{14}C$ signatures identical to the bulk DOC pool (we note here that our ^{1}H-NMR spectra are not consistent with a large contamination by bulk DOC, but we cannot rule out contamination by a small amount of aliphatic DOC - likely with C:N values greater than those used in the calculation). The potential fractional contribution from DOC adsorption is calculated first using measured (samples) and assumed (ambient, bulk

DOC) C:N values, and then potential shifts in our experimental Δ^{14}C-DNA values are calculated based on a two endmember mass balance using Δ^{14}C values for bulk DOC in the mesopelagic North Central Pacific (Druffel *et al*., 1992; Table 3.5).

Table 3.1. Isotopic composition and sample sizes of carbon sources and microbial DNA from ^{14}C process blanks.

Sample	Date	Carbon Source	Culture Volume (L)	$\Delta^{14}C$ (‰)	Size (µg C)	UCIG#	$\delta^{13}C$ (‰)
glucose				+87 ± 2	819	3411	-10.7
microbial DNA	10/03	glucose	20	+63 ± 5	1015	1565	-11.7
microbial DNA	6/07	glucose	8	+64 ± 4, +62 ± 4	120, 200	38655, 38656	-11.7
acetate				-988 ± 4, -986 ± 4	800, 250	38653, 38654	-35.3
microbial DNA	6/07	acetate	8	-821 ± 4	110	39810	-34.5

Table 3.2. Collection dates, isotopic composition, size of ^{14}C samples, and C:N of DNA samples. All samples were collected at the Natural Energy Lab in Kona, HI located at 19.74ºN, 156.05ºW. $\Delta^{14}C$ errors are propagated AMS errors and blank corrections. *average surface Pacific 0.2-0.5 µm DNA C:N (n = 3). †n.a., not available (amount of N in submitted sample too small for accurate measurement); $\Delta^{14}C$-DNA corrections for this sample were calculated using the highest C:N ratio (5.32 for 915 m > 0.5 µm size fraction) as an upper bound.

Sample	Collection date	$\delta^{13}C$ (‰)	Measured $\Delta^{14}C$ (‰)	Size (µgC)	UCIG#	C:N	Corrected $\Delta^{14}C$ (‰)
21 m 0.2-0.5 µm	12/03 & 5/04	-19.0	+36 ± 2	450	5397	4.05*	+60
670 m 0.2-0.5 µm	2/06	-20.1	-187 ± 14	22, 24	11272, 10539	n.a.†	-140†
670 m > 0.5 µm		-20.0	-106 ± 4	150	38657	4.31	-73
915 m 0.2-0.5 µm	2/06	-19.7	-87 ± 10	28	10540	5.06	-15
915 m > 0.5µm		-19.4	-27 ± 4	80, 110	38658, 38659	5.32	+69

Table 3.3. General phylogenetic classification and accession numbers of nearest BLAST hits of bacterial 16S rRNA clone libraries from extracted DNA samples.

Sample	Phylogenetic classification	No. clones	Accession no. of nearest BLAST hits	% identity
21 m 0.2-0.5 µm	alpha proteobacteria (41/49)	20	EU805169	99
		8	EU8054456	100
		3	EU805169	100
		2	EF573034	99
		1	EU861201	99
		1	EU805167	99
		1	EU346850	95
		1	EU804260	99
		1	EF572240	99
		1	EF572229	99
	delta proteobacteria (3/49)	1	DQ070808	97
		1	DQ395309	98
		1	EU249716	93
	cyanobacteria (3/49)	3	EU804473	99
	chloroflexi (1/49)	1	AB295005	99
	bacteriodetes (1/49)	1	EU010168	98
670 m 0.2-0.5 µm	delta proteobacteria (2/8)	2	DQ396254	97
	SAR406 (2/8)	1	AB193918	98
		1	DQ300753	99
	alpha proteobacteria (1/8)	1	AB193895	98
	gamma proteobacteria (1/8)	1	AF469226	99
	planctomycetes (1/8)	1	AY381291	87
	actinobacteria (1/8)	1	EU361010	99

Table 3.3, continued. General phylogenetic classification and accession numbers of nearest BLAST hits of bacterial 16S rRNA clone libraries from extracted DNA samples.

Sample	Phylogenetic classification	No. clones	Accession no. of nearest BLAST hits	% identity
915 m 0.2-0.5 µm	gamma proteobacteria (2/7)	1	DQ513059	99
		1	DQ906763	91
	alpha proteobacteria (1/7)	1	U75258	97
	delta proteobacteria (1/7)	1	DQ396048	99
	planctomycetes (1/7)	1	AY381291	87
	SAR406 (1/7)	1	EU092071	95
915 m > 0.5 µm	gamma proteobacteria (5/14)	1	EU361544	99
		1	AF469226	99
		1	DQ396109	96
		1	AY907800	99
		1	AF434117	98
	alpha proteobacteria (2/14)	1	AF469348	99
		1	EF574992	87
	verrucomicrobia (2/14)	2	EU686604	97
	bacteriodetes (1/14)	1	AY279054	99
	actinobacteria (1/14)	1	DQ396300	99
	acidobacteria (1/14)	1	EU491382	95
	delta proteobacteria (1/14)	1	EF646130	99
	cyanobacteria (1/14)	1	EF574918	99

Table 3.4. General phylogenetic classification and accession numbers of nearest BLAST hits of archaeal 16S rRNA clone libraries from extracted DNA samples.

Sample	Phylogenetic classification	No. clones	Accession no. of nearest BLAST hits	% identity
21 m 0.2-0.5 µm	group I crenarchaeota (27/36)	25	EU283425	99
		1	AY627460	98
		1	DQ300510	100
	group II euryarchaeota (9/36)	7	DQ299286	99
		1	DQ156396	100
		1	EF106797	100
670 m 0.2-0.5 µm	group I crenarchaeota (20/20)	13	EU486950	99
		1	EU686615	99
		2	U46680	99
		1	EF414502	99
		2	EF645850	99
		1	AB193963	96
670 m > 0.5 µm	group II euryarchaeota (3/15)	3	AB193995	99
	group I crenarchaeota (12/15)	4	EF645850	99
		2	EU791558	99
		1	AF121995	99
		3	EU686615	99
		1	EU696620	88
		1	EU686642	99

Table 3.4, continued. General phylogenetic classification and accession numbers of nearest BLAST hits of archaeal 16S rRNA clone libraries from extracted DNA samples.

Sample	Phylogenetic classification	No. clones	Accession no. of nearest BLAST hits	% identity
915 m 0.2-0.5 µm	group I crenarchaeota (16/18)	1	DQ641746	99
		1	AB193977	99
		14	EU791558	99
	group II euryarchaeota	1	DQ270603	98
		1	AB193995	99
915 m > 0.5 µm	group I crenarchaeota (5/15)	2	EF645850	99
		3	EU791558	99
	group II euryarchaeota (9/15)	9	AB193995	100
	group III euryarchaeota (1/15)	1	AB177280	98

Table 3.5. Salinity and temperature data for sample collection depths, provided by NELHA (http://www.nelha.org; War, 2006), and inputs for Δ^{14}C-DNA corrections based on bulk DOC adsorption (C:N from Bronk, 2002; Δ^{14}C from Druffel *et al*., 1992). *n.a., not available (amount of N in sample was too low for accurate measurement); Δ^{14}C-DNA corrections for this sample were calculated using the highest C:N ratio (5.32 for 915 m > 0.5 µm size fraction) as an upper bound.

Sample	Salinity	Temp. (˚C)	Sample C:N	Bulk DOC C:N, Δ^{14}C	Measured Δ^{14}C (‰)	% DOC adsorption	Corrected Δ^{14}C (‰)
21 m 0.2-0.5 µm	34.7	24.5-27.5	4.05	14, -191‰	+36	10%	+60
670 m 0.2-0.5 µm	34.4	6.5	n.a.*	16, -405‰	-187	--	-140*
670 m > 0.5 µm			4.31	16, -405‰	-106	10%	-73
915 m 0.2-0.5 µm	34.5	5.8	5.06	16, -470‰	-87	16%	-15
915 m > 0.5 µm			5.32	16, -470‰	-27	18%	+69

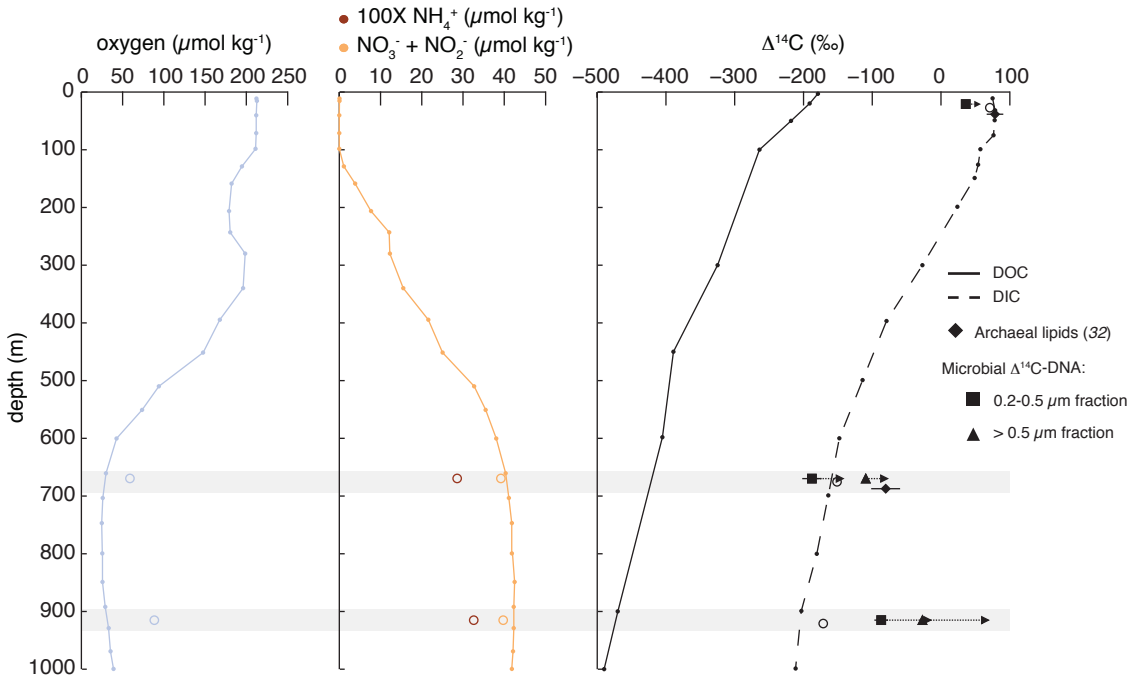

Figure 3.1. $\Delta^{14}C$ values for 0.2-0.5 µm (squares) and > 0.5 µm (triangles) size fractions of DNA extracted from 21 m, 670 m, and 915 m, along with vertical profiles of $\Delta^{14}C$ of DOC (at 31˚N, 159˚W; Druffel *et al*., 1992) and DIC (at 35˚N, 155˚E; Kumamoto *et al*., 2002). $\Delta^{14}C$-DNA error bars are propagated AMS errors and blank corrections, and if not visible are less than the width of the marker. The shift in $\Delta^{14}C$ values of DNA once corrected for possible contamination from DOC adsorption is shown by arrows originating at each measured point and traveling in the direction of more modern $\Delta^{14}C$ values. These latter arrows represent the minimum contribution from autotrophy to each depth (see text and Supporting Information for calculations). Also included are abundance-weighted averages and error of archaeal lipid $\Delta^{14}C$ from 0.2-0.5 µm size fraction at 21 m and > 0.2 µm size fraction at 670m (diamonds; Ingalls *et al*., 2006). Vertical profiles of oxygen and nitrate + nitrite data are for nearby station ALOHA (http://hahana.soest.hawaii.edu). Open circles represent data specific to the NELHA sampling site (at 19.74˚N, 156.05˚W; Ingalls *et al*., 2006; War, 2006).

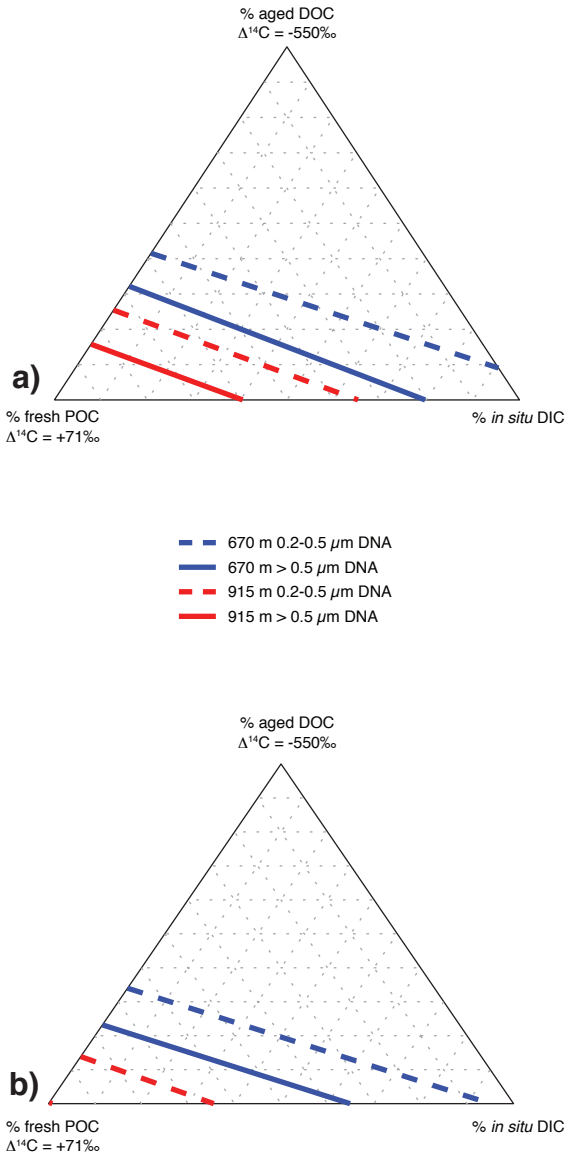

Figure 3.2. Ternary plot of possible % contributions of fresh POC ($\Delta^{14}C$ = +71‰), aged DOC ($\Delta^{14}C$ = -550‰), and in situ DIC ($\Delta^{14}C$ = -151‰ at 670 m and -171‰ at 915 m) to microbial $\Delta^{14}C$-DNA (a). $\Delta^{14}C$-DNA values corrected for possible DOC contamination (see Supporting Information) are plotted in (b). Gridlines indicate 10% increments.

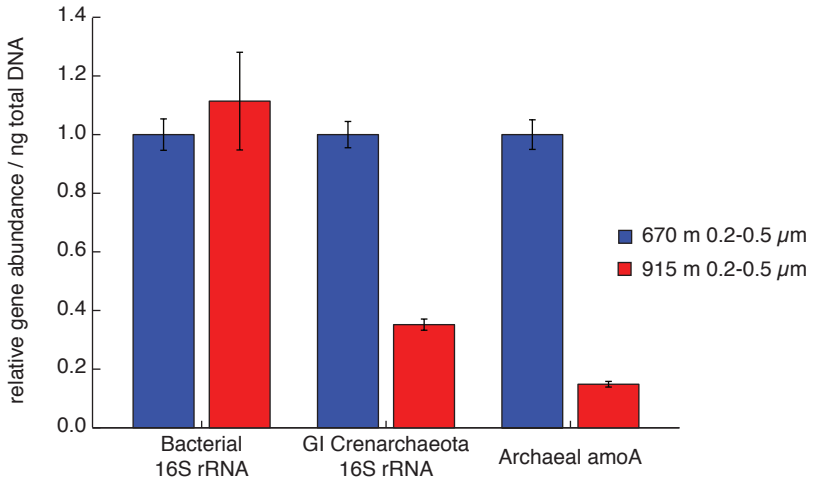

Figure 3.3. qPCR data for 0.2-0.5 µm size fraction DNA samples showing average (n = 3) relative abundances of bacterial 16S rRNA, group I Crenarchaeota 16S rRNA, and archaeal amoA gene copies per ng total DNA as quantified by PicoGreen and normalized to the 670 m 0.2-0.5 µm sample. Error bars indicate standard deviations.

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IV. Examining the importance of methane-derived carbon in supporting planktonic microbial communities above cold methane seeps

Summary

Sequences coding for a subunit of the particulate methane monooxygenase gene (*pmoA*) and 16S rRNA genes specific to type I methanotrophs were amplified from microbial DNA isolated from the water column above the methane seeps. However, δ^{13}C-DNA values indicate methane-derived carbon does not contribute significantly to the total microbial community carbon at the sites studied, consistent with qPCR data suggesting only a small percentage of the bacterial community in these environments possess *pmoA*.

Introduction

Planktonic marine microbes regulate many of the processes involved in the biogeochemical cycling of compounds throughout the water column, including mediating the release of methane from the ocean to the atmosphere. Methane is a significant greenhouse gas, and its cycling is an important component of the global carbon budget. Therefore, quantifying the amount of CH_4 that actually reaches the atmosphere remains a priority (Valentine *et al*., 2001). Microorganisms that oxidize methane have been identified as methanotrophs, capable of utilizing methane as both a carbon and energy source (Hanson and Hanson, 1996), and have been studied in a wide variety of terrestrial and marine environments.

Methane dominates the fluids of cold seeps, and both anaerobic and aerobic methane oxidation in marine sediments have been identified as important processes in removing methane in and around active seeps; however, in some areas methane vents

into the water column where it is available for oxidation by the planktonic microbial community under aerobic conditions (Reeburgh, 2007). Evidence for methane oxidation in the water column near active methane vents in the Eel River basin was demonstrated by Valentine *et al*. (2001). $\delta^{13}C\text{-}CH_4$ measurements revealed an observed value of -40‰, isotopically heavier than CH_4 emitted from the sediments (-50 to -60‰). These changes in $\delta^{13}C\text{-}CH_4$, in combination with CH_4 concentration and CH_4 oxidation rate data collected at the site, indicated CH_4 oxidation was occurring in the water column in the area around the methane seeps. These authors estimated that $\sim 2 \times 10^6$ g CH_4 yr^{-1} was being oxidized in the water column above the venting field. Similar techniques combined with fluorescent *in situ* hybridization identified bacterial methanotrophy in the Black Sea water column (Durisch-Kaiser *et al*., 2005).

Methanotrophs use methane monooxygenase enzymes to catalyze the oxidation of methane to methanol, and the presence of the gene coding for a subunit of the particulate form of this type of enzyme (*pmoA*) has been commonly used to determine the genetic potential for methane oxidation in environmental samples as well as to survey general methanotroph phylogeny. Tavormina *et al*. (2008) determined that *pmoA* sequences recovered from the water column were distinct from those found in the sediment community, implying that a separate community of planktonic methane oxidizers exist and are not merely resuspended organisms from the sediments.

Although it has been demonstrated that no significant amounts of vent-released methane escape from the ocean into the atmosphere (Heeschen *et al*., 2005) due to

oxidation in the water column (Grant and Whiticar, 2002), the extent to which this oxidized methane supports the total microbial community above these seeps has not been determined. This study aimed to quantify the importance of methane-derived carbon in supporting the total microbial community present above actively venting methane seeps, compared to that of surface-derived photosynthetically-fixed carbon. By capitalizing on the $\delta^{13}C$-depleted signature of methane released from these seeps, we hoped to trace its incorporation into microbial biomass through natural abundance $\delta^{13}C$ measurements of extracted DNA. Blair *et al*. (1985) showed that stable carbon isotopic fractionation during nucleic acid biosynthesis by heterotrophic bacteria was negligible from the $\delta^{13}C$ of substrate carbon, and the utility for using $\delta^{13}C$-DNA to trace carbon assimilation in microbes was demonstrated in estuarine environments by Coffin *et al*. (1990). Additionally, the genetic potential for methane oxidation and the presence of methanotrophs were probed through *pmoA* and 16S rRNA molecular assays.

Results and discussion

Sample sites and ancillary data

Samples were collected from depths approximately 50 m above the sea floor from cold methane seep locations in the Eel River basin, at Hydrate Ridge offshore Oregon, and from two sites along the continental margin off Costa Rica (Mounds 11&12 and Jaco Scarp) (Figure 4.1). Temperature and salinity data for the collection sites are shown in Table 4.1. Average oxygen concentrations ranged from a low of

12.2 μmol kg^{-1} at Hydrate Ridge to 66.6 μmol kg^{-1} at Jaco Scarp (Table 4.1). Active methane venting was identified by visible bubbling and / or the presence of chemosynthetic communities that included filamentous bacterial mats, clam beds, and tubeworms as observed from HOV *Alvin* prior to sampling (L. Levin and K. Brown, personal communication).

While $[CH_4]$ and $\delta^{13}C$-CH_4 values were not obtained during this study, evidence for the presence of methane in the water column and its significantly ^{13}C-depleted signature has been previously documented at these sites (Table 4.2, and references therein). Benthic biomass collected concurrently with our water column sampling from both Hydrate Ridge and Eel River had minimum $\delta^{13}C$ values of -78.7‰ and -65.7‰, respectively (Table 4.3; L. Levin and A. Thurber, personal communication), indicating that methane-derived carbon was incorporated into at least a portion of the macrofauna found in the sediments at these sites during the time of sampling. The importance of chemosynthetically fixed methane-derived carbon in supporting macrobenthic organisms has been previously demonstrated here through light carbon isotopic signatures (Levin and Michener, 2002).

Elemental and stable isotopic analyses

$\delta^{13}C$ values for extracted microbial DNA samples were significantly heavier than the previously reported values for methane (Table 4.4). DNA extracted from the 0.2-1.2 μm size fraction collected in the water column above the Eel River seep area had a $\delta^{13}C$ value of -19.1‰, and the larger > 1.2 μm sample $\delta^{13}C$ value was -19.4‰.

The $\delta^{13}C$ values for the same size fractions collected at Hydrate Ridge were -18.3‰ and -19.5‰, respectively. The integrated surface sample 0.2-1.2 μm size fraction $\delta^{13}C$-DNA value was -19.3‰. Results from samples isolated from the Costa Rica margin were similar: $\delta^{13}C$-DNA values were -18.2‰ and -17.0‰ from the Jaco Scarp 0.2-0.5 μm and > 0.5 μm size fractions, respectively, and -17.3‰ from the 0.2-0.5 μm fraction collected over Mounds 11 and 12. C:N ratios ranged from 4.08 to 7.02 (Table 4.4), elevated from the expected 3 for pure DNA. The increased C:N values may be due to co-isolation of other cellular biochemicals, such as carbohydrates, that are carbon-rich. Co-isolation of carbohydrates will also modify the measured $\delta^{13}C$; for example, Teece and Fogel (2007) observed a 7-10‰ enrichment in the total carbohydrate fraction relative to total cellular carbon in the heterotroph *Shewanella putrefaciens*, and ribose, which should be included in extracted nucleic acids, showed enrichment of 10‰. In addition, carbohydrates from autotrophs (including marine phytoplankton) can be enriched by 2-7‰. However, even with such enrichment in carbohydrate $\delta^{13}C$, the isolated samples do not exhibit the depletion expected from methane incorporation. Another possible explanation for the elevated C:N ratios is the adsorption of dissolved organic matter to the filters that subsequently persists in the sample through the DNA extraction procedure. Marine DOM has an average $\delta^{13}C$ value of about -21‰ (Bauer, 2002), slightly more depleted than our sample DNA values. Mass balance calculations using fairly conservative C:N ratios for marine DOM (16 at depth and 14 in surface waters; Bronk, 2002) to correct for possible DOM adsorption would shift $\delta^{13}C$-DNA values up only marginally +0.3-1.1‰ and thus

would not mask any isotopic signal from methane incorporation.

$\delta^{13}C$ values of suspended particulate organic carbon (POC) isolated from the water column do not indicate the presence of methane-derived carbon (Table 4.3). With average $\delta^{13}C$ values of -18.4‰ and -19.4‰, POC from the Hydrate Ridge water column as well as the off-seep OMZ site, respectively, appears to be of marine origin. While the more ^{13}C-depleted value of -25.9‰ in the Eel River basin could suggest the influence of methane-derived carbon, we believe it to be the result of terrestrial riverine input at this site as the $\delta^{13}C$ of suspended POC in the Eel River is -25.3 ± 0.7‰, driven primarily by the terrestrial vascular plant end-member of -26.5 ± 1.1‰ (Blair *et al*., 2003). DOM fractions isolated from this site had marine $\delta^{13}C$ values between -18‰ to -21.8‰ (de Jesus, 2008) and likely serve as substrates for heterotrophic bacteria in the water column.

Phylogenetic analysis of 16S rRNA and pmoA

Similar to the results of Tavormina *et al*. (2008), recovered sequences using primers specifically targeting type I methanotrophic 16S rRNA genes (Type IF and IR; Chen *et al*., 2007) were primarily non-methanotrophic, and differences among sites were not observed (Table 4.5). Less than a tenth (8.5%) of recovered sequences representing 8 OTUs had 90-93% similarity to known type I methanotrophs (Figure 4.2), but because these sequences are only moderately related it is difficult to classify them accurately as methanotrophic species. The remaining non-methanotroph sequences were dominated by Gammaproteobacteria (80% of all non-methanotrophic

sequences), with nearest NCBI BLAST hits including the genera *Vibrio*, *Psychromonas, Alteromonas*, *Alcanivorax*, and *Moritella*. Other represented classes included members of the *Chloroflexi*, *Verrucomicrobiae*, and *Acidobacteria*. The affiliated genera of recovered non-methanotrophs were similar to those found by Tavormina *et al*. (2008), but this is likely an artifact of the primers used.

Recovered *pmoA* sequences obtained using the water column specific *pmoA* primer set (Tavormina *et al*., 2008) all clustered into two major groups, OPU 1 and OPU 3/4 (Figure 4.3), which contain other *pmoA* sequences previously identified in the water column both above methane seeps (Tavormina *et al*., 2008) and in oxygen minimum zones (Hayashi *et al*., 2007). These results further the conclusions of Tavormina *et al*. (2008) in that the *pmoA* sequences recovered from the water column are distinct from those previously identified in sedimentary environments. This appears to hold true regardless of the sampling location and its characteristics, as similar *pmoA* sequences were isolated from the Jaco Scarp > 0.5 μm size fraction sample, which was collected at a significantly deeper depth than the Eel River and Hydrate Ridge samples. Additionally, RFLP patterns indicated that the same *pmoA* sequences are present in the 0.2-0.5 μm size fractions isolated from both Jaco Scarp and Mounds 11 & 12 (data not shown). *pmoA* sequences initially recovered using primers A189f and mb661r (Costello and Lidstrom, 1999), commonly used to identify *pmoA* in sediment communities, all clustered into the OPU 3/4 group, so use of the water column specific primers designed by Tavormina *et al*. (2008) expanded the recovered diversity slightly. However, this strategy still demonstrated that planktonic

pmoA sequences display limited diversity across a range of sampling locations, and are distinct from those previously found in sediment environments. The DNA extraction protocol we employed used an inorganic lysis treatment of sodium perchlorate, in contrast to the more common chemical and enzymatic methods used by Tavormina *et al*. (2008) that included lysozyme, proteinase K, and SDS exposure, yet still provided similar results for recovered sequences.

Importance of methane-derived carbon

Our δ^{13}C-DNA data indicate that no significant amount of methane-derived carbon is being assimilated in the nucleic acids of the microbial communities above these seeps. The presence of the *pmoA* gene is only an indication of metabolic potential, so it is possible that the gene is not expressed or that the aerobic methanotrophs in this community are not actively oxidizing or incorporating methane. In a study of methane oxidation in water samples taken from the southeastern Bering Sea, Griffiths *et al*. (1982) saw no evidence of incorporated carbon from labeled $^{14}CH_4$ in biomass > 0.45 μm, even though it was determined oxidation was occurring from the generation of labeled $^{14}CO_2$. This suggested that the methane was being used as an energy source though very little was being used in biosynthesis. The likely explanation for our samples is that even if methane oxidation is occurring in the planktonic microbial community above the methane seeps, the incorporation of methane-derived carbon into cellular biomass is very small when compared to the assimilation of photosynthetically-derived carbon, delivered by sinking POC, by the

rest of the microbial community.

Evidence of methanotrophic bacteria in the oxic Black Sea water column has indicated these organisms comprise only 2.5% of total DAPI-stained cells on average, while changes in δ^{13}C-CH_4 and rate measurements with tritiated-CH_4 indicated intense methane oxidation was occurring here (Durisch-Kaiser *et al*., 2005). If a similar proportion of the total microbial community at the sites studies here are methanotrophs, incorporation of ^{13}C-depleted methane would only decrease the δ^{13}C-DNA value from a marine δ^{13}C signature by < 2‰. This depletion, if present in our samples, could be masked by the inclusion of enriched microbial carbohydrates co-extracted with the DNA. Although Valentine *et al*. (2001) estimated 2 x 10^6 g C yr^{-1} was oxidized in the 25-km^2 venting area at Eel River basin, that could only supply enough carbon to satisfy less than 1% of the bacterial carbon demand (BCD) for a similar-sized area (using estimates for BCD from the mesopelagic in the northeastern Pacific at Station ALOHA; Steinberg *et al*., 2008). Our data also demonstrate that any methane-derived carbon assimilated by the presumably small fraction of methanotrophs does not accumulate or persist as substrates for heterotrophs within the microbial community above the seeps.

Quantitative PCR analysis

Quantitative PCR (qPCR) demonstrated that more copies of the *pmoA* gene were present per ng of total DNA in the two samples from Hydrate Ridge (0.2-1.2 μm and > 1.2 μm size fractions) as compared to the samples from the Eel River basin and

the integrated surface sample (Figure 4.4a). When normalized to the number of bacterial 16S rRNA gene copies in each sample, the Hydrate Ridge samples again had higher values (Figure 4.4b). The number of bacterial 16S rRNA gene copies per ng total DNA in the integrated surface sample was nearly 3-fold higher than that of the 0.2-1.2 μm size fractions at both Hydrate Ridge and Eel River. The smaller size fraction (0.2-1.2 μm) seep samples had more than twice as many bacterial 16S rRNA gene copies per ng total DNA than their > 1.2 μm counterparts (Table 4.6).

While significantly more *pmoA* copies are present in the samples from Hydrate Ridge compared to those from Eel River and the surface, no evidence of greater methane oxidation and assimilation is apparent from the δ^{13}C-DNA data. By looking at the ratio of *pmoA* to bacterial 16S rRNA genes, only a maximum 3.6% of bacteria have a copy of *pmoA* in any of the samples collected (assuming 1 copy of *pmoA* per methanotroph, though 2 or more per cell may actually be the case; Stolyar *et al*., 1999; Auman *et al*., 2000), and as mentioned above, such a small percentage of methanotrophic activity is unlikely to be detected in the δ^{13}C-DNA value. Because these assays were conducted on DNA, they only serve as indicators of metabolic potential and not gene expression or enzyme activity, and thus cannot confirm methane oxidation.

Although qPCR detected *pmoA* gene copies in the integrated surface sample, in quantities similar to the samples from Eel River, we were unable to amplify this sample by regular PCR, possibly due to concentration discrepancies or inhibition, and thus did not recover and identify any *pmoA* sequences for phylogenetic analysis.

However, the qPCR data suggests that *pmoA* may be ubiquitous in the water column regardless of methane concentration.

Conclusion

Through stable isotopic analysis of extracted microbial DNA, we determined that methane-derived carbon is not a significant source of carbon supporting the total microbial community present in the water column above methane seeps. Although genes coding for a subunit of particulate methane monooxygenase and 16S rRNA moderately related to known type I methanotrophs were present in the analyzed DNA, it appears these organisms are either not actively oxidizing and incorporating methane, or comprise only a small fraction of the total microbial community, as suggested by qPCR data, and thus contribute an insignificant portion of the carbon sustaining prokaryotic production in these venting seep environments. As previously observed (Tavormina *et al*., 2008), planktonic *pmoA* exhibits low diversity and appears phylogenetically distinct from sediment systems, and these observations hold true across a range of water column environments.

Experimental procedures

Sample collection

Water was collected during cruises on the R/V *Atlantis* using a rosette of 23 10-L Niskin bottles from a depth approximately 50 m above bottom at locations within the Costa Rice Dome system (Mounds 11 &12 and Jaco Scarp; June 2005) and both

the Eel River basin (CA) and Hydrate Ridge (OR) seep systems (July 2006) (Table 4.1; Figure 4.1). Once on board, the water was filtered directly from the Niskin bottles through two large-volume capacity cartridge filters of pore size 1.2 μm (Eel River and Hydrate Ridge) or 0.5 μm (Costa Rica sites) and 0.2 μm plumbed in-line. Approximately 3,100 L were filtered from above Mounds 11 & 12 near Costa Rica, 2,800 L from the Jaco Scarp, 5,600 L from the Eel River basin, and 9,050 L from Hydrate Ridge. Filters were frozen at -80˚C until extraction back in the laboratory. Ancillary data (temperature, salinity, and oxygen concentrations) were measured with a Seabird SBE9+ CTD and SBE43 oxygen sensor.

DNA extraction

Nucleic acids were extracted from the filters using a modified method from Blair *et al.* (1985) to minimize carbon contamination for isotopic analyses (Hansman *et al*., submitted). Filters were cut open using a combusted hacksaw, unfolded, and extracted in ~1.5 L of 1.5 M $NaClO_4$ at 4˚C for 48 h. The resulting solution was concentrated by ultrafiltration (Pellicon, MWCO 5000), and then an organic extraction using chloroform:isoamyl alcohol (24:1) was performed. Nucleic acids were then precipitated and purified with ethanol.

Stable isotope analysis

The majority of isolated DNA (~ 85%) was submitted to the UC-Davis Stable Isotope Facility for elemental and stable isotope analysis. Nucleic acids in solution

were transferred to pre-weighed tin cups, dried to solids at 50˚C, and analyzed for $\delta^{13}C$ and $\delta^{15}N$ using EA-IRMS. Stable isotope values are reported relative to Pee Dee Belemnite (marine carbonate) or atmospheric nitrogen, and elemental data are the molar ratios of carbon to nitrogen (C:N) in each sample.

PCR, cloning, sequencing, and phylogenetic analysis

Genes coding for *pmoA* specific to the water column were amplified using primers wcpmoA189f and wcpmoA661r using the cycling parameters described in Tavormina *et al*. (2008). Primers Type IF and Type IR were used to amplify 16S rRNA genes specific to type I methanotrophs as described in Chen *et al*. (2007). PCR products were cleaned using the Qiagen PCR Purification kit and eluted in 30 µl sterile water. 4 µl of cleaned product was used in a TOPO cloning reaction with vector pCR2.1 as described by the manufacturer (Invitrogen). White colonies were picked and grown statically in 200 µl Luria-Bertani media with kanamycin and 10% glycerol for 12 h at 37˚C in 96-well plates. Resulting glycerol stocks were frozen immediately at -80˚C until single-pass sequencing with primers M13f&r by Agencourt Bioscience (Beverly, MA).

DNA sequences were assembled using VectorNTI software (Invitrogen), checked for chimeras using the Bellerophon server (Huber et al., 2004), and grouped into OTUs using DOTUR (Schloss and Handelsman, 2005). 16S rRNA sequences were aligned to the Silva database (Pruesse *et al*., 2007), while *pmoA* nucleotide sequences were first translated into amino acids using NCBI's open reading frame

finder, and then aligned using MUSCLE (Edgar, 2004). Aligned sequences were analyzed phylogenetically in ARB (Ludwig et al., 2004).

We note as word of caution that many *pmoA* clones contained inserts of the incorrect size (both larger and smaller than the expected size) when checked by colony PCR (e.g., Figure 4.5). While this was most likely due to insufficient cleanup of PCR products prior to cloning, of the genes examined in this thesis, this problem appeared to only occur for this particular gene and across all samples. Selected sequencing of the incorrectly-sized inserts found no significant similarity to GenBank sequences using BLAST.

Quantitative PCR

For the qPCR assays, each 25 µl reaction consisted of 12.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.4 µM each primer, 2 ng BSA, and 1-8 ng DNA as determined by PicoGreen (Molecular Probes). All assays were run in triplicate. Bacterial 16S rRNA genes were quantified as described in Allen *et al*. (2005). *pmoA* assays were run using water-column specific primers wcpmoA189f and wcpmoA661r (Tavormina *et al*., 2008) and the following cycling parameters: 15 min at 95˚C, followed by 40 cycles of 40 s at 95˚C, 30 s at 56˚C, and 40 s at 72˚C, with a 10 s detection step at 78˚C at the end of each cycle. Standard curves were constructed with 10-fold dilutions of PCR products from 10^7 to 10^2 gene copies. PCR efficiencies and correlation coefficients were 88% and $r^2 = 0.998$ for bacterial 16S rRNA, and 85% and $r^2 = 0.998$ for *pmoA*. Quantification was determined as gene copies per ng total

DNA, and relative abundances are normalized to the Hydrate Ridge 0.2-1.2 μm DNA sample.

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Table 4.1. Locations, depth, temperature, salinity, oxygen concentrations, and volumes of collected water column samples. Samples were integrated onto the same filter set at each particular seep site.

Methane Seep System	Location	Depth (m)	Avg. Temp. (˚C)	Avg. Salinity	Avg. [O_2] (µmol kg^{-1})	Volume filtered (L)
Eel River basin, CA	40˚48.7'N, 124˚36.7'W	500	6.3	34.15	38.6	4600
	40˚47.1'N, 124˚35.8'W	518	6.0	34.17	30.8	1000
Hydrate Ridge, OR	44˚34.2'N, 125˚8.8'W	703	4.7	34.28	12.2	5760
	44˚40.0'N, 125˚6.0'W	555	5.3	34.15	25.4	3290
Mounds 11& 12, Costa Rica	8˚55.7'N, 84˚18.8'W	900	5.3	34.58	24.0	3100
Jaco Scarp, Costa Rica	9˚7.1'N, 84˚50.5'W	1650	2.9	34.63	66.6	2800

Table 4.2. Published methane concentration and stable carbon isotope ranges from the water column above various seep systems. n.a., not available. * $\delta^{13}C$ of methane hydrate in Mound 11.

Methane seep site	$[CH_4]$ (nmol l^{-1})	$\delta^{13}C$-CH_4 (‰)	Reference
Eel River	20-300	-50 to -60	Valentine *et al*., 2002
Hydrate Ridge	up to 4400	-63 to -66	Heeschen *et al*., 2005
Mounds 11&12	5.3-107.3	-45.2 to -43.3*	Schmidt *et al*., 2005; Mau *et al*., 2006
Jaco Scarp	68.8-178.5	n.a.	Mau *et al*., 2007

Table 4.3. Average $\delta^{13}C$ values of water column suspended particulate organic carbon (POC) and benthic fauna biomass.

Sample Site	Average $\delta^{13}C$ (‰)	
	POC	**benthic fauna (low)**
Eel River	-25.9	-26.7 (-65.7)
Hydrate Ridge	-18.4	-32.4 (-78.7)
off-seep OMZ	-19.4	-19.0 (-33.2)

Table 4.4. Stable isotopic measurements and C:N of extracted microbial DNA samples. n.d., not determined (amount of N in samples too small for accurate measurement)

DNA Sample	$\delta^{13}C$ (‰)	C:N
Eel River 0.2-1.2 µm	-19.1	5.40
Eel River > 1.2 µm	-19.4	5.59
Hydrate Ridge 0.2-1.2 µm	-18.3	4.08
Hydrate Ridge > 1.2 µm	-19.5	7.02
integrated surface 0.2-1.2 µm	-19.3	5.19
Jaco Scarp 0.2-0.5 µm	-18.2	n.d.
Jaco Scarp > 0.5 µm	-17.0	n.d.
Mounds 11&12 0.2-0.5 µm	-17.3	n.d.

Table 4.5. Summary of phylogenetic classes of nearest NCBI BLAST hits of non-methanotrophic bacterial 16S rRNA sequences recovered with Type IF and Type IR primers (Chen *et al*., 2007) and distribution amongst various sample sites and size fractions. Values indicate fraction of total non-methanotroph clones for each sample. 8.5 percent of total recovered sequences had ≥ 90% sequence similarity to known type I methanotrophs (Figure 4.2) and are not included in these data.

Class of nearest NCBI BLAST hits	surface	Eel River		Hydrate Ridge		Jaco Scarp		Mounds 11/12
	0.2-1.2 µm	0.2-1.2 µm	> 1.2 µm	0.2-1.2 µm	> 1.2 µm	0.2-0.5 µm	> 0.5 µm	0.2-0.5 µm
Gammaproteobacteria	27/30	25/29	20/23	18/22	17/22	13/22	20/23	14/22
Deltaproteobacteria	--	1/29	2/23	--	5/22	--	--	2/22
Chloroflexi	--	1/29	--	4/22	--	8/22	2/23	6/22
Acidobacteria	--	1/29	1/23	--	--	1/22	--	--
Verrucomicrobiae	--	1/29	--	--	--	--	1/23	--
unclassified	3/30	--	--	--	--	--	--	--

Table 4.6. Quantitative PCR data for *pmoA* and bacterial 16S rRNA genes in extracted DNA samples from Eel River and Hydrate Ridge above-seep sites and surface waters.

	gene copies / ng DNA			relative abundance / ng DNA	
Sample	***pmoA***	**bacterial 16S rRNA**	***pmoA* / bacterial 16S rRNA**	***pmoA***	**bacterial 16S rRNA**
Eel River 0.2-1.2 µm	1785	542349	0.0033	0.175	0.912
Eel River > 1.2 µm	296	226076	0.0013	0.029	0.38
Hydrate Ridge 0.2-1.2 µm	10178	594941	0.0171	1.000	1.000
Hydrate Ridge > 1.2 µm	10115	280768	0.0360	0.994	0.472
integrated surface 0.2-1.2 µm	1011	1615825	0.0006	0.099	2.716

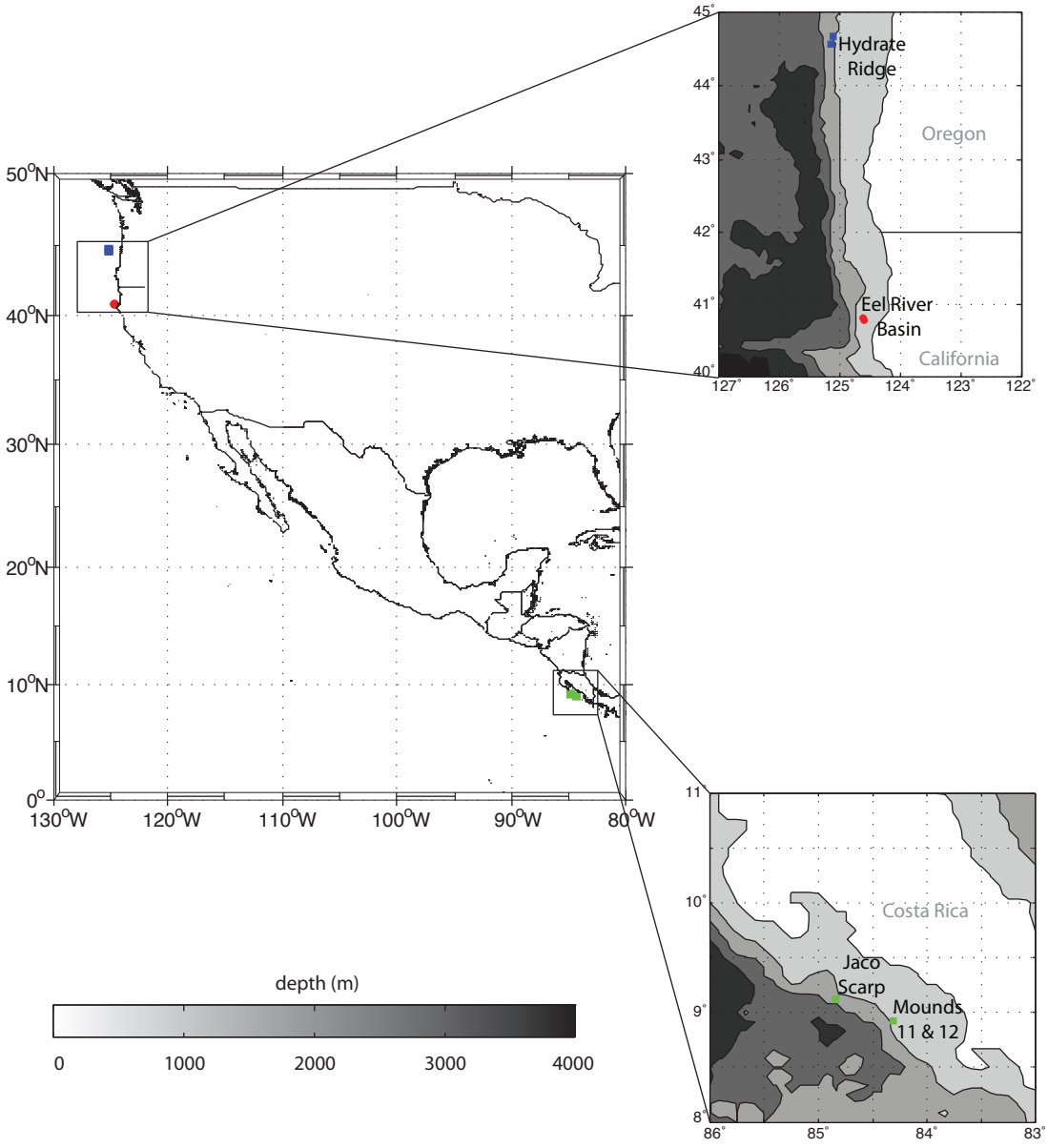

Figure 4.1. Map of sampling locations.

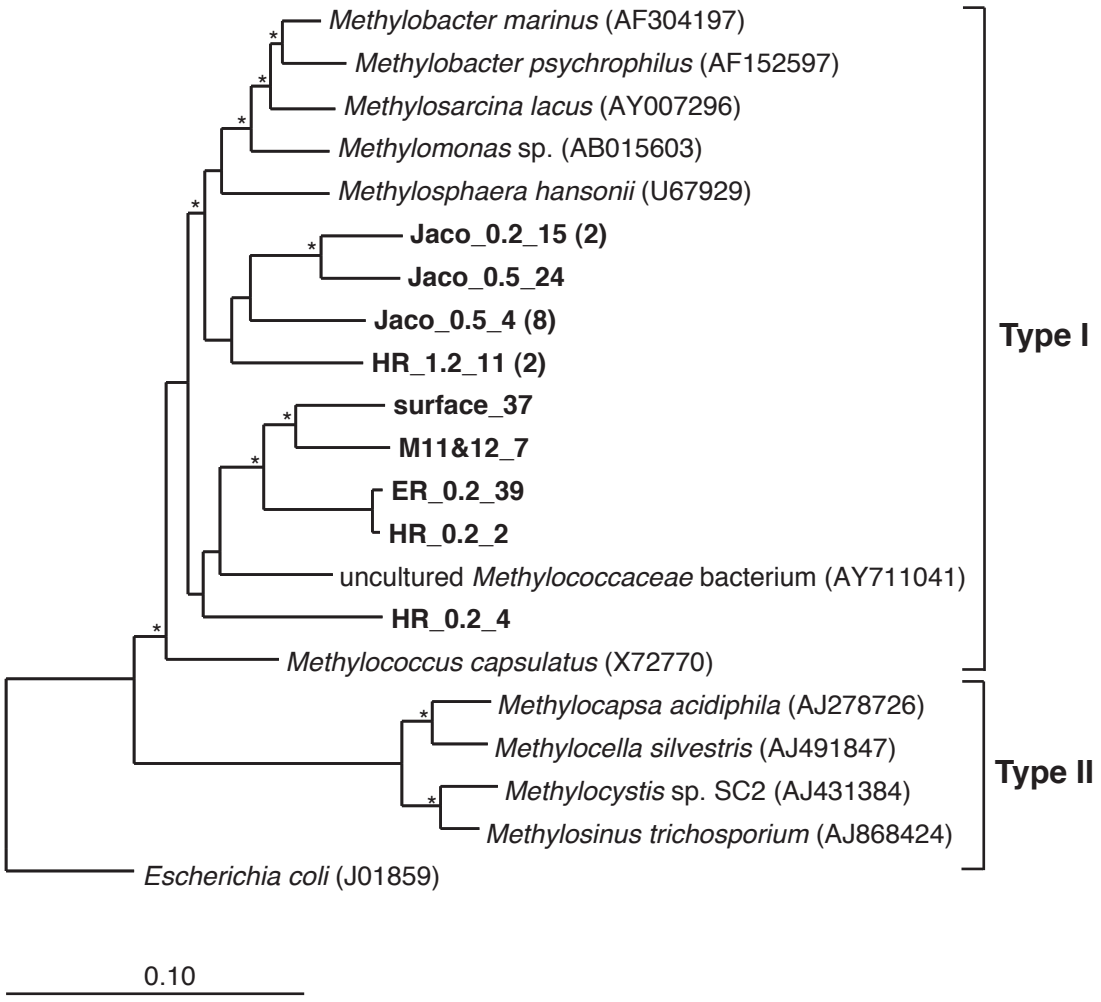

Figure 4.2. Phylogenetic relationships of 16S rRNA gene sequences recovered from water column samples using primers specific for type I methanotrophs (Chen et al., 2007). Only sequences with ≥ 90% similarity to known methanotrophs are included. Parenthetical numbers next to bolded clones indicate quantity of 97% similar OTUs from that particular sample, asterisks denote > 50% bootstrap values, and the scale bar represents 0.1 substitution per site.

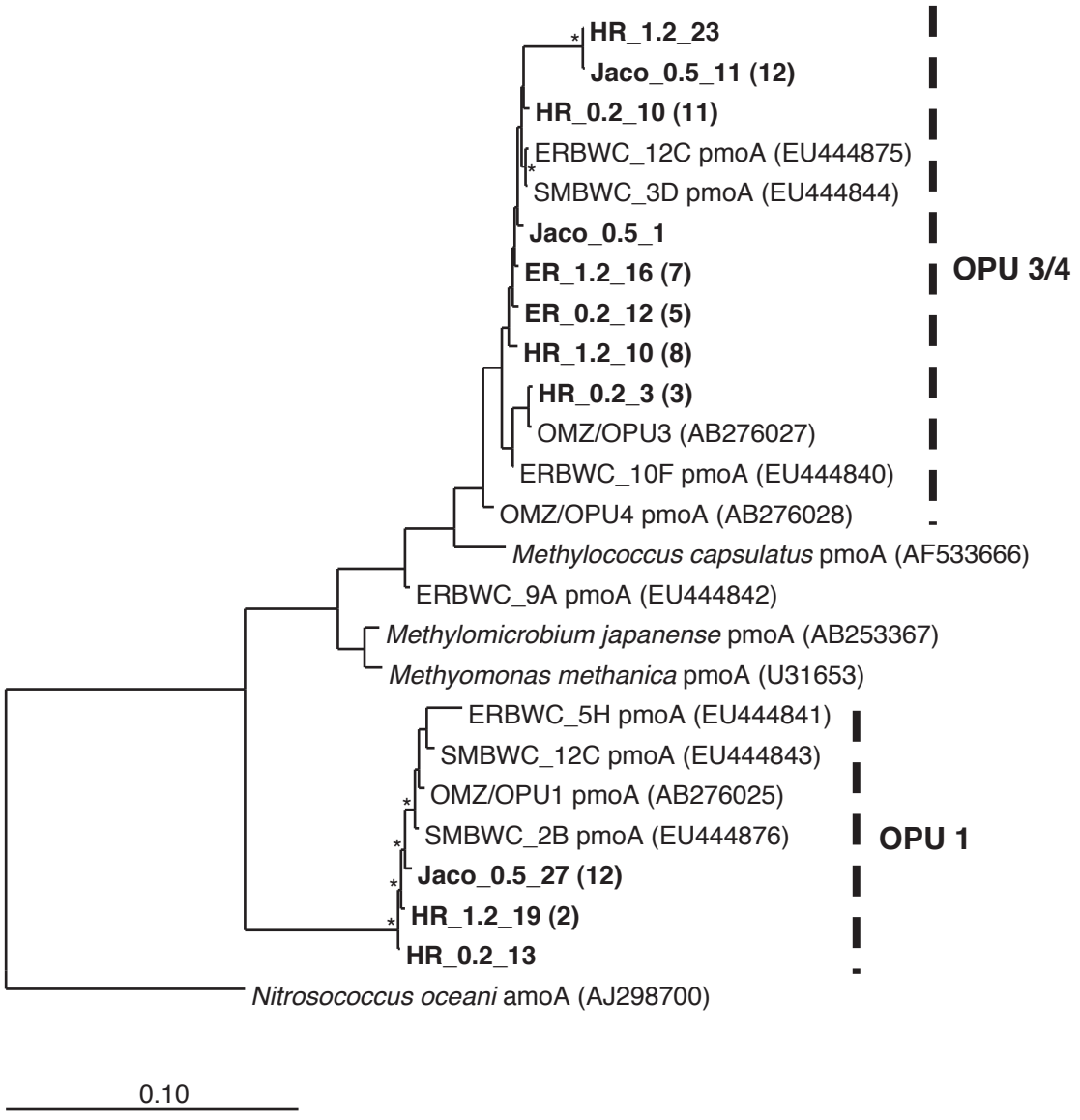

Figure 4.3. Phylogenetic tree based on the amino acid sequences of the α-subunit of particulate methane monooxygenase as inferred from pmoA gene sequences recovered from the water column above cold methane seeps. Asterisks mark bootstrap values > 50%, and the scale bar represents 10% sequence divergence. Number of 97% identical sequences from each sample are indicated in parentheses next to bolded clones.

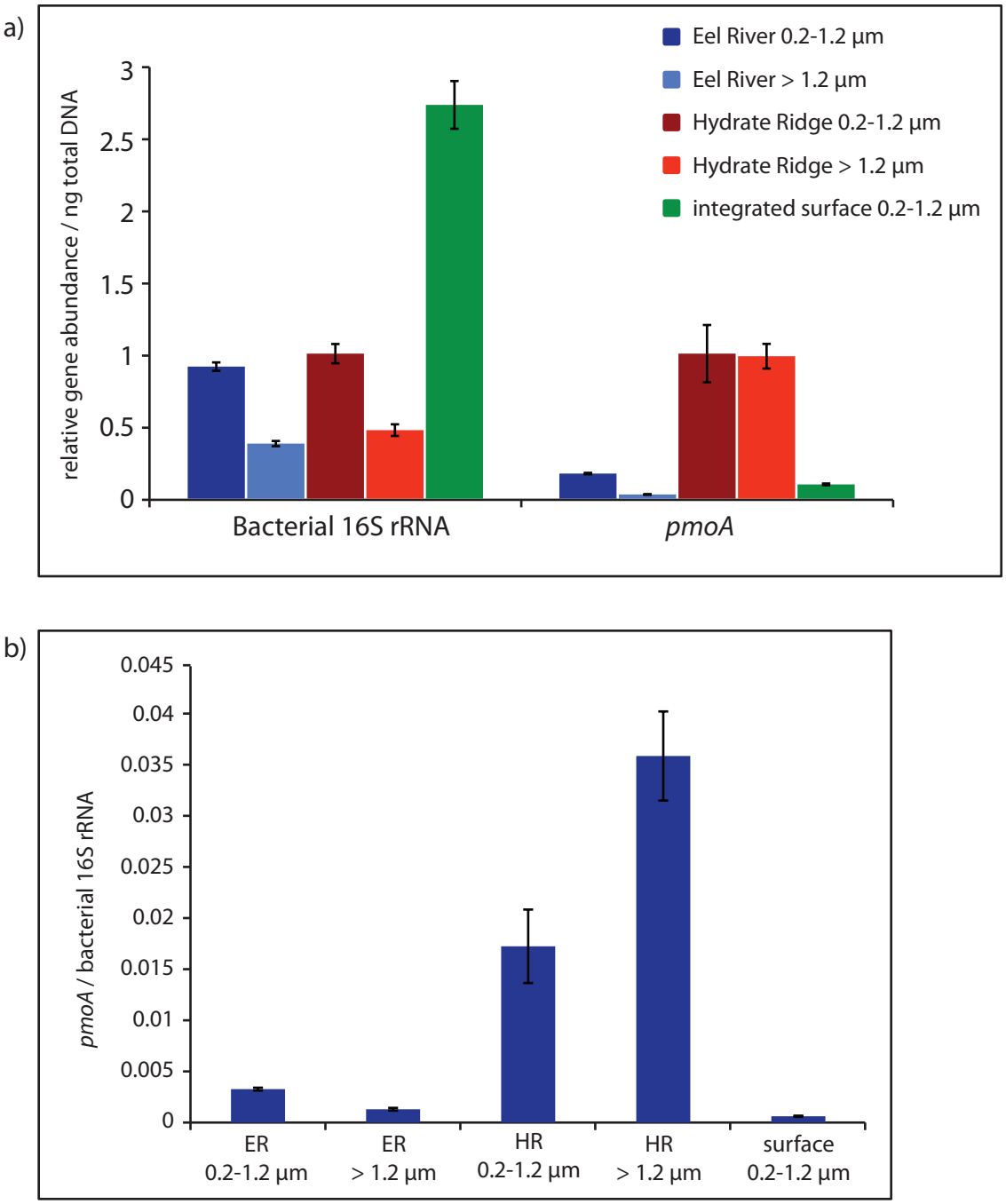

Figure 4.4. Quantitative PCR results for bacterial 16S rRNA and *pmoA* gene copies in DNA samples isolated from above methane seeps in the Eel River basin and Hydrate Ridge, as well as an integrated surface sample. Average (n = 3) relative gene abundances per ng total DNA are normalized to the Hydrate Ridge 0.2-1.2 µm sample (a), and the ratio of *pmoA* to bacterial 16S rRNA gene copies per ng total DNA are plotted in (b). Error bars indicate standard deviations.

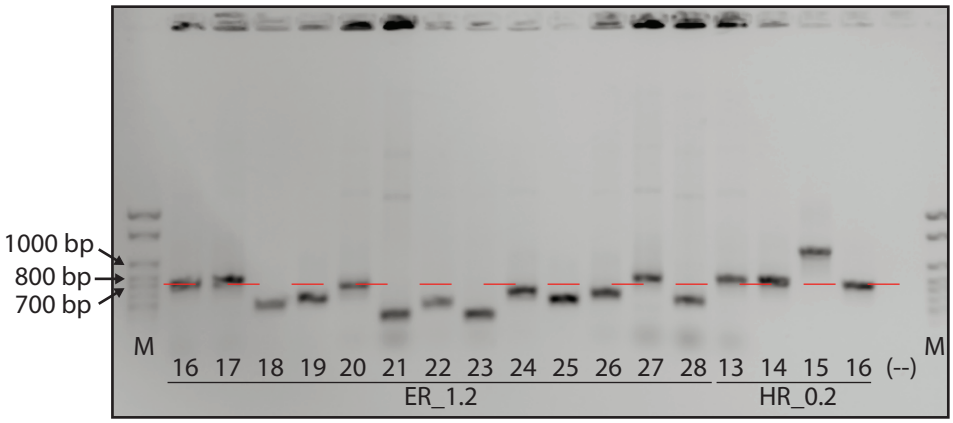

Figure 4.5. Sample agarose gel of white pmoA colonies amplified with primers M13f&r to determine size of PCR insert in vector. Expected band for pmoA with these primers is ~ 700 bp and indicated by dashed line. Wells of numbered colonies from the Eel River > 1.2 µm and Hydrate Ridge 0.2-1.2 µm samples are indicated, as well as lanes with a 100 bp DNA marker (M; AllStar Scientific) and the negative control (--).

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V. Diversity and distribution of heterotrophic assimilatory nitrate reductase in the marine water column

Abstract

Recovered sequences coding for a subunit of heterotrophic assimilatory nitrate reductase (*nasA*) identified the presence of this gene in the bathypelagic realm and revealed previously undetected *nasA* diversity in the subsurface ocean. Quantitative PCR results indicate, however, that *nasA* genes are present in only a very small fraction of the bacterial community throughout the marine environments sampled, though that fraction generally increases with depth in the water column.

Introduction

Microbes play critical roles in the biogeochemical cycles of the marine environment, including that of nitrogen. Generally, heterotrophic bacteria release inorganic nitrogen through the decomposition of dissolved organic material, while specialists mediate other processes such as nitrogen fixation, nitrification and denitrification. With the marine *Crenarchaeota* being recently identified as major players in the marine nitrogen cycle as nitrifiers (Könneke *et al*., 2005; Francis *et al.,* 2005; Wuchter *et al*., 2006), questions arise as to the availability of reduced nitrogen in the deep ocean for the total prokaryotic community, including both heterotrophic bacteria and chemoautotrophic archaea. Under the current paradigm, the entire microbial community of the dark ocean relies on freshly produced dissolved organic matter released from sinking particles for production, for although archaea appear to be fixing *in situ* inorganic carbon the fuel for this autotrophy comes from reduced nitrogen. On the other hand, free-living heterotrophic bacteria appear to rely primarily

on the organic carbon delivered from sinking particles (Hansman *et al*., submitted), but their nitrogen requirements in the deep ocean are not known.

Although the traditional role for heterotrophic bacteria in the marine environment has been the recycling of organic material to release ammonium, studies have shown bacteria utilize a variety of dissolved inorganic nitrogen (DIN) species including nitrate (NO_3^-), ammonium (NH_4^+), and urea. They generally prefer NH_4^+ over NO_3^-, as only 1 NADH is required to utilize NH_4^+ compared to 5 for NO_3^- (Vallino *et al*., 1996), but have been shown to be able to compete with phytoplankton in surface waters for both NH_4^+ and NO_3^- (Kirchman, 2000). For example, a study in the Barents Sea showed that the percentage of DIN assimilation by bacteria increased with depth down to 80 m, with bacteria accounting for 16-40% of the total NO_3^- uptake (Allen *et al*., 2002).

Nitrate assimilation by bacteria is mediated by assimilatory nitrate reductases (Moreno-Vivián *et al*., 1999), which are phylogenetically widespread among sequenced genomes (Richardson *et al*., 2001). Primers specific for the catalytic subunit of heterotrophic assimilatory nitrate reductase (*nasA*) have been used to survey for the genetic potential for heterotrophic bacterial nitrate utilization in the marine environment, which has been found to be widely distributed in surface waters (Allen *et al*., 2001). The relative abundance of *nasA* genes specific to *Marinobacter* sp. was positively correlated with nitrate availability and bacterial DIN uptake in the euphotic zone of the Barents Sea (Allen *et al*., 2005). *nasA* genes were also found with varying degrees of diversity at stations along a transect from the mouth of the Pearl River

estuary out into the South China Sea at depths down to 200 m, though in very low abundances (0.001 to 0.080 gene copies per ml of seawater) (Cai and Jiao, 2008). Based on the relationship between the abundance of the *nasA* gene and nitrate availability observed by Allen *et al*. (2005), and given the assumed scarcity of reduced N in the oxygenated ocean, this study was designed to address the hypothesis that the genetic capability for assimilatory nitrate reduction was more widespread in heterotrophic bacteria of the deep ocean. Thus far, the presence of *nasA* at depths below 200 m in the water column has not been documented. In this study, the diversity, distribution, and abundance of *nasA* genes were surveyed to assess the genetic potential for heterotrophic nitrate assimilation from a range of depths throughout the marine water column in a variety of locations in the eastern North Pacific.

Materials and Methods

Sample collection and DNA extraction

Samples were collected by filtering large volumes (9,050-48,000 L) of seawater through cartridge filters of either 0.5- (NELHA 915 m) or 1.2-µm pore size (composed of cellulose esters; Millipore Opticap) to remove eukaryotes and large particles, plumbed in-line with a 0.2-µm polyethersulfone filter (Pall Gelman or Millipore Opticap) for trapping microbial cells. The large-volume pumping capabilities at the Natural Energy Laboratory (NELHA) in Kona, HI, were used to filter seawater from 915 m. Samples CCE-P0605 and AT15-7 were collected using a

diaphragm pump to pump surface water onboard through tubing extended off the side of the R/Vs *Knorr* and *Atlantis*, respectively. The Hydrate Ridge sample was collected by deploying a rosette of 23 10-L Niskin bottles to a depth approximately 50 m above the bottom. Water was then filtered directly from the bottles once brought onboard. The two 3000 m samples were collected *in situ* using a battery-powered modified WTS-LV pump (McLane Labs, Falmouth, MA) deployed on the hydrowire of the R/V *Sproul*. Filters were frozen at -80˚C until extraction.

Microbial DNA was extracted from the 0.2-µm filters using a method modified from Blair *et al*. (1985) (Hansman *et al*., submitted). Briefly, filters were cut open with a combusted hacksaw and extracted in a lysis solution of 1.5 M $NaClO_4$ for 48 h at 4˚C. This solution was concentrated by ultrafiltration (5,000 MWCO; Millipore Pellicon), then an organic extraction was performed with chloroform and isoamyl alcohol (24:1). Nucleic acids were precipitated from the aqueous fraction with ethanol, pelleted by centrifugation, and re-dissolved in sterile, nuclease-free water.

PCR, clone libraries, and phylogenetic analysis

nasA genes were amplified by PCR using the nested primer set and protocol detailed in Allen *et al*. (2001). Products from 3 to 6 reactions were pooled, cleaned using the QIAquick PCR purification kit (Qiagen), then inserted into vector pCR®2.1-TOPO (Invitrogen). TOP10 competent cells were transformed as recommended by the manufacturer (Invitrogen), spread onto LB-kanamycin agar plates, and incubated overnight at 37˚C. 24 white colonies were picked for each sample and grown

statically in 200 µl LB media with kanamycin and 10% glycerol for 12 h at 37˚C in 96-well plates. The resulting glycerol stocks were immediately frozen at -80˚C until single-pass sequencing with primers M13f&r by Agencourt Bioscience (Beverly, MA).

DNA sequences were assembled using VectorNTI (Invitrogen), checked for chimeras using the Bellerophon server (Huber *et al*., 2004), and translated into amino acids using NCBI's open reading frame finder. Sequences were then aligned using MUSCLE (Edgar, 2004), grouped into OTUs using DOTUR (Schloss and Handelsman, 2005), and analyzed phylogenetically in ARB (Ludwig *et al*., 2004).

Quantitative PCR (qPCR)

qPCR was performed on 1-8 ng extracted DNA with 12.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.4 µM each primer, and 2 ng BSA for each reaction in a final volume of 25 µl. DNA concentrations were determined by PicoGreen (Molecular Probes) assay, according to the manufacturer's protocol. Bacterial 16S rRNA genes were quantified as described in Allen *et al*. (2005), using primers 932F and 1062R. Primers nas964 and nasA1735 (Allen *et al*., 2001) were used to quantify *nasA* genes using cycling parameters as detailed in Cai and Jiao (2008). PCR products generated using the qPCR primers were used as standards in tenfold dilution series from 10^8 to 10^2 copies. PCR efficiencies and r^2 values were, respectively, 88% and 0.998 for bacterial 16S rRNA, and 88% and 0.993 for *nasA*.

Results

DNA samples were extracted from a variety of locations and depths in the eastern North Pacific, including surface, mesopelagic, and deep waters (Table 5.1). The NELHA sample was isolated in February 2006 from 915 m depth off the coast of Kona, HI. The surface CCE-P0605 sample was collected during the May 2006 process cruise of the California Current Ecosystem (CCE) LTER, while the AT15-7 sample is integrated from surface waters in the Eel River basin offshore California and Hydrate Ridge offshore Oregon, collected in July 2006. During that same cruise, the Hydrate Ridge sample was collected from the water column approximately 50 m above bottom in an area of active methane seeps. 3000 m samples were collected at two different time points, July 2007 and May 2008, from the same location in ~ 3800 m water depth off the coast of San Diego, CA.

Inferred amino acid phylogeny of recovered *nasA* sequences is presented in Figure 5.1. Of the 71 sequences recovered from 3 different samples, 7 OTUs of sequences sharing 97% identity were identified. The majority (> 83%) of the sequences recovered within each sample were identical. While the inferred amino acids sequences of recovered *nasA* genes from the surface AT15-7 sample were all > 90% similar to previously described sequences, the majority of the sequences isolated from the mesopelagic and deep depths were only 67-79% identical to those found in GenBank.

Abundances of *nasA* and bacterial 16S rRNA gene copies, as determined by qPCR, are shown in Figure 5.2a. *nasA* abundances ranged from 141 to 1,152 gene

copies, in sharp contrast to the nearly 300,000 to 1.6 x 10^6 bacterial 16S rRNA copies per ng total DNA. The proportions of *nasA* to bacterial 16S rRNA gene copies in each sample, ranging from 0.0001 to 0.0039, are plotted in Figure 5.2b. Quantitation was calculated per ng total DNA, and relative abundances are normalized to the surface AT15-7 sample (Table 5.2).

Discussion

This study presents the first reported *nasA* sequences recovered from the deep ocean. Although other functional gene sequences and phylogenetic markers were amplified from DNA isolated from the > 1.2 μm filter we were unable to amplify any *nasA* gene sequences from this size fraction. From the limited number of sequenced clones from the DNA isolated in the smaller size fraction (0.2 -1.2 μm) at each depth, diversity appears very low within each sampling location (Figure 5.1). For example, > 20 (of 24) sequences in each sample were identical. However, among sites and depths, the majority (63%) of recovered sequences were < 90% similar than those found in GenBank, and the results indicated that most of the sequences recovered from the meso- and bathypelagic had not been previously observed in surface samples. Very few studies have explored heterotrophic assimilatory nitrate reductase in the marine environment, and it appears there is diversity in the *nasA* gene here that has yet to be detected and documented, specifically in subsurface waters. The majority of previously recovered sequences appear to be phylogenetically related to gamma-Proteobacteria, including *Vibrio* sp. and *Marinobacter* sp. Sequences closely related

to *Roseobacter* sp. and other alpha-Proteobacteria have also been described (Allen *et al*., 2001; Allen *et al*., 2005; Stepanauskas and Sieracki, 2007; Cai and Jiao, 2008). In this study, the sequences that displayed significant similarity to previously classified *nasA* genes were related to gamma-Proteobacteria, though distant identity to *nasA* sequences from alpha-Proteobacteria was also observed.

Allen *et al.* (2005) examined the diversity of *nasA*, the relative abundance of *nas*A genes specific to *Marinobacter*, and the relative importance of bacterial nitrate reductase at 5 m and 80 m at a variety of locations in the Barents Sea. In this Allen *et al*. study, at every station nitrate, and in all but one case ammonium, was higher at 80 m. In two out of the five stations, nitrate uptake by the bacterial community was significantly elevated at 80 m; the qPCR assay showed consistently higher relative expression of *nasA* at 80 m; and variations in the composition of the *nas*A population was best correlated with nitrate variability. Based on these lines of evidence Allen *et al*. (2005) concluded that the nitrate concentration had the greatest influence on bacterial nitrate uptake dynamics. On the other hand, culture experiments have shown that the presence of ammonium inhibits nitrate metabolism (Kirchman, 2000), suggesting that ammonium concentrations should control heterotrophic nitrate assimilation. However, the high concentration of ammonium used in many laboratory experiments is unlikely to be representative of ammonium dynamics in open ocean environments.

Nitrate and ammonium concentrations are only available for some of the sites examined in the current study. During the collection of the CCE-LTER sample nitrate

concentrations were less than 1 μM. Ammonium concentrations at this site were also low, < 0.58 μmol kg^{-1}. At NELHA, nitrate concentrations were 40.1 μmol kg^{-1} and ammonium concentrations were 0.33 μmol kg^{-1}. The AT15-7 surface sample was collected during July 2006 and nitrate concentrations were probably greater than or equal to concentrations observed during CCE-LTER. Measurable concentrations of nitrate are expected in surface waters at this site because summer upwelling events are typical in this region of the eastern North Pacific (e.g., Bane *et al*., 2007). Concentrations at Hydrate Ridge are not known, but the water depth at this station (650 m) suggests that nitrate concentrations would be much higher than surface waters and similar to NELHA 915 m. Finally, the two 3000 m samples from the eastern North Pacific are expected to have very high nitrate concentrations, between 38 and 42 μmol $kg^{-1,}$, based on the radiocarbon signature of the dissolved inorganic carbon (DIC) at this depth (-232 ± 3‰; Hansman, unpublished) and the measured nitrate concentration at a nearby site in a water mass of similar radiocarbon age (Masiello *et al*., 1998; Loh and Bauer, 2000). The large range in nitrate concentrations expected at the various study sites allows us to further test the hypothesis presented in Allen *et al*., (2005) based on the finding that the number of *nasA* genes are positively correlated with nitrate concentrations. From the qPCR data generated in the current study, no apparent trend with the abundance of *nasA* gene copies and depth exists (Figure 5.2a). However, when normalized to the quantity of bacterial 16S rRNA genes in each sample, the proportion of *nasA* genes generally increases with depth (and nitrate concentrations) and is much greater at NELHA 915 m and 3000 m compared to

surface waters (Figure 5.2b). The difference between the two surface samples is consistent with the hypothesis that nitrate concentrations were likely higher during the collection of AT15-7 than CCE-LTER.

The sample from Hydrate Ridge does not follow the above trend; however, it was collected from the water column directly above actively venting methane seeps and as such the nitrogen-acquiring strategies of the microbial community might be influenced more strongly by other environmental factors. Enhanced mineralization of organic material was observed in the surface sediments here as compared to a reference site by Valentine *et al*. (2005), so sufficient organic nitrogen may be available to the water column heterotrophs. Additionally, recent evidence for nitrogen fixation in the anaerobic sediments of methane vents in the Eel River Basin, a site with characteristics very similar to Hydrate Ridge, determined through $^{15}N_2$ incorporation and the presence of nitrogenase genes (Pernthaler *et al*., 2008) suggests the possibility of an alternative N source to the planktonic microbial community above the seeps. Further, Pernthaler *et al.* (2008) report that pore water ammonium concentrations within the Eel River Basin methane seeps are ~ 40 μM, which would certainly result in a positive flux of ammonium into the deep water column. If similar conditions exist within the Hydrate Ridge methane seeps then such high ammonium concentrations may select against nitrate assimilating planktonic heterotrophic bacteria.

Although the ratios of *nasA* gene copies to bacterial 16S rRNA gene copies for both 3000 m samples were greater than those of the samples collected from shallower depths, there were significant differences between the two deep samples that were

collected at different time points. Nearly double the number of *nasA* gene copies were present per ng of total DNA in the May 2008 sample, and the ratio of *nasA* to bacterial 16S rRNA was more than two times that of the July 2007 sample. This implies that the mechanisms for nitrogen acquisition and assimilation by the total prokaryotic community differed between the two time points. qPCR data also demonstrated that 5 times as many group I *Crenarchaeota* 16S rRNA and archaeal *amoA* genes were present in the sample collected in July 2007 as compared to the May 2008 sample per ng of total DNA (Figure 5.3). Marine *Crenarchaeota* are believed to be chemoautotrophs that fix inorganic carbon through ammonia oxidation, and the temporal difference in their abundance may imply that the availability of ammonium was greater during July 2007 than May 2008, consistent with the lower relative abundance of *nasA*. (No significant difference in the absolute abundance of bacterial 16S rRNA genes was observed between the two years (Figure 5.2a)). These data further support the notion that the metabolic potential of the microbial community differed significantly between the two sampling periods. This marked interannual variation in the abundance of *nasA* in this deep ocean site where nitrate concentrations are expected to be static suggests that in the deep ocean nitrate concentrations are not the only factor that controls the abundance of nitrate assimilating heterotrophic bacteria.

Ratios of *nasA* genes to bacterial 16S rRNA genes were up to 1000-fold higher than those found by Allen *et al*. (2005) in surface waters of the Barents Sea for *Marinobacter* sp. *nasA* genes, though this is not surprising as the majority of

recovered sequences were not associated with *Marinobacter* sp. These ratios are in agreement with the findings of Stepanauskas and Sieracki (2007), who demonstrated through whole-genome multiple displacement amplification that < 1% of the bacterioplankton population they sampled from coastal surface waters contained *nasA* genes.

Early assumptions that heterotrophic bacteria were unable to compete with phytoplankton for nitrate have been questioned by several recent studies which show that bacteria can represent a substantial fraction of total community nitrate uptake (Kirchman, 2000; Table 5.3), particularly when chlorophyll *a* concentrations are low or phytoplankton are in the senescent period (Rodrigues and Williams, 2002; Fouilland *et al*., 2007). Bacteria were expected to be able to satisfy their nitrogen requirement in surface waters by relying primarily on ammonium, free amino acids and other fractions of the dissolved organic matter reservoir, and potentially urea (Kirchman, 2000), without needing access to the nitrate reservoir. In addition, the cosmopolitan cyanobacterium *Prochlorococcus* sp. is unable to utilize nitrate (Moore *et al*., 2002), and the cultured representative of the ubiquitous SAR11 clade, *Pelagibacter ubique* (a heterotroph), does not appear to have the *nasA* gene (based on a BLAST search of its genome) or any genes coding for nitrate uptake and assimilation (Mills *et al*., 2008). However, some studies have observed rates of bacterial nitrate and ammonium uptake to be similar during certain times of year (Rodrigues and Williams, 2002), and urea uptake appears to be dominated by phytoplankton (Kirchman, 2000; Fouilland *et al*., 2007). While free amino acids and

other nitrogen-rich dissolved compounds are likely to be available to support some of the bacterial N demand in surface waters, their concentrations decrease rapidly with depth (e.g., Lee and Bada, 1977; Bronk, 2002; Yamashita and Tanoue, 2003). In addition, the production rate of amino acids in the deep ocean is likely much lower than in surface waters, and so the flux through this reservoir is probably small as well. In general, amino acids may make only a small contribution toward supporting bacterial N demand in the deep ocean. Furthermore, dissolved organic matter becomes increasingly recalcitrant with depth (e.g., Druffel *et al*., 1992; McCarthy *et al*., 1997; Aluwihare *et al*., 2005), making unlikely that this vast reduced reservoir of nitrogen is bioavailable. Recent direct studies of chemoautotrophy by *Crenarchaeota* suggest that carbon fixation is fueled by ammonia oxidation (e.g., Könneke *et al*., 2005; Wuchter *et al*., 2006; Beman *et al*., 2008), and thermodynamic calculations and field observations indicate that between 2 and 10 molecules of ammonium are required per carbon atom fixed (Ward *et al*., 1989; Ingalls *et al*., 2006; Wuchter *et al*., 2006). Based on archaeal carbon fixation rates measured in field incubations (Herndl *et al*., 2005), and ammonium regeneration rates calculated for the deep ocean (based on sinking particulate organic carbon flux measurements and assuming Redfield stoichiometry), all of the ammonium delivered to the deep ocean would be needed to sustain archaeal chemoautotrophy in this environment (Ingalls *et al.*, 2006; Wuchter *et al*., 2006). These data suggest that free-living bacteria face stiff competition for access to regenerated nitrogen in the deep ocean, and bacteria adapted to using nitrate – the dominant form of bioavailable N in the deep ocean – should occupy an

ecological niche that allows them to outcompete other heterotrophic bacteria. Therefore, the low abundance of *nasA* genes relative to total bacterial 16S rRNA genes in the subsurface ocean, despite the observed differences between surface and deep waters, was unexpected. The results obtained in this study suggest that bacteria capable of assimilatory nitrate reduction represent a very small fraction of the total bacterial community even in the deep ocean. Therefore, the question of whether enough ammonia and organic nitrogen is available to support both chemoautotrophic and heterotrophic microbial growth in the deep ocean remains an open and important question.

This study further demonstrates the ubiquitous distribution of *nasA* genes throughout the marine environment down to depths of 3000 m, and implies *nasA* diversity has not yet been fully explored. The results presented here only examine the genetic potential for nitrate assimilation through PCR and qPCR, though Allen *et al*. (2001) found that the presence of *nasA* in marine isolates was generally correlated with the ability to grow aerobically on nitrate as a sole N source. Generally, the data presented here support the findings of Allen *et al*. (2005) that higher relative abundances of *nasA* are correlated with nitrate concentrations. The amino acid sequence data in Figure 5.1 also corroborate the hypothesis that *nasA* community composition varies with nitrate concentrations (Allen *et al*., 2005), or potentially, depth. qPCR data suggest *nasA* abundances are small relative to the total bacterial community, but further research is needed to investigate gene expression and quantify the extent of nitrate utilization throughout the water column. Finally, as observed with

other functional genes, the data presented here show that particle-attached and free-living communities have distinct metabolisms in the deep ocean environments examined in this study.

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Table 5.1. Locations, filtration volumes, and average temperatures and salinities for DNA samples.

Sample	Location	Volume filtered (L)	Avg. Temp. (˚C)	Avg. Salinity
7/06 AT15-7 surface	40.8˚N, 124.6˚W; 44.6˚N, 125.1˚W	~10,000	14.3	32.0
5/06 CCE-LTER surface	32.9˚N, 120.3˚W	10,390	14.7	32.7
7/06 Hydrate Ridge 650 m	44.6˚N, 125.1˚W	9,050	4.9	34.2
2/06 NELHA 915 m	19.7˚N, 156.1˚W	48,000	5.8	34.5
7/07 3000 m	31.5˚N, 120.0˚W	15,000	1.6	34.7
5/08 3000 m	31.5˚N, 120.0˚W	20,000	1.6	34.7

Table 5.2. Quantitative PCR data for *nasA* and bacterial 16S rRNA genes in extracted DNA samples from various sampling locations and depths.

	gene copies / ng DNA			relative abundance / ng DNA	
Sample	***nasA***	**bacterial 16S rRNA**	***nasA* / bacterial 16S rRNA**	***nasA***	**bacterial 16S rRNA**
7/06 AT15-7 surface	148	1248129	0.0001190	0.132	0.772
5/06 CCE-LTER surface	1126	1615825	0.0006970	1.000	1.000
7/06 Hydrate Ridge 650 m	141	594941	0.0002370	0.125	0.368
2/06 NELHA 915 m	566	461252	0.0012270	0.503	0.285
7/07 3000 m	608	345769	0.0017580	0.540	0.214
5/08 3000 m	1152	292217	0.0039430	1.023	0.181

Table 5.3. Summary of heterotrophic bacterial inorganic nitrogen uptake studies, adapted from Kirchman, 2000.

Location	Total Uptake by Bacteria (%)		Comments	Reference
	NH_4^+	NO_3^-		
North Atlantic	22-39	4-14	Spring bloom	Kirchman *et al*., 1994
Georgia coastal waters	78	0	Used inhibitors	Wheeler and Kirchman, 1986
Subarctic Pacific	31	32	Station P; 4-month average	Kirchman and Wheeler, 1998
Georges Bank (North Atlantic)	38	27	Coast to open ocean transect	Harrison and Wood, 1988
Boothbay Harbor, ME	34	10	Flow cytometry	Lipschultz, 1995
Menai Strait	40	24	Seasonal avg. for May to Sept.	Rodrigues and Williams, 2002
Barents Sea	12-40	16-40	5, 30, and 80 m depth	Allen *et al*., 2002
Southern California Bight	3	9	Nearshore + offshore avg.	Bronk and Ward, 2005
North Water, northern Baffin Bay	40	25	NO_3^- uptake w/ low Chl *a* (< 2 μg L^{-1})	Fouilland *et al*., 2007
Average	36	18		

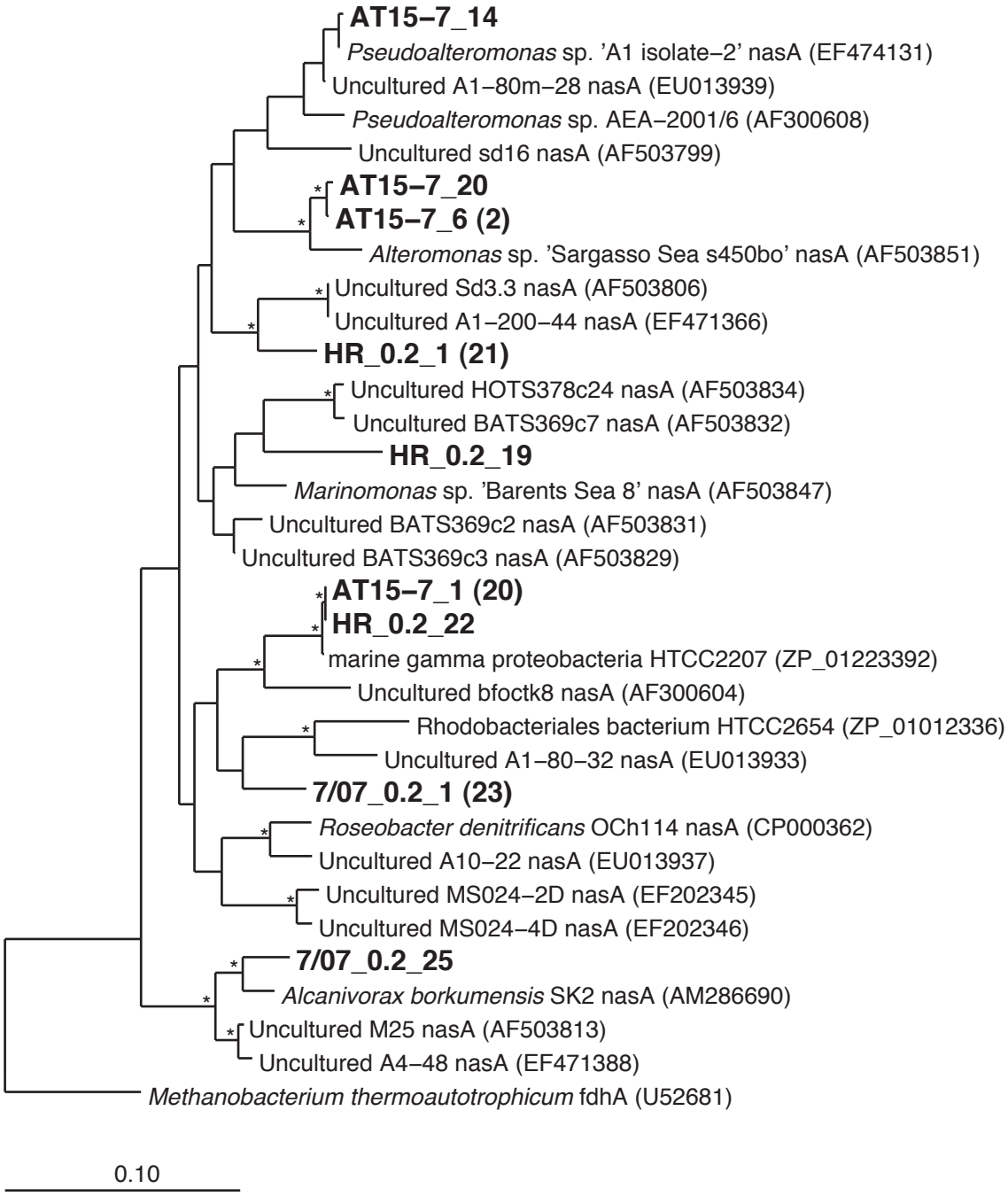

Figure 5.1. Inferred amino acid phylogeny from recovered nasA sequences. The scale bar represents 0.1 substitution per site, and the asterisks indicate bootstrap values ≥ 50%. Numbers in parentheses next to bolded clones are the number of identical amino acids sequences from that sample. HR, Hydrate Ridge; AT15-7, integrated surface sample from AT15-7 cruise; 7/07, 3000 m sample collected in July 2007.

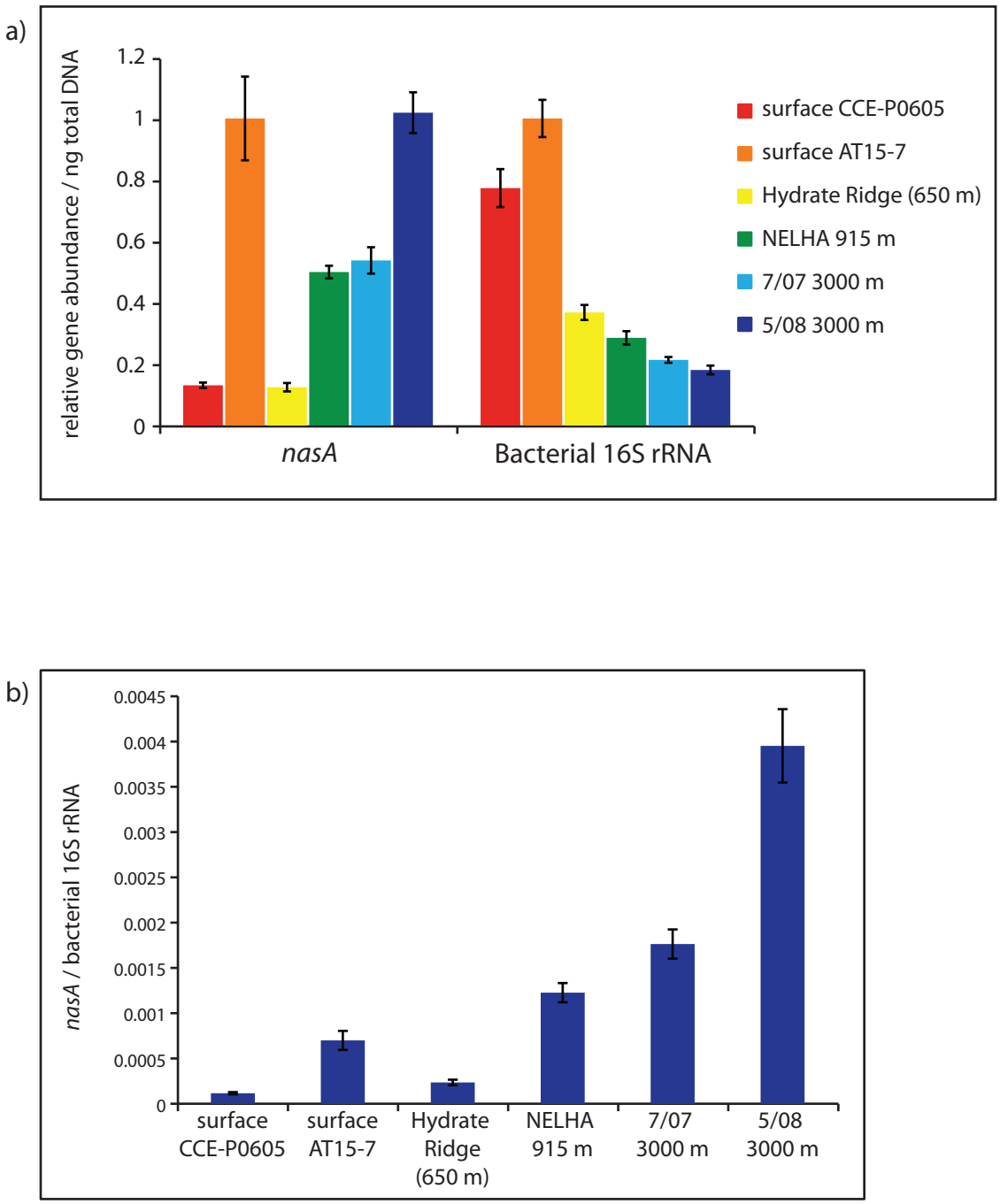

Figure 5.2. Quantitative PCR results for *nasA* and bacterial 16S rRNA gene copies in DNA samples isolated from a variety of depths and locations in the eastern North Pacific. Average (n = 3) relative gene abundances per ng total DNA are normalized to the surface AT15-7 sample (a), and the ratios of *nasA* to bacterial 16S rRNA gene copies per ng total DNA are plotted in (b). Error bars represent standard deviations.

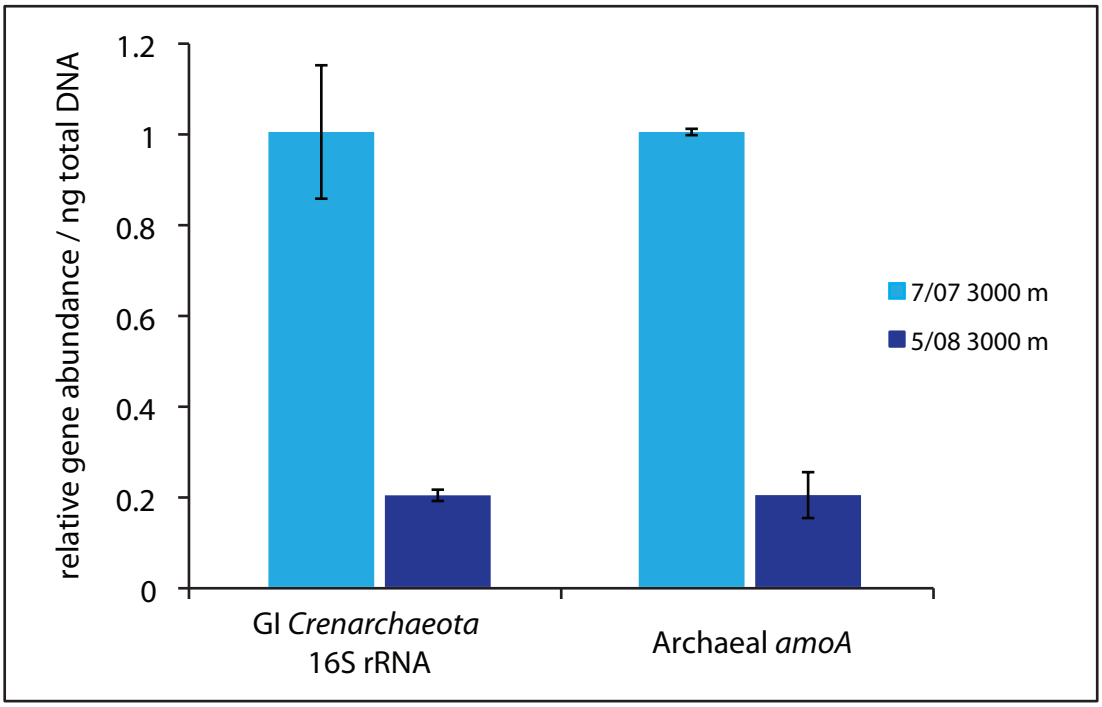

Figure 5.3. Average (n = 3) relative abundance of GI *Crenarchaeota* 16S rRNA and Archaeal *amoA* gene copies in 3000 m samples collected in July 2007 and May 2008, as determined by qPCR and normalized to the 7/07 3000 m sample. Error bars indicate standard deviations.

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VI. Conclusions

In recognizing the major role microorganisms play in the biogeochemical cycling of carbon and nutrients in the marine environment, this thesis focused on microbial metabolism in the deep ocean, a largely under-explored and poorly constrained field. Prokaryotes are the most numerous organisms throughout the water column, and with abundances of approximately 10^4 cells ml^{-1} in the deep ocean, an estimated 10^{28} microbes are contained in the world ocean (Karner *et al*., 2001). The majority of these are believed to be heterotrophic bacteria that utilize fresh dissolved organic carbon released from sinking particles (Azam and Long, 2001), regulating oceanic carbon flux through the 'microbial loop' (Azam *et al.*, 1983; Azam, 1998). However, having only been discovered relatively recently to be ubiquitous in the marine environment (DeLong, 1992; Fuhrman *et al*., 1992), we know now archaea exist in comparable numbers to bacteria in the deep ocean (Karner *et al*., 2001), are capable of fixing inorganic carbon as well as take up amino acids (Ouverney and Fuhrman, 2000; Pearson *et al*., 2001; Wuchter *et al*., 2003; Herndl *et al*., 2005; Ingalls *et al*., 2006), and could be major players in the marine nitrogen cycle as nitrifiers oxidizing ammonia for energy (Könneke *et al*., 2005; Wuchter *et al*., 2006; Beman *et al*. 2008). These new discoveries lead to many questions regarding the cycling of carbon and nitrogen by the total prokaryotic community in the marine water column, and this thesis attempted to examine some of those questions through a combination of geochemical analyses and molecular biological techniques.

Chapter 2 detailed the development of a method for extracting microbial nucleic acids (NA) suitable for radiocarbon analysis. This required isolating microbes

from large volumes of seawater, while minimizing carbon contamination from reagents and extracellular components. Process blanks were key in verifying the reflection of the carbon source isotopic signature in the NA, and in identifying possible contaminants. The isolated NA was also of sufficient quality for use in a number of molecular biological techniques to examine the composition and metabolic potential of the microbial community present in the analyzed samples. Using the same large volume samples a variety of other techniques such as individual nucleoside purification, intracellular lipid isolation, protein isolation (not discussed), and fosmid library generation (not discussed) were also developed and applied with mixed success. The cell isolation and extraction method was also applied in a collaborative study to examine archaeal metabolism in the mesopelagic ocean (Ingalls *et al*., 2006)

The work in **Chapter 3,** which commanded the bulk of my effort, utilized the distribution of natural abundance radiocarbon in the marine environment to identify the sources of carbon fueling prokaryotic production in the mesopelagic ocean, using the method developed above. This represented the first study to examine *in-situ* microbial community metabolism in the subsurface ocean. While fresh organic material delivered from sinking particles was directly confirmed as an important carbon source for free-living microbes, the extent of autotrophic carbon fixation was also very significant and variable with depth. If nitrification (oxidation of ammonium to nitrite and eventually nitrate) supports the bulk of this carbon fixation, then this study further highlights the significant demand for reduced nitrogen, presumably delivered from sinking organic material, by the total microbial community in the deep

ocean, and stresses the importance of constraining particle flux down into the dark ocean. Compound specific radiocarbon measurements showed conclusively that particle-associated and free-living microbial communities isolated from the same depth had distinct carbon metabolisms on the timescales examined here. However, the small number of phylogenetic sequences retrieved for each sample did not identify differences in the composition of the microbial community between depths or size fractions.

The importance of methane in supporting the planktonic microbial community in the water column above cold methane vent systems was examined in **Chapter 4**. The same technique developed in Chapter 2 was used to isolate NA, but this time for stable carbon isotope measurements. The sites examined included one low oxygen environment, environments that receive considerable organic matter input from surface waters, and a deep water site. Aerobic methane oxidation has been demonstrated to occur in the water column above cold methane seeps (Valentine *et al*., 2001), and genes coding for particulate methane monooxygenase were found in all samples from these environments. Yet, the stable isotope and qPCR data suggested methane-derived carbon was not a significant carbon source to the total prokaryotic community in these systems. Despite the diverse sampling environments methane monooxygenase sequences retrieved from each site were highly conserved and showed little diversity, falling within two major groups. In addition, water column sequences were only distantly related to sequences retrieved from sediments by other investigators. Phylogenetic (16S) rRNA gene sequences were also only between 90-

93% related to known methane oxidizing bacteria. While phylogenetic sequences specific for methane oxidizing bacteria were rare in these environments, inter-site differences in the abundance of gene sequences coding for particulate methane monooxygenase were observed. This finding indicates that this specialized but rare community does respond to fluctuations in the geochemistry at these different sites.

Chapter 5 focused on nitrogen metabolism through a survey for the potential of heterotrophic nitrate assimilation by bacteria in the marine water column. The recovery of previously undocumented *nasA* sequences suggested undetected diversity still remains throughout the marine environment, particularly in the subsurface ocean – sequences retrieved from surface and deep waters were distinct. However, intra-site diversity was very low. qPCR results displayed the significant differences in the proportion of *nasA* genes to bacterial 16S rRNA from different sampling depths, locations, and times, but overall the abundance of *nasA* gene copies is less than 1% of bacterial 16S rRNA genes, indicating heterotrophic assimilation of nitrate is not a dominant mode of nitrogen acquisition by the microbial community throughout the water column. Variations in the relative abundance of the *nasA* gene were correlated with nitrate concentrations, with a few notable exceptions. Additionally, the *flux* of ammonium through the N reservoir at any given site may also help to determine the relative abundance of *nasA* containing populations. For example, interannual variations in the *nasA* gene at the 3000 m depth appeared to be inversely correlated with archaeal *amoA* gene sequences. Variations observed at the 3000 m site suggest that even in this relatively static environment, the metabolic potential of the microbial

community is fluid and responds to what are likely to be very small changes in water column geochemistry. As with results from Chapter 3, the composition of the free-living and particle-attached communities isolated from the same depth were distinct in the deep ocean.

Summary of Major Research Findings.

(1) Ambient DOC was not a major source of carbon to the microbial population of the subsurface ocean at the sites examined in this study.

(2) Carbon derived from sinking POC and ambient DIC fuels the microbial food web of the meso- and bathypelagic ocean to different extents, and the contribution of each varied with both space and time.

(3) At one depth in the mesopelagic, carbon derived from the ambient DIC pool supported ~95% of the microbial community.

(4) The extent of total community DIC utilization was correlated with *Crenarchaeota* 16S rRNA gene abundance and archaeal *amoA* gene abundance.

(5) In the three methane seep environments examined, the stable carbon isotopic composition of total prokaryotic NA indicated that aerobic methane oxidation was not a significant source of carbon for the microbial community inhabiting the water column above these seeps.

(6) Consistent with the stable carbon isotope results, qPCR results indicated that the gene coding for particulate methane monooxygenase was present in only low abundance in these water column communities.

(7) Gene sequences coding for particulate methane monooxygenase retrieved from these water column environments were not closely related to sequences of the same gene retrieved from sediment aerobic methane oxidizing communities. In addition, 16S rRNA gene sequences of water column communities were not closely related to those of known methane oxidizing bacteria.

(8) Higher relative abundances of the heterotrophic bacterial assimilatory nitrate reductase gene (*nasA*) were identified in the mesopelagic and bathypelagic suggesting that a greater percentage of the bacterial community in deep ocean environments were able to access the nitrate reservoir. However, these genes still represented < 1% of total 16S rRNA genes indicating that bacterial nitrate utilization is not widespread in the deep ocean.

(9) The *nasA* gene sequences retrieved from free-living bacteria in the deep ocean were not closely related to sequences previously retrieved from surface ocean environments.

(10) The qPCR results consistently showed that particle-attached and free-living communities sampled at the same depth had different compositions, and the geochemical evidence showed marked differences in their carbon metabolism as well.

Future Research Directions

What fuels the observed extensive chemoautotrophy in the deep ocean? If transformations of nitrogen provide the major energy source for this process then how is the N cycle balanced in the deep ocean given the demand for reduced nitrogen by

both autotrophic and heterotrophic microbial communities.

Both heterotrophic production and chemoautotrophic production in the deep ocean are ultimately fueled by particle flux from overlying surface waters, but the spatial decoupling of these process observed in the current study (greater autotrophy at 670 m and greater heterotrophy of particle derived carbon at 915 m) suggests that there must be some spatial or temporal decoupling of the supply of reduced N and labile C to these communities from passive and active particle flux.

In general, C and N fluxes to the deep ocean need to be better constrained and the *in-situ* growth rates of heterotrophic and autotrophic organisms must be determined before a comprehensive budget of C and N cycling in the deep ocean can be attempted.

What is the ammonium requirement of ammonium oxidizing archaea?

Carbon sources fueling microbial production in deep ocean environments, where particle flux is expected to be very low, should be examined as well to determine whether data from the mesopelagic ocean is consistent with microbial metabolism in the bathypelagic. Certainly, the radiocarbon technique should be applied in other deep ocean environments.

In the mesopelagic population where 95% of the carbon fueling microbial production was derived from DIC, bacterial 16S rRNA gene sequences were abundant. What is the metabolic profile of this community? Certainly, some fraction of these bacteria is likely to be autotrophic (e.g., nitrite oxidizing bacteria), but what is the relative abundance of this autotrophic bacterial community and what fuels their

metabolism?

What controls the abundance of nitrate assimilating heterotrophic bacteria in the marine environment? In the deep ocean where nitrate concentrations are high and stable, and where heterotrophic organisms experience no competition from phytoplankton, *nasA* genes were expected to be abundant. Yet, they represented < 1% of bacterial 16S rRNA genes. Do archaea compete effectively with both ammonium oxidizing bacteria *and* heterotrophic bacteria for ammonium?

Marked differences were observed between the aerobic methane oxidizing communities inhabiting the water column and sediments. These differences were apparent at both the 16S rRNA gene sequence level, and at the level of sequences coding for particulate methane monooxygenase. How diverse are these populations of methane oxidizing bacteria, and are water column methane oxidizing communities found throughout the ocean (even in regions where there is no active venting)?

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