

DISTRIBUTIONS AND ACTIVITIES OF CHEMOLITHOAUTOTROPHIC  
BACTERIA IN APHOTIC WATERS OF THE NORTH PACIFIC SUBTROPICAL  
GYRE

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## Abstract

The dark, interior waters of the world's oceans form some of the largest habitable space on Earth, yet knowledge of deep-sea biology and biogeochemistry remains rudimentary. In this thesis, I quantified and sequenced form II *cbbM* ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) genes in seawater samples from the surface through the bathypelagic (0-4000 m) of the North Pacific Subtropical Gyre (NPSG) to determine the distributions of chemotrophic bacteria in the ocean. In addition, I conducted two substrate addition experiments to determine whether an increase in a reduced sulfur substrate (thiosulfate) would change rates of carbon fixation (based on assimilation of  $^{14}\text{C}$ -bicarbonate), bacterial production (based on incorporation of  $^3\text{H}$ -leucine), or the abundance of *cbbM* genes. Polymerase chain reaction (PCR) amplification and sequencing of *cbbM* genes revealed a diverse assemblage of bacteria, including members of  $\gamma$ - and  $\delta$ -proteobacteria, in the mesopelagic waters of the NPSG. Quantitative PCR (qPCR) analyses of *cbbM* genes revealed low abundances in the epipelagic transitioning to high abundances throughout the meso- and bathypelagic. Results from the thiosulfate addition experiments indicated that in one of the two experiments, elevated concentrations of thiosulfate stimulated rates of both  $^{14}\text{C}$ -bicarbonate assimilation and  $^3\text{H}$ -leucine incorporation, but resulted in no significant changes in *cbbM* abundances. Such results hint that mixotrophy may be a common strategy among chemotrophic bacteria dwelling in the energy-limited waters of the deep sea. Moreover, my results indicate that largely unexplored chemotrophic

microbial metabolisms may play an important role in biogeochemical transformations of the dark ocean.

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# Chapter 1

## Introduction

Microorganisms play crucial roles in ocean ecosystems. Collectively these largely invisible organisms constitute the majority of biomass in the sea and form the base of the ocean food web (Kirchman, 2008). Moreover, their diverse metabolic activities sustain life on this planet through their regulation of numerous elemental cycles. Oceanographic research over the past several decades has revealed that metabolically diverse microbes inhabit the oceans (DeLong et al, 2006; DeLong and Karl, 2005; Giovannoni et al., 2000). Partly for logistical reasons, much of the research on microbial ecology and biogeochemistry has focused on the well-lit waters of the upper ocean; however, the vast, dark waters of the ocean's interior harbor a significant fraction of the global ocean's prokaryotes (Whitman et al., 1998), and the metabolic capabilities, energy sources, and contribution of these organisms to carbon cycling remain largely unknown.

The aphotic zone constitutes the largest habitable space on Earth. This realm is subdivided into the mesopelagic (200-1000 m), the bathypelagic (1000-4000 m) and abyssal zones (> 4000 m). The residence time of the water masses comprising the mesopelagic is on the order of decades to centuries, and centuries to millennia for water in depths >1000 m (England, 1995). Unlike the epipelagic, or sunlit layer of the sea, the dark ocean is characterized by the absence of light, high pressure, low temperature, and elevated inorganic nutrient concentrations. In addition, it contains a majority of the

global ocean's prokaryotic biomass with more than half (65%) of all pelagic bacteria and archaea occupying the water column below 200 m (Whitman et al., 1998).

The dark pelagic water column also contains  $\geq$  98% of the ocean's dissolved inorganic carbon (DIC; Gruber et al., 2004). Approximately 5-20% of photosynthetically fixed carbon is exported from the euphotic zone to the mesopelagic waters in the form of sinking particles (Martin et al., 1987; Eppley and Petersen, 1979). These sinking particles transfer photosynthetically-derived energy and reduced material to aphotic waters, fueling microbial metabolisms in the deep sea (Hansell and Ducklow, 2003). Yet, the flux of sinking particles decreases exponentially with depth (Buesseler et al., 2007; Martin et al., 1987). A number of studies suggest the energy demands of the microbial community below the euphotic zone exceed the flux of sinking particulate organic matter (Herndl and Reinhaler, 2013; Swan et al., 2011; Verela et al., 2011; Reinhaler et al., 2010; Ingalls et al., 2006; Herndl et al., 2005) and suggest underestimation of sinking particle flux (Michaels et al., 1994; Buesseler, 1991), or microbial energy demands are overestimated by current methods.

Microbial metabolism can be classified based on the forms of energy (photo- or chemo-), reducing power (litho- or organo-), and carbon substrate (auto- or hetero-) utilized to support growth (**Table 1.1**). In the well-lit upper ocean, sunlight appears to be the major source of energy fueling photolithoautrophic growth by diverse groups of phytoplankton (Falkowski and Raven 2007; Raven, 1996). However, various methodological approaches have identified diverse groups of chemolithoautotrophs involved in carbon fixation in the dimly-lit meso- and bathypelagic waters (Herndl and Reinhaler, 2013; Middleberg, 2011; Hansman et al., 2009; DeLong et al., 2006; Ingalls

et al., 2006; Elsaïd and Naganuma, 2001). Many of these organisms appear mixotrophic, relying on both inorganic and organic substrates as sources of energy and carbon.

### *The North Pacific Subtropical Gyre*

The North Pacific Subtropical Gyre (NPSG) is the largest of the five gyres that together comprise ~40% of Earth's surface. The NPSG extends from approximately 15°N to 35°N latitude and 135°E to 135°W longitude, covers  $\sim 2 \times 10^7 \text{ km}^2$ , making it one of the largest continuous ecosystems on Earth (**Figure 1.1**; Karl and Lukas, 1996; Sverdrup et al., 1946). The prevailing anticyclonic circulation of the upper ocean within the NPSG maintains a persistently oligotrophic habitat, characterized by low nutrients and low biomass standing stocks. In 1988 the Hawaii Ocean Time-series (HOT) program established the field sampling site Station ALOHA (A Long-term Oligotrophic Habitat Assessment; 22.45°N 158°W) which is located in the deep ocean (~4,800 m depth) 100 kilometers north of the Hawaiian Island O'ahu (22.45°N 158°W). Members of the HOT program have sampled this location approximately monthly over the past 25+ years, with the primary goal of gaining a comprehensive understanding of temporal dynamics in biogeochemical and physical properties and processes in the ocean.

The repeated oceanographic measurements conducted at Station ALOHA over the last quarter century have been essential for the observation of cyclical (seasonal, annual) and non-cyclical (climate change) variations in the physics, chemistry and biology of the ocean that would otherwise be missed during stand-alone expeditions. The program's sustained sampling has provided improved understanding of the phenomena and characteristics that define the ocean water column in this region. This includes providing

time-resolved climatology of the vertical structure of key ocean properties like temperature, dissolved oxygen, and DIC (**Figure 1.2**). The anticyclonic circulation and associated Ekman downwelling results in a stable, deep thermocline (typically occurring at ~150 m depth) forming a mixing barrier between the warm, nutrient-poor upper ocean waters and the cooler, relatively nutrient-enriched but light-limited deep waters (Karl and Lukas, 1996). This physical structure partly maintains steep vertical gradients in DIC concentrations, with DIC increasing steadily from the surface ocean into the mesopelagic waters. This enrichment of DIC with depth is linked to the biological carbon pump, the collective set of processes that result in the vertical transport of photosynthetically fixed carbon downward, largely as particulate organic matter, and its subsequent remineralization in the dark ocean.

The HOT program's sampling effort has provided unique insight into microbial community dynamics occurring in the euphotic zone at Station ALOHA. HOT program measurements of carbon fixation based on the assimilation of  $^{14}\text{C}$ -bicarbonate reveals that a majority of the primary productivity occurs in the upper 75 m of the ocean (Church et al., 2013; Karl et al., 2002; Karl and Lukas, 1996; Letelier et al. 1996). The decrease of photosynthetic activity with depth is attributed to the exponential decrease of photosynthetically active radiation (PAR; **Figure 1.3**). Despite the oligotrophic nature of this ecosystem, the  $^{14}\text{C}$ -tracer approach suggests organic matter production (depth-integrated 0-175 m) at Station ALOHA ranges between 32.0 to 52.6 mmol C m $^{-2}$  day $^{-1}$ , with rates typically ~2 fold greater in the summer (**Figure 1.3**). Historically (1989-2000), the HOT program also measured rates of  $^{14}\text{C}$ -bicarbonate assimilation in dark bottles, with depth-integrated (0-175 m) rates ranging between 1.5 to 2.3 mmol C m $^{-2}$

day<sup>-1</sup>. Similar to <sup>14</sup>C-bicarbonate assimilation measured in the light, the dark rates typically increase in the summer months (**Figure 1.3**). Microbial assimilation of <sup>14</sup>C-bicarbonate in the dark presumably derives from diverse chemoautotrophic metabolisms or anaplerotic uptake of inorganic carbon by both autotrophic and heterotrophic organisms (Alonso-Saez et al., 2010; Sorokin, 1964).

During a 5 year study at Station ALOHA between 1988-1993, Karl et al. (1996) determined that the average carbon export to the deep sea was 6% of surface primary production (where primary production averaged 38.5 mmol C m<sup>-2</sup> day<sup>-1</sup> and the average particulate carbon (PC) flux observed at 150 m was 2.4 mmol C m<sup>-2</sup> day<sup>-1</sup>). Similarly, more recent measurements (2008-2012), reveal primary productivity averages 46.3 mmol C m<sup>-2</sup> day<sup>-1</sup> and PC export averages 1.7 mmol C m<sup>-2</sup> day<sup>-1</sup> at 150 m, implying particulate carbon export was equivalent to 4% of the contemporaneous <sup>14</sup>C-bicarbonate based productivity. Assuming sediment trap collections of sinking particles approximates the total material flux to the mesopelagic waters, and that waters advected into this region contain low concentrations of reduced material capable of supporting microbial growth, the downward particle flux should represent an upper limit on the metabolic activity that could occur in the dark ocean in this ecosystem.

Active and metabolically diverse microbial assemblages below the euphotic zone have been identified (DeLong et al., 2006; Herndl et al., 2005; Reinhaler et al., 2010; Swan et al., 2011), challenging earlier assertions that deep-sea microbial communities are slow growing (Jannasch and Wirsén, 1973), because the pool of organic matter in the dark ocean is dominated by refractory DOC (Hansell and Carlson, 1998; Barber, 1968). The metabolism of deep-sea microorganisms depends on the availability of both electron

donors (reduced substrates) and electron acceptors (oxidized substrates). Electron donors in the deep sea include, but are not limited to, organic matter (including CH<sub>4</sub>), ammonium, reduced sulfur substrates (including S<sub>2</sub>O<sub>3</sub><sup>2-</sup>, H<sub>2</sub>S, S<sup>0</sup> and S<sup>2-</sup>), and H<sub>2</sub> (Orcutt et al. 2011). Deep-sea hydrothermal vent communities are perhaps the most well known example of diverse and complex ecosystems supported by chemolithautotrophy. Aside from regions where geothermal input of these reduced compounds is important, delivery of reduced material, via gravitational settling of particulate matter, is the major source of electron donors fueling deep sea metabolism (Karl et al. 1984).

#### *Microbial carbon fixation in the dark pelagic sea*

Knowledge of the microbial community catalyzing carbon cycling in the dark ocean remains rudimentary. While prokaryotic plankton are recognized as an important component to biogeochemical cycling in the ocean's interior, their diversity, distribution, abundance, and contribution to carbon cycling remain largely unknown. Over the past decade, researchers have identified some of the major groups of microorganisms within the domains Archaea and Bacteria that are metabolically active in the ocean's mesopelagic and bathypelagic regions. Microautoradiography coupled with catalyzed reporter deposition fluorescent *in-situ* hybridization (Micro CARD-FISH) revealed that a considerable fraction of deep-sea prokaryotes are capable of assimilating radiolabeled amino acids (leucine) and bicarbonate (Herndl et al., 2005). In addition, <sup>14</sup>C-bicarbonate fixation and <sup>3</sup>H-leucine incorporation measurements by Reinhaler et al. (2010) suggested chemoautotrophic DIC fixation within the dark ocean serves as a source of autochthonously produced organic carbon and occurs at rates comparable to heterotrophic

production. Initially thought to live heterotrophically, abundant members of the phylum Thaumarchaeota have been found to use inorganic carbon, likely fueled in part by oxidation of ammonia (Reinthal et al., 2010; Ingalls et al., 2006; Könneke et al., 2005; Pearson et al., 2001). These nitrifying microorganisms account for ~20% of prokaryotic biomass in the sea (Karner et al., 2001) and thus appeared to account for a significant fraction of inorganic carbon fixation in the ocean's interior (Pearson et al., 2001; Herndl et al., 2005; Ingall et al., 2006). However, archaeal nitrification appears insufficient to solely support measured inorganic carbon fixation rates (Reinthal et al., 2010; Varela et al., 2011), suggesting additional metabolic strategies and energy sources must also contribute to a significant portion of dark carbon fixation.

There are five so-called ‘alternative’ carbon fixation pathways among major phylogenetic lineages that appear to contribute towards carbon cycling in the ocean (**Table 1.2**; Hugler and Sievert, 2011). The first is the reversal of the oxidative Krebs cycle (termed the rTCA cycle) that describes a pathway that permits various groups of bacteria to assimilate inorganic carbon during the formation of organic matter. This cycle may be limited to anaerobic or microaerophilic bacteria due to the oxygen sensitive enzymes, 2-oxoglutarate synthase and pyruvate synthase, involved. The second alternative carbon fixation pathway is the reductive acetyl-CoA pathway, which combines two molecules of CO<sub>2</sub> to acetyl-CoA in a noncyclic way via the key enzyme CO dehydrogenase/acetyl-CoA synthase. This pathway, also known as the Wood-Ljundhal cycle, is the only carbon fixation pathway identified in both bacteria and archaea (Hugler and Sievert, 2011; Berg et al. 2010). An additional carbon fixation pathway, the 3-hydroxypropionate (3-HP) cycle, describes two cycles that together form

pyruvate from three molecules of bicarbonate via the acetyl-CoA and propionyl-CoA carboxylase enzymes. So far, the complete genes for this cycle have only been identified in the green non-sulfur bacteria Chloroflexaceae (Hügler and Sievert, 2011). An additional pathway of carbon fixation is the 3-hydroxypropionate/4-hydroxybutyrate cycle. (3-HP/4HB). The first part of the 3-HP/4HB consists of the reaction sequence from acetyl-CoA to succinyl-CoA, but the second part regenerates acetyl-CoA from succinyl-CoA after forming 4-hydroxybutyrate as an intermediate (Hügler and Sievert, 2011). This is the carbon fixation pathway that appears to be used by ammonia oxidizing thaumarchaea (Hallam et al., 2006). Finally, the recently described dicarboxylate/4-hydroxybutyrate (DC/4-HB) cycle (Huber et al. 2008) involves enzymes from both the rTCA cycle and the latter part of the 3-HP/4-HB cycle, in addition to three other enzymes required for the conversion of acetyl-CoA to oxaloacetate (Hügler and Sievert, 2011).

#### *Ribulose-1, 5-Bisphosphate Carboxylase/Oxygenase*

The most prevalent and best characterized carbon fixation pathway is the Calvin-Benson-Bassham (CBB) cycle. The enzyme involved in the CBB cycle, ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO), is estimated to be the most abundant protein in the world (Watson and Tabita, 1997) and continues to be intensively studied since it was first described in the 1950's. Microbial assemblages in the dark pelagic possess the genetic capacity for RuBisCO synthesis, suggesting this pathway may be important to carbon cycling in the deep sea. RuBisCO catalyzes the assimilation of carbon dioxide ( $\text{CO}_2$ ) into the first step of the CBB reductive pentose phosphate cycle. The enzyme drives the reaction of  $\text{CO}_2$  with the five-carbon sugar ribulose-1, 5-

bisphosphate (*RuBP*), creating an unstable six-carbon compound which rapidly forms two molecules of D-phosphoglyceric acid (PGA) (Parry et al., 2003; Kellogg and Juliano, 1997). RuBisCO also catalyzes the combination of oxygen ( $O_2$ ) with *RuBP* instead of  $CO_2$  resulting in one molecule of each 3-phosphoglycerate and 2-phosphoglycolate, with three and two carbon atoms, respectively (Parry et al., 2003; Kellogg and Juliano, 1997; Watson and Tabita, 1997). This oxygenation reaction is known as photorespiration because  $CO_2$  is released by the breakdown of phosphoglycolate (Kellogg and Juliano, 1997) and is not productive in terms of carbon fixation. Because both the carboxylation and oxygenation reactions occur at the same active site,  $CO_2$  and  $O_2$  are competitive substrates for RuBisCO enzymatic activity. The oxygenation reaction may be favorable under elevated  $pO_2$ :  $pCO_2$  conditions (Keys, 1986) but overall the ability of RuBisCO to discriminate between  $CO_2$  and  $O_2$  depends on a specificity factor,  $\tau$  (Watson and Tabita, 1997).

All RuBisCO protein forms are unique in their structure, amino acid composition, and  $\tau$ . Form I is composed of eight large catalytic subunits, encoded by *rbcL* or *cbbL* genes, and eight small subunits, encoded by *rbcS* or *cbbS* genes (Pichard et al., 1997). The prefix “*cbb*” denotes Calvin Benson-Bassham pathway structural genes whereas “*rbc*” notes the lack of other structural genes of the CBB cycle for cyanobacteria (Tabita, 1999). Form I occurs in all eukaryotic algae and cyanobacteria and is the primary type found among oceanic phytoplankton (Paul et al., 2000). Forms IA and IC occur only in prokaryotes while forms IB and ID are also found in eukaryotes (Tabita 2004). *Prochlorochoccus* and some *Synechococcus* found in oligotrophic oceans contain form IA. Form IB is found in green algae, cyanobacteria, and higher plants (Paul et al., 2000).

The highest affinities for CO<sub>2</sub> are found amongst form IB, while the greatest  $\tau$  values (the ability to discriminate between CO<sub>2</sub> and O<sub>2</sub>) occur in eukaryotes containing form ID (Falkowski and Raven, 2007). Form ID is also found in the chromophytes, including diatoms, prymnesiophytes, cryptophytes, pelagophytes, and red algae (Paul et al., 2000). Unlike form I, isolated sequences from form II show very close identity preventing them from sub-classification (Tabita, 1999). Form II RuBisCO contains only 2 large subunits (L<sub>2</sub>) and is known to exist in diverse taxa of eubacteria ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -proteobacteria) and various dinoflagellates (Elsaied et al., 2002; Paul et al., 2000; Kellogg and Juliano, 1997). The specificity ( $\tau$ ) of form II for CO<sub>2</sub> is low, leading to a higher likelihood of catalyzing the oxygenase reaction and suspicion that form II is unable to sustain growth in aerobic environments (Lorimer et al., 1993). Additionally, Watson and Tabita (1997) suggest that form II is the most ancient of RuBisCO because it likely arose in an anaerobic environment similar to hypothesized conditions on pre-biotic Earth.

The occurrence of forms I and II has been documented in various chemoautotrophic bacteria, including the symbionts of invertebrates at hydrothermal vents (Tabita, 1999). RuBisCO form I or form II, encoded by *cbbL* and *cbbM* genes, respectively, are now known to occur in  $\delta$ - and  $\gamma$ -proteobacteria inhabiting the dark, oxygenated pelagic water column of the global ocean (Swan et al., 2011). Interestingly, both *cbbL* and *cbbM* have been described in the ubiquitous, but largely uncultivated members of the  $\delta$ -proteobacteria (specifically phylotypes in the SAR324 clade), while deep-sea  $\gamma$ -proteobacteria (including uncultivated phylotypes belonging to mostly uncultivated bacteria of the Agg47, ARCTIC96BD-19, and SUP05 rRNA gene clusters) contain only *cbbM* genes (Swan et al., 2011; Walsh et al., 2009).

Archaeal form III RuBisCO genes were reported by Tabita (1999) and have since been confirmed by others (Berg et al. 2010). Yet the role of this form III RuBisCO is unclear because the second enzyme of the Calvin cycle, phosphoribulokinase, is absent in Archaea (Berg et al., 2010; Finn and Tabita, 2003). There is also a form IV RuBisCO found in both bacteria and archaea that has been termed a “RuBisCO-like protein” or RLP and appears to be involved in sulfur metabolism (Berg et al. 2010; Tabita et al., 2008).

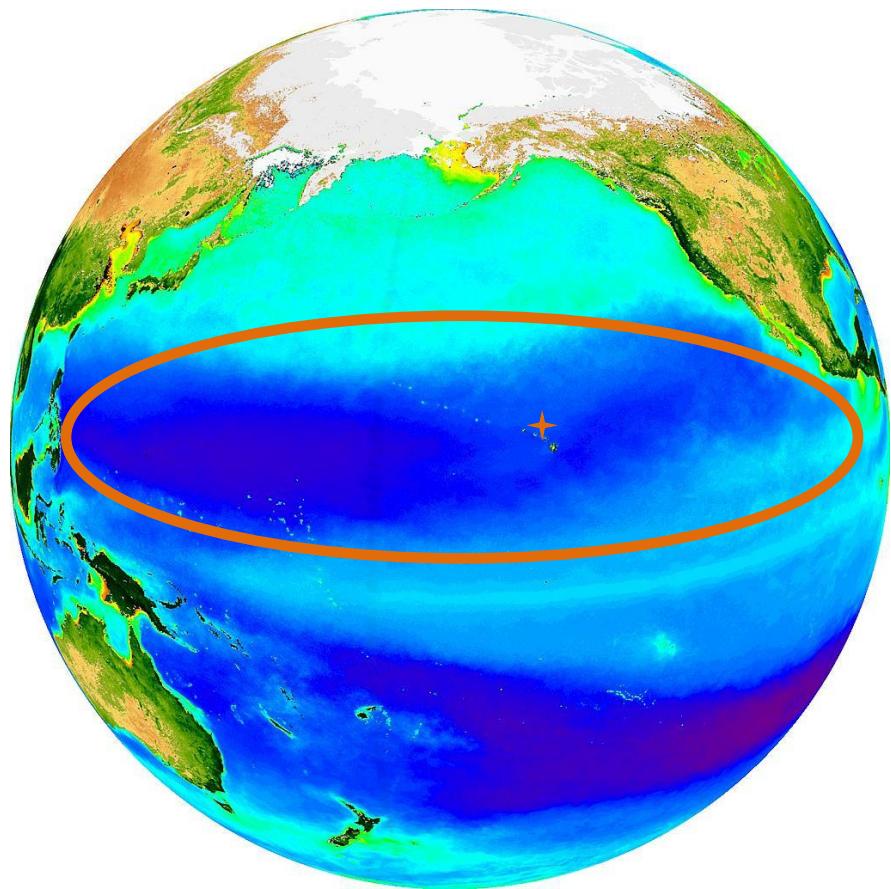
The widespread distribution of RuBisCO is evidence that it plays a critical role in marine ecosystems. It is found throughout the entire water column from the surface ocean to the seafloor. In the euphotic zone, transcriptional activity of form I RuBisCO is strongly correlated to diel patterns of photosynthetic activity (Pichard et al., 1997) and is responsible for at least 95% of carbon fixation by phytoplankton (Paul et al., 2000). The remaining <5% is attributable to non-RuBisCO carboxylases involved in the essential biosyntheses of purines and lipids (Raven, 1996). These photoautotrophic organisms utilize sunlight as an energy source to fix carbon. On the seafloor, chemolithoautotrophic, symbiotic bacteria of epifauna allow complex ecosystems to exist at deep-sea hydrothermal vents (Elsaied et al., 2007; Elsaied et al., 2002; Elsaied and Nagauma, 2001; Robinson et al., 1998). These chemoautotrophs use hydrothermal plume water enriched in reduced chemicals to fuel autotrophy. RuBisCO genes were also found to be actively expressed in the dimly lit regions of the euphotic zone (Pichard et al., 1997) and, more recently, present throughout the mesopelagic waters (Swan et al., 2011). Dark pelagic microbial communities are displaced from an immediate energy source (*i.e.* light or hydrothermal plume/seep water), and although plumes can extend laterally and

vertically for hundreds of kilometers (Orcutt et al., 2011) the volumetric contributions of these geothermal sources are likely too small to support significant microbial growth throughout the deep sea. As a consequence, autotrophy occurring in the dark ocean must ultimately be fueled by energy originally harvested from sunlight in the upper ocean and transported to the ocean's interior in the form of reduced substrates. Oxidation of reduced sulfur compounds, including S<sup>0</sup>, H<sub>2</sub>S<sup>-</sup>, S<sup>2-</sup>, and S<sub>2</sub>O<sub>3</sub><sup>2-</sup>, is common among ubiquitous groups of prokaryotes that rely on RuBisCO for CO<sub>2</sub> fixation (Swan et al., 2011). Single amplified genomes (SAGs) revealed that prominent, largely uncultivated members of the  $\delta$ - and  $\gamma$ -proteobacteria possess metabolic machinery that suggests that they use sulfur oxidation to supply the energy for carbon fixation via the CBB cycle. RuBisCO activity of these widespread proteobacteria may significantly contribute to carbon cycling and help fill the gap between carbon supply and demand in the dark ocean.

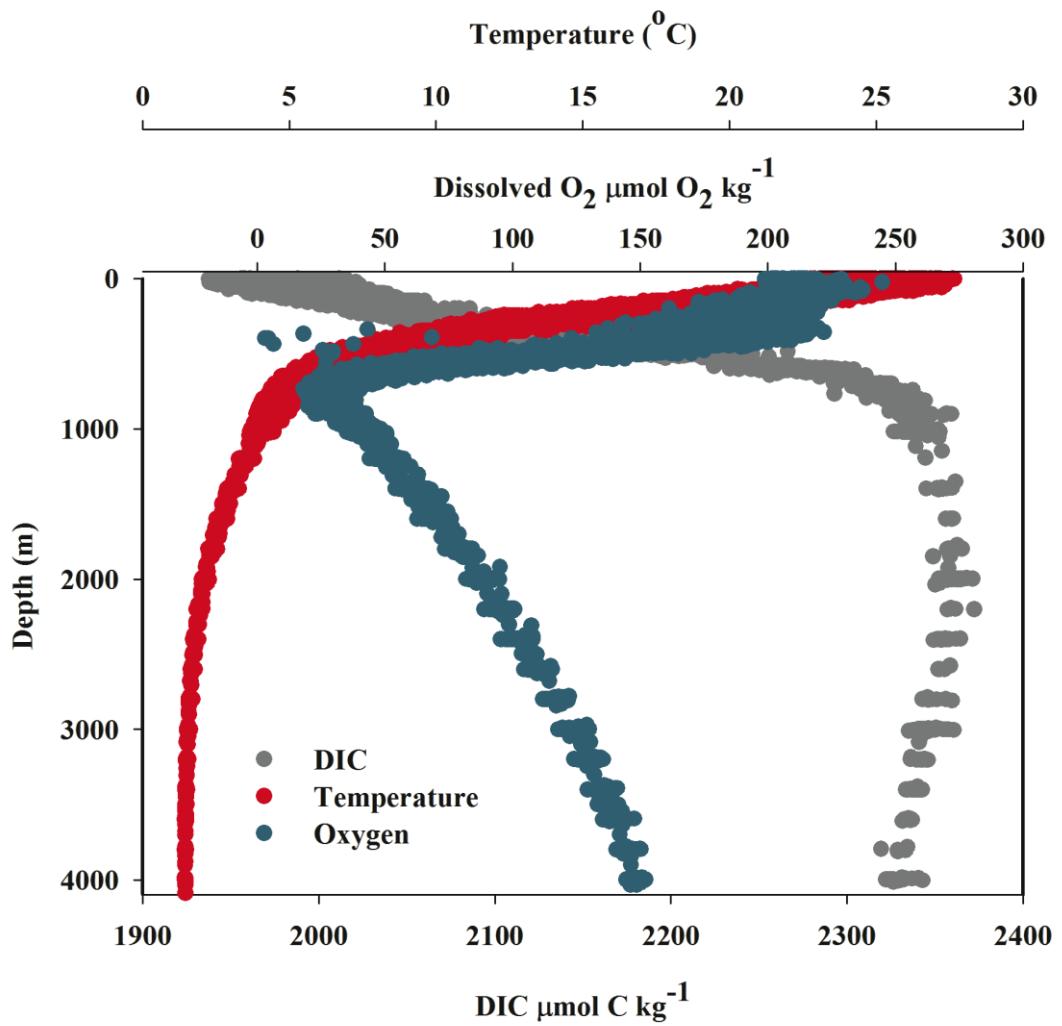
#### *Motivation for the research described herein*

I have investigated the diversity, abundance, and distribution of dark, mesopelagic chemoautotrophic bacterial lineages using RuBisCO genes as molecular markers. This research is timely because, although it is known that metabolically active microorganisms are abundant below the euphotic zone, these organisms remain poorly characterized. The discovery of RuBisCO genes occurring in SAGs that also contained novel sulfur oxidation genes provided insight into the physiologies of ubiquitous, but largely uncultivated proteobacterial lineages (Swan et al. 2011). However, it remains unclear whether there are additional, uncharacterized RuBisCO-containing microbes in the deep

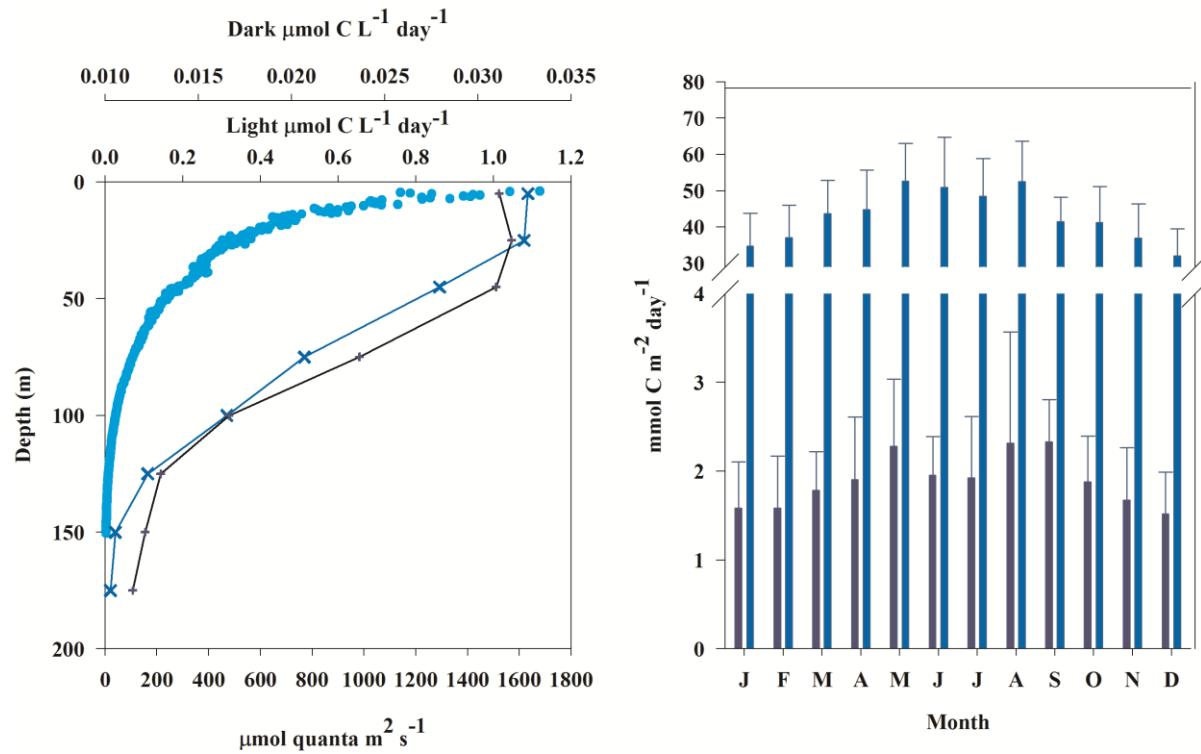
sea of the NPSG, and to date there is limited information available on the abundance or vertical distribution of these organisms. In addition to evaluating the diversity and distributions of these organisms, I sought to examine the role of reduced sulfur substrates in supporting growth and carbon fixation by microorganisms in the dark, pelagic waters of the NPSG. My work contributes to a growing body of information on the roles chemoautotrophic microorganisms have in carbon cycling in the ocean's interior.



**Figure 1.1.** Earth-orbiting satellite composite view of ocean color in the North Pacific Subtropical Gyre (NPSG) with star showing the approximate location of Station ALOHA. Satellite ocean color image from SeaWiFS Globe Design Tool ([http://oceancolor.gsfc.nasa.gov/cgi/biosphere\\_globes.pl](http://oceancolor.gsfc.nasa.gov/cgi/biosphere_globes.pl))



**Figure 1.2.** HOT program measurements (1988-2012) of temperature (red,  $^{\circ}\text{C}$ ), dissolved oxygen ( $\text{O}_2$ ) and dissolved inorganic carbon (DIC; cyan and gray, respectively,  $\mu\text{mol kg}^{-1}$ ) at Station ALOHA.



**Figure 1.3.** Vertical profiles of carbon fixation measured in the light (light blue) and in the dark (dark blue) at Station ALOHA (1988–2012). Photosynthetically active radiation (PAR; light blue circles) and rates of carbon fixation in both the light and in the dark based  $^{14}\text{C}$ -bicarbonate assimilation (left). Monthly binned, depth integrated (0–175 m) rates of  $^{14}\text{C}$ -bicarbonate assimilation in the light and dark (right) - note the scale break.

**Table 1.1.** Classification of microbial metabolisms (electron donor examples adapted from Karl, 1995).

Classification	Energy	Electrons	Carbon	Example organisms
Photolithoautotroph	Light	inorganic $\text{H}_2\text{O}$ , $\text{H}_2\text{S}$ , $\text{S}^0$ , $\text{H}_2$	inorganic	Cyanobacteria ( <i>Prochlorococcus</i> spp.)
Photoorganoheterotroph	Light	organic $\text{CH}_2\text{O}$	organic	Erythrobacter ( <i>Erythrobacter longus</i> )
Chemolithoautotroph	Chemical	inorganic $\text{H}_2$ , $\text{H}_2\text{S}$ , $\text{S}_2\text{O}_3^{2-}$ , $\text{S}^0$ , $\text{NH}_4^+$ , $\text{Fe}^{2+}$	inorganic	Thaumarchaea ( <i>Nitrosopumilus maritimus</i> )
Chemolithoheterotroph	Chemical	inorganic $\text{H}_2$ , $\text{H}_2\text{S}$ , $\text{S}_2\text{O}_3^{2-}$ , $\text{S}^0$ , $\text{NH}_4^+$ , $\text{Fe}^{2+}$	organic	Roseobacteria ( <i>Silicibacter pomeroyi</i> )
Chemoorganoheterotroph	Chemical	organic $\text{CH}_2\text{O}$	organic	Rickettsiales ( <i>Pelagibacter ubique</i> ; SAR11)

**Table 1.2. Pathways for carbon fixation and occurrences in major prokaryote phylogenetic lineages.**

Pathway	Abbreviation	Occurrence in major phylogenetic lineages	
		Bacteria	Archaea
<b>Calvin-Benson-Bassham cycle</b>	CBB	Cyanobacteria Chloroflexi Firmicutes $\gamma$ -, $\beta$ -, and $\alpha$ -proteobacteria	
<b>Reductive Tricarboxylic Acid cycle</b>	rTCA	$\gamma$ -, $\alpha$ -, and $\delta$ -, $\varepsilon$ -proteobacteria Nitrospirae Aquificales Chlorobiales	
<b>Reductive acetyl-CoA/ Wood-Ljundahl pathway</b>	WL	$\delta$ -proteobacteria Spirochaetes Firmicutes Planctomycetes	Euryarchaeota
<b>3-Hydroxypropionate bicycle</b>	3-HP	Chloroflexi	
<b>3-Hydroxypropionate/4-Hydroxybutyrate cycle</b>	3-HP/4-HB		Thaumarchaeota and Crenarchaeota
<b>Dicarboxylate/ 4-Hydroxybutyrate cycle</b>	DC-4HB		Crenarchaeota

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## Chapter 2

### Introduction

Carbon cycling in the global ocean is dynamic, catalyzed by countless organisms through diverse metabolic pathways and fueled by numerous energy sources. In the surface ocean, phytoplankton reduce carbon dioxide ( $\text{CO}_2$ ) to organic matter using chemical energy harvested from the sun via photosynthesis. A significant portion of primary production is respiration back to  $\text{CO}_2$  within the euphotic zone, with <20% of the organic matter sinking to the dark ocean in the form of particulate organic matter (POM; [Karl et al., 2011](#)). In addition, dissolved organic matter (DOM) is delivered to the deep sea through vertical mixing and overturn of the water column ([Carlson et al., 1994](#)). Microorganisms inhabiting the deep sea comprise the largest pool of living biomass in the ocean, and their active growth serves as a major pathway for both organic matter remineralization and biogenic carbon production ([Whitman et al., 1998](#)). The delivery of organic matter in the form of sinking particles is the major pathway fueling the metabolic demands of deep-sea organisms ([Karl et al., 1984](#)). Subsequent remineralization of this sinking organic carbon maintains elevated concentrations of dissolved inorganic carbon (DIC) in the ocean's interior. Collectively these processes are termed the biological carbon pump, which ultimately moves atmospheric  $\text{CO}_2$  to the deep sea through biological processes.

The global ocean contains almost as much dissolved organic carbon (DOC) as  $\text{CO}_2$  in the atmosphere (~750 Pg C), with the total oceanic DOC inventory estimated to be ~662 Pg C ([Hansell et al., 2009](#)). Deep sea DOC concentrations range from 34-50

$\mu\text{mol C kg}^{-1}$  (Hansell et al., 2009), approximately half the concentration seen in the surface ocean, yet deep sea DOC inventories comprise significant carbon reservoirs because of the vast volume below the base of the euphotic zone. Total inventories of DOC in the surface ocean (0-200 m) are estimated to be ~47 Pg C, with an additional ~138 Pg C found throughout the mesopelagic (200-1000 m) and ~477 Pg C throughout bathypelagic waters (4000+ m; Hansell et al., 2009). DOC becomes increasingly refractory with depth, as reflected by its increasing age, with bulk pools estimated to be between 4,000-6,500 years old (Hansman et al., 2009; Williams and Druffel, 1987). Hence, despite its large size, bulk pools of DOC appear largely unreactive on biologically meaningful time scales and thus do not appear to sustain a significant fraction of microbial metabolism in the deep sea. Both suspended and sinking POM appear younger than bulk pools of DOM, as reflected by nutrient stoichiometry and radiocarbon ages, and hence POM pools often appear to have greater nutritional value than DOC (Williams and Druffel, 1987). Consistent with these results, a study on the relationship between DOC and apparent oxygen utilization indicated that turnover of DOM supported ~10% of microbial respiration in the mesopelagic (Aristegui et al., 2002). Comparisons between DOC and POM provide strong evidence that the POM flux supports the bulk of dark ocean microbial respiration. Consistent with such results, studies have shown that heterotrophic biomass production follows the attenuation of particle flux with depth, suggesting a balance between carbon supply and demand in the ocean's interior (Anita et al., 2001; Nagata et al., 2000).

In contrast, some studies have concluded that microbial carbon demand, the sum of heterotrophic biomass production and respiration, is greater than that supplied from

particle flux, and propose ‘primary production’ via chemoautotrophy as a source of new organic carbon in the deep sea (Herndl et al., 2008; Reinthal et al., 2006; Herndl et al., 2005). However, the carbon budget can not simply be balanced by *in-situ* chemoautotrophic activity if energy fueling carbon fixation below the euphotic zone ultimately originates from photosynthetic production. The potential mismatch between microbial carbon and energy supply and demand suggests that current measurements of particle flux underestimate the organic matter sinking to the dark ocean (Buesseler, 1991) and/or deep sea metabolic demands are overestimated by current methods.

Evidence is accumulating that the dark ocean harbors a diverse community of metabolically active prokaryotes. In particular, both isotopic and genetic methodologies have provided new evidence that microorganisms in the ocean’s interior waters rely on diverse metabolic strategies for growth, notably including obligate or facultative chemoautotrophy. Based on stable carbon ( $\delta^{13}\text{C}$ ) isotopic measurements of archaeal membrane lipids, the marine archaea were hypothesized as potential chemoautotrophs (Ingalls et al., 2006; Pearson et al., 2001; Schouten et al., 1998; Hoefs et al., 1997). Shortly after, the autotrophic capacity within Archaea was confirmed by incubation studies using stable- and radio-labeled inorganic carbon (Varela et al., 2011; Herndl et al., 2005; Wutcher et al., 2003), which demonstrated the assimilation of inorganic carbon into biomass. More recently, numerous and diverse bacterial assemblages in the meso- and bathypelagic waters were reported to rely on chemoautotrophic metabolisms and they appear to be functionally important components of dark ocean biogeochemistry (Swan et al., 2011; Herndl et al., 2005). Despite these advances, knowledge of the specific

reduced compounds utilized as energy sources and the metabolic pathways used by dark ocean autotrophic prokaryotes remains rudimentary.

Investigating the contribution of deep-sea microbial metabolisms to carbon cycling is essential for a comprehensive understanding of carbon transformations in the dark ocean. Various cultivation-independent methodologies, mostly focused on rRNA gene analyses, provided tools for classifying the diversity of microorganisms dwelling in the aphotic zone of various oceans (Giovannoni et al. 1996; Field et al. 1997; DeLong et al., 1989; DeLong 1993; Fuhrman et al. 1993; Amann et al., 1995). These initial studies revealed that members of the deep-sea community that are phylogenetically related to diverse groups within the domains Bacteria (including Bacteroidetes, Marine Group A, and  $\alpha$ -,  $\gamma$ -,  $\delta$ -, and  $\varepsilon$ - proteobacteria) and Archaea (including Thaumarchaeota, Crenarchaeota, and Euryarchaeota). These molecular approaches provided insight into the evolutionary relationships among marine microbes and enabled the use of tools for quantifying abundances and growth of some of these major lineages. For example, using MICRO-CARD-FISH (microradiography combined with catalyze reporter deposition, fluorescence in situ hybridization) Herndl et al. (2005) showed that while the abundance of archaea, specifically members of the Thaumarchaeota, was consistently greater than bacteria, the growth rates of bacteria were often greater in the meso- and bathypelagic waters of the Atlantic Ocean based on  $^3\text{H}$ -leucine incorporation. This suggests that substantial contributions to carbon cycling can be made by groups of bacteria that are not numerically dominant in the dark ocean.

More recently, advancements in molecular techniques have provided new insights into previously unrecognized forms of microbial metabolisms utilized by deep sea

organisms, and many of these studies have found evidence that oceanic cycles of carbon and sulfur are tightly coupled through microbial metabolism. The redox potential of sulfur (ranging from -2 in sulfide to +6 in sulfate) makes sulfur-containing compounds potentially important in biogeochemical processes. Microbial sulfur oxidation and reduction reactions appear to be widespread in the deep sea, with sulfate reduction a major process driving organic matter decomposition in marine sediments and low oxygen pelagic waters, and sulfur-oxidation important throughout the meso- and bathypelagic waters of oxygenated ocean systems and at hydrothermal vents. In such environments, sulfur redox reactions appear to factor prominently into the framework of microbial metabolism. Recent creation of single amplified genomes (SAGs) from individual cells collected in the North Atlantic and Pacific Oceans and isolated by flow cytometry revealed that ubiquitous groups of  $\gamma$ - and  $\delta$ -proteobacteria in the dark ocean contain the metabolic machinery needed for chemolithoautotrophy. Specifically, SAGs from uncultivated members of  $\delta$ -proteobacteria, previously characterized based on amplification of 16S rRNA gene sequences from the mesopelagic waters in the Sargasso Sea (ribotypes were termed SAR324; [Wright et al. 1997](#)), contained functional genes for both carbon fixation and sulfur oxidation ([Swan et al. 2011](#)). Previous studies have determined that members of the SAR324 clade of  $\delta$ -proteobacteria are widely distributed throughout the dark oxygenated water column of the global ocean ([DeLong et al., 2006](#); [Morris et al., 2005](#); [Wright et al., 1997](#)).

Similarly, the Swan et al. (2011) SAG analyses identified diverse members of  $\gamma$ -proteobacteria, including ribotypes previously identified only through 16S rRNA genes (sequence-types termed ARCTIC96BD-19 and Agg47) that also possessed the genetic

capacity to grow as sulfur oxidizing chemoautotrophs (Swan et al., 2011). The ARCTIC96BD-19 was first described from a sample collected from below the mixed layer in the Arctic Ocean, while AGG47 was first described as an aggregate associated bacterium (Bano and Hollibaugh, 2002; DeLong et al., 1993). The ARCTIC96BD-19 and AGG47 clusters are phylogenetically closely related to a cluster of  $\gamma$ -proteobacteria termed SUP05 (Swan et al., 2011).

Members of the  $\gamma$ -proteobacteria belonging to the SUP05 clade, named after the Suiyo seamount plume where these 16S rRNA gene sequences were first retrieved, have most often been described from gene surveys originating from anoxic or oxygen-deficient environments (Sunamura et al. 2004; Walsh et al., 2009; Canfield et al. 2010). However, members of the SUP05 clade have recently been isolated from surface waters of Puget Sound and the deep chlorophyll maximum in the North Pacific by Marshall and Morris (2013). The authors' laboratory studies confirmed that this isolate has the potential to oxidize sulfur by identifying  $S^0$  in extracellular globules and based on detection of *aprA* genes. The authors suggest this isolate may live heterotrophically, with highest observed growth rates in the presence of thiosulfate and glucose; notably carbon fixation genes (*cbbM*) could not be amplified from this organism using previously published PCR primers (Marshall and Morris, 2013). A recent study conducted in the oxygen minimum zone waters of the Chilean upwelling region revealed a seasonal bacterial community including the SUP05 clade within the oxygen minimum zone capable of both oxidative and reductive sulfur cycling (Canfield et al. 2010). This cycling of sulfur within the OMZ was termed cryptic because there was no *in-situ* chemical expression of microbial sulfur utilization.

Some of these  $\gamma$ -proteobacteria appear distantly related to sulfur oxidizing bacterial endosymbionts sampled from hydrothermal vents or cold seeps (Wright et al. 1997; Suzuki et al. 2004). These  $\gamma$ -proteobacteria belonging to the ARCTIC96BD-19, AGG47, and SUP05 clades may represent important components of the dark pelagic ocean's microbial assemblages. In addition to their presence in oxygen minimum or deficient zones (Glaubitz et al., 2013; Anantharaman et al., 2012; Walsh et al., 2009; Steven and Ulloa, 2008; Suzuki et al., 2004), two of these clades, ARCTIC96BD-19 and AGG47, have been described as abundant members of the bacterioplankton in the Arctic Ocean (Bano and Hollibaugh 2002) and Monterey Bay (Suzuki et al. 2004). More recently, all three of these  $\gamma$ -proteobacteria clades have been reported in oxygenated waters (Marshall and Morris, 2013; Swan et al., 2011) suggesting these organisms may be more widespread than previously thought. Given their potential for carbon and sulfur cycling, these organisms may be important players in biogeochemistry in the dark ocean.

For my Master's thesis research, I investigated the distributions, abundances, and activities of chemoautotrophic bacterial lineages at Station ALOHA in the North Pacific Subtropical Gyre. The work relied on two complementary approaches: 1) targeted studies on the vertical distributions and abundances of genes encoding the large subunit of the RuBisCO protein among two distinct groups of chemoautotrophs; and 2) experimental evaluation of the influence of a reduced sulfur substrate (specifically thiosulfate,  $S_2O_3^{2-}$ ) on rates of biomass production and growth of chemoautotrophic microorganisms. To date, there is limited information available on the abundance or vertical distribution of these organisms. In addition, through substrate perturbation experiments, I sought to examine the role of reduced sulfur substrates in supporting

growth and carbon fixation by microorganisms in the dark, pelagic waters of the subtropical North Pacific Ocean. This work contributes to a growing body of information on the roles chemoautotrophic microorganisms have in carbon cycling in the ocean's interior.

## Methods

Sampling for this project relied on several oceanographic research cruises in the NPSG where I collected samples and conducted field-based experiments. These cruises were organized and led by the Hawai'i Ocean Time-series program (HOT) and the Center for Microbial Oceanography; Research and Education (C-MORE). A central objective of the project was to characterize the diversity of microorganisms dwelling in aphotic waters that contain form II RuBisCO by amplifying and sequencing the *cbbM* genes from plankton community DNA extracts. Using quantitative PCR (qPCR), I also examined the vertical distributions of two phylogenetically distinct groups of proteobacteria (members of the  $\delta$  and  $\gamma$ ) that had been previously identified as possessing functional genes involved in carbon fixation and sulfur oxidation (Swan et al. 2011). I then used qPCR to investigate the responses of these microorganisms to additions of  $S_2O_3^{2-}$  in seawater incubation experiments. During these experiments, seawater was subsampled for measurements of radiolabeled  $^{14}C$ -bicarbonate assimilation and  $^3H$ -leucine incorporation rates, and samples were collected for subsequent extraction of planktonic nucleic acids. Together, these analyses provided insight into carbon cycling and possible sources of energy supporting carbon fixation in the dark pelagic ocean.

### *Nucleic acid collection and extraction*

Seawater was collected aboard the University of Hawai'i research vessel R/V *Kilo Moana* on HOT cruises in June, 2012 (HOT 243); June, 2013 (HOT 253); and October, 2013 (HOT 256) from discrete depths using rosette sampler equipped with 24 twelve-liter

sampling bottles (Karl and Lukas 1996) and a suite of *in situ* sensors for characterizing the hydrography and biogeochemistry of the water. Seawater was subsampled from the rosette bottles into acid-washed polycarbonate bottles. Two to five liters were filtered using a peristaltic pump onto inline, 25 mm diameter 0.2 µm pore size Supor ® filters and placed into 2-mL Sarstedt microcentrifuge tubes containing appropriate buffers. Filters for subsequent DNA extraction were immersed in 400 µL buffer AP1 (Qiagen Plant kit). Approximately 0.2 g of zircon beads were added to microcentrifuge tubes containing DNA buffer. Immediately following filtration, filters were flash frozen in liquid nitrogen and stored at -80 °C until processed in the shore-based laboratory.

In the laboratory, cells caught on filters were mechanically and chemically disrupted as part of the appropriate nucleic acid extraction protocol. DNA was extracted following a modified version of the Qiagen DNeasy Plant kit, as described in Paerl et al. (2008). Briefly, cells caught on filters were freeze/thawed in lysis buffer then homogenized via bead beating. DNA was then purified by the addition of RNase A before proteins and polysaccharides were precipitated by the addition of acetic acid. Samples were then centrifuged and eluted following the manufacturer specification, including the binding of DNA to DNeasy minicolumns with buffer containing ethanol. DNA concentrations were quantified flourometrically using the Quant-iT DNA High-sensitivity assay kits (Invitrogen, Carlsbad, CA, USA).

#### *PCR conditions and cloning*

RuBisCO form II *cbbM* genes were amplified from the resulting DNA extracts by polymerase chain reaction (PCR) using oligonucleotide primers described by Campbell

and Cary (2004) and Swan et al. (2011; **Table 2.1**). The PCR master mixes were prepared to a final concentration of 1x AmpliTaq Gold 360 Buffer, 3.0 mM MgCl<sub>2</sub>, 200 μM dNTPs each, 1.0 μM forward primer, 1.0 μM reverse primer, and 1.25 units AmpliTaq Gold® (Applied Biosystems ®). Two microliters of DNA template was added to each reaction for a total volume of 50 μL. Thermal cycling conditions were: 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 30 seconds, 45 °C for 1 minute, 72 °C for 2 minutes; with a final extension at 72 °C for 15 minutes.

The PCR products were visualized on a 1% agarose gel stained with SYBR® Safe DNA Gel Stain (Life Technologies) under blue light, and the correct size PCR product was excised and purified using a Qiagen® QIAquick Gel Purification kit. Amplicons were ligated into either an Invitrogen PCR-TOPO, Lucigen CloneSMART, Promega pGEM-TR Easy, or Qiagen PCR Cloning<sup>Plus</sup> vectors and transformed into *Escherichia coli* JM109 competent cells according to the manufacturer's manual. Clones were sent for sequencing at the Advanced Studies in Genomics, Proteomics and Bioinformatics facility located on the University of Hawai'i at Manoa campus.

Nucleic acid sequences obtained from clone libraries were imported into Geneious® where they were edited, trimmed, and aligned using the Geneious alignment tools. Aligned DNA sequences were then imported into ARB for subsequent construction of phylogenetic distance trees.

#### *Quantitative PCR primers, thermocycling conditions, and quantification*

Two SYBR Green-based quantitative PCR (qPCR) assays targeting *cbbM* genes were adopted from Swan et al. (2011). The first assay targeted specific γ-proteobacterial

*cbbM* genes using the primer pair Plank1cbbM65F and Plank1cbbM269R with an annealing temperature 59 °C, while the second assay targeted specific δ-proteobacterial *cbbM* genes using the primers Plank2cbbM68f and Plank2cbbM270r and an annealing temperature at 57°C (**Table 2.1**). Reactions contained 1X KAPA SYBR Fast buffer, 0.2 μM of each primer, 3X 10 mg/ mL bovine serum albumin, and 2 μL DNA template for a total reaction volume of 20 μL. Thermocycling conditions included 2 minutes at 50 °C, followed by a denaturing step at 95 °C for 2 minutes before 40 cycles of 95 °C for 15 seconds and annealing for 1 minute.

The qPCR reactions were analyzed using an Eppendorf Mastercycler® ep RealPlex instrument. Samples were quantified against 10-fold dilutions of plasmid standards containing the appropriate PCR amplified and cloned gene fragment. Gene copies within these plasmids were quantified as;

$$\text{Gene copies}/\mu\text{l} = (\bar{A} * \text{grams DNA}/\mu\text{l}) / (\text{plasmid length} * 660)$$

where  $\bar{A}$  is Avogadro's number ( $6.022 \times 10^{23}$  gene copies  $\text{mol}^{-1}$ ), grams of DNA is per  $\mu\text{l}$  plasmid plus insert as determined fluorometrically on a Qubit® 2.0 (Life Technologies), plasmid length refers to the base pair length of the cloning vector plus the amplified insert, and assuming 660 is the molecular weight ( $\text{g mol}^{-1}$ ) of a nucleic acid base pair. A 10-fold dilution of each nucleic acid sample was also analyzed to test potential qPCR inhibition as suggested by Bustin et al. (2009).

The efficiency of qPCR reactions was calculated via the following;

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

### *Thiosulfate enrichment incubations*

In June 2012 and October 2013, seawater was collected from 200 m during HOT program cruises to Station ALOHA. Water was subsampled from the Niskin rosette bottles and transferred into 6 acid-washed 20 L polycarbonate carboys. Carboys were covered with black plastic sheeting to minimize light perturbation and incubated in the dark at *in situ* temperatures using indoor incubators. To initiate the experiments, three replicate carboys received an addition of 5  $\mu$ M sulfur (final concentration) as sodium thiosulfate. The remaining three replicate carboys were left unamended to serve as controls. All carboys were subsampled every three days for rates of bacterial production (derived from measurements of  $^3$ H-leucine incorporation), carbon fixation (based on  $^{14}$ C-sodium bicarbonate assimilation), and seawater was filtered for subsequent extraction of plankton nucleic acids. During the June 2012 HOT cruise (HOT 243), the control and  $S_2O_3^{2-}$  treatment carboys were treated as true replicates, such that only one sample for  $^{14}$ C-bicarbonate and  $^3$ H-leucine was collected from each carboy (no analytical replication). During the October 2013 HOT cruise (HOT 256), analytical triplicates for  $^{14}$ C-sodium bicarbonate and  $^3$ H-leucine were collected from each carboy. Approximately one liter of seawater was filtered for each nucleic acid sample at each time point for both HOT cruises. Nucleic acid samples were then analyzed as described above. Details of the sampling and processing scheme for the  $^{14}$ C-bicarbonate and  $^3$ H-leucine rate measurements are described below.

### *<sup>3</sup>H-leucine incorporation rates*

Seawater (1.5 mL) was subsampled from each carboy during S<sub>2</sub>O<sub>3</sub><sup>2-</sup> enrichment incubations into 2.0 mL Axygen microcentrifuge tubes. A final concentration of 20 nM radiolabeled <sup>3</sup>H-leucine was added to triplicate 1.5 mL seawater samples and a blank (tubes contained 100 µl 100% Trichloroacetic Acid, TCA). Samples were placed in the same dark incubator containing the carboys for 3 to 12 hours before triplicates were killed with the addition of 100 µl 100% TCA. Samples were then frozen and stored at -20 °C until processed following the microcentrifuge method ([Smith and Azam, 1992](#)). Upon thawing, samples were centrifuged for 15 minutes at 25,000 x g at 4 °C. Seawater was then decanted, 1 mL of ice cold 5% TCA was added, and tubes were centrifuged at 25,000 x g for 5 minutes. The TCA was decanted and the lip of each tube was dabbed on a dry paper towel, followed by the addition of 1 mL ice cold 80% ethanol. Tubes were spun for an additional 5 minutes at 25,000 x g. Ethanol was decanted, the lip of each tube was dried, and then samples were left to vent uncapped overnight in a fume hood. The next day 1 mL of PerkinElmer UltimaGold™ scintillation cocktail was added to each vial before samples were vigorously vortexed and analyzed on a Perkin Elmer Tri-Carb 2800TR liquid scintillation analyzer. The activity of the blank tubes was subtracted from the appropriate triplicates, and a conversion factor of 1.5 kg C per mol leucine incorporated ([Kirchman, 2001](#)) was utilized to derive a final rate of bacterial production in units of nmol C L<sup>-1</sup> day<sup>-1</sup>.

### *<sup>14</sup>C-bicarbonate assimilation*

Rates of carbon fixation were derived from measurements of <sup>14</sup>C-bicarbonate assimilation (Steeman-Nielsen, 1952). Seawater was subsampled from each carboy during thiosulfate enrichment experiments into 250 mL ( $V_{\text{filtered}}$ ) polycarbonate bottles. A 500  $\mu\text{L}$  aliquot of 200  $\mu\text{Ci m}^{-1}$  radiolabeled <sup>14</sup>C-sodium bicarbonate was added to the seawater and incubated between 12 and 24 hours at *in situ* temperature in the dark. After incubation, 250  $\mu\text{L}$  ( $V_{\text{specific activity}}$ ) from each subsample was added to a 20 mL glass scintillation vial containing 500  $\mu\text{L}$   $\beta$ -phenylethlyamine to determine each subsample's specific activity ( $^{14}\text{C}_{\text{specific activity}}$ ). The remaining seawater was gently vacuum filtered onto 25 mm diameter 0.2  $\mu\text{m}$  polycarbonate filters and placed in an empty 20 mL glass scintillation vial. Sample vials were stored at -20 °C until processing. Upon processing, filters were acidified by the addition of 1 mL 2N Hydrochloric acid (HCl) and allowed to vent, uncapped for 12-24 hours in a fume hood. After acidification, the <sup>14</sup>C activity remaining on the filters was assumed to represent the radiolabeled bicarbonate that had been assimilated into organic carbon by the microbial community ( $^{14}\text{C}_{\text{filtered}}$ ). The resulting radioactivity in the samples was measured by liquid scintillation; 10 mL PerkinElmer UltimaGold™ scintillation cocktail was added to the filter and specific activity vials and radioactivity was analyzed on a Perkin Elmer Tri-Carb 2800TR liquid scintillation analyzer. Carbon assimilation rates were calculated as follows;

$$\text{C assimilation} = \left\{ \left( \frac{^{14}\text{C}_{\text{filtered}}}{V_{\text{filtered}}} \right) / \left( \frac{^{14}\text{C}_{\text{specific activity}}}{V_{\text{specific activity}}} \right) \right\} * [\text{DIC}] * 12.011 * 1.06$$

Where [DIC] is the concentration of dissolved inorganic carbon at 200 m during the June 2012 cruise as measured by the HOT program (2057  $\mu\text{mol kg}^{-1}$ ), 12.011 is the

molecular weight of carbon ( $\text{g mol}^{-1}$ ), and 1.06 is the  $^{14}\text{C}/^{12}\text{C}$  isotopic fractionation factor as determined by Steeman-Nielsen (1952).

#### *Analytical omitting criteria*

Triplet measurements were collected for  $^3\text{H}$ -leucine and  $^{14}\text{C}$ -bicarbonate analyses. Variability among the triplicate measurements was on occasion large (coefficient of variation  $>49\%$ ); hence I followed the following criteria for omitting measurements believed to be erroneous due to analytical artifacts: If a rate measurement from one of the triplicate samples was more than twice the value of the other two samples from that triplicate, then the value furthest from the median was omitted. This was strictly followed unless the sample was consistently omitted for three or more time points, in which case it was determined that the observed variability was not analytical, but rather the result of true variability between triplicates and the measurements were included in the analyses. In total  $<15\%$  of the measurements were omitted from subsequent analyses.

**Table 2.1.** Polymerase Chain Reaction (PCR) and quantitative PCR primer sequences and details.

Primer	Sequence (5'- 3')	Annealing temp. (C)	Product size (bp)	Target organisms	Reference
<b>PCR</b>					
cbbM_F	TTCTGGCTGGGBGGHGAYTTYATYAARAAYGACGA	45	~330	Form II RuBisCO bacterial diversity	Campbell and Cary, 2004
cbbM_R	CCGTGRCCRGCVCGRTGGTARTG				
cbbMF	ATCATCAARCCSAARCTSGGCCTGCGTCCC	45	~400	Form II RuBisCO bacterial diversity	Swan et al., 2011
cbbMRb	AGAAGTTACTGCACCATGCCAGCTCTATG				
<b>qPCR</b>					
Plank1cbbM65f	CACAAGGAAATCAAGTATATGCAAG	59	~200	$\gamma$ -proteobacteria containing <i>cbbM</i>	Swan et al., 2011
Plank1cbbM269r	TCAACTAGKAAGGCAACATGGTG				
Plank2cbbM68f	AAGGTAATCAAATTGGGTCC	57	~200	$\delta$ -proteobacteria containing <i>cbbM</i>	Swan et al., 2011
Plank2cbbM270r	ATCAACAAAGAAATGCAATGTTTC				

## Results

### *Habitat variability at Station ALOHA*

I examined the vertical distributions of selected groups of chemoautotrophic bacteria throughout the epi-, meso-, and bathypelagic waters (0-4000 m) at Station ALOHA based on qPCR amplification of specific *cbbM* genes. In addition to examining the vertical distributions of chemoautotrophs, I sought to examine the response in carbon fixation and bacterial production to additions of  $\text{S}_2\text{O}_3^{2-}$ , a reduced sulfur substrate hypothesized to serve as an energy source supporting chemoautotrophic growth in the sea (Swan et al. 2011). Previous investigations into chemoautotrophic microbial growth suggest such organisms are most abundant and active in the mesopelagic waters (200-1000 m); hence,  $\text{S}_2\text{O}_3^{2-}$  manipulation experiments targeted the upper mesopelagic waters (200 m).

Mixed-layer temperatures at Station ALOHA typically vary between 22.3 to 27.4 °C, with peak temperatures occurring in the late summer (August-September), and lowest temperatures in the late winter (February-March). Below the mixed layer, temperatures decrease rapidly through the main thermocline, with temperatures in the upper mesopelagic waters (200 m) varying between 15.2 to 21.6 °C, decreasing ~4 °C near the base of the mesopelagic zone (1000 m). Temperatures continue to decline gradually through the bathypelagic waters, decreasing to ~1.4 °C at 4000 m. Upper ocean (<200 m) concentrations of dissolved  $\text{O}_2$  vary from ~160 to 245  $\mu\text{mol O}_2 \text{ kg}^{-1}$  in the upper ocean (<200 m), decreasing to <40  $\mu\text{mol O}_2 \text{ kg}^{-1}$  in the mid-mesopelagic, and then increasing to ~150  $\mu\text{mol O}_2 \text{ kg}^{-1}$  through the bathypelagic waters. Concentrations of DIC also demonstrate large depth-dependent gradients, ranging from 1938 to 2086  $\mu\text{mol C kg}^{-1}$  in

the epipelagic waters, increasing sharply through the mesopelagic zone with concentrations peaking ( $>2350 \mu\text{mol C kg}^{-1}$ ) near the top of the bathypelagic zone. Deeper in the bathypelagic zone, concentrations of DIC remain elevated and demonstrate relatively low temporal variability; for example, HOT program measurements (1988-2012) suggest DIC concentrations range between 2311 to  $2343 \mu\text{mol C kg}^{-1}$  at 4000 m (**Figure 2.1**).

At Station ALOHA, fluxes of sinking particulate matter (measured at 150 m, near the base of the euphotic zone over since the inception of the HOT program, 1989-2012) range between 0.9 to  $4.7 \text{ mmol C m}^{-2} \text{ day}^{-1}$ , are generally elevated ( $1.5$  to  $4.7 \text{ mmol C m}^{-2} \text{ day}^{-1}$ ) between May and August and lower (0.9 to  $4.5 \text{ mmol C m}^{-2} \text{ day}^{-1}$ ) in the winter months (Dec.-Feb; **Figure 2.2**). These moderate to weak seasonal fluctuations in particulate matter export are consistent with seasonal patterns observed in euphotic zone rates of  $^{14}\text{C}$ -bicarbonate assimilation (measured in the light and hence inclusive of both photoautotrophic and chemoautotrophic production). The  $^{14}\text{C}$ -based measurements of productivity indicate  $\sim 3$  fold changes over the course of the year, with rates increasing during the summer, averaging  $51.31 \text{ mmol C m}^{-2} \text{ day}^{-1}$ , decreasing to  $34.73 \text{ mmol C m}^{-2} \text{ day}^{-1}$  in the winter (**Figure 2.2**). Euphotic zone rates of  $^{14}\text{C}$ -bicarbonate assimilation at Station ALOHA measured in the dark, and hence presumably reflecting anaplerotic carbon assimilation and chemoautotrophic production, show similar seasonal variability, with elevated rates in the summer, decreasing in the winter. The measured rates of dark  $^{14}\text{C}$ -bicarbonate assimilation are considerably lower than those measured in the light; upper ocean (0-200 m) depth-integrated dark rates typically range between 0.8 to 5.8

$\text{mmol C m}^{-2} \text{ day}^{-1}$ , equivalent to ~2% to 11% of rates measured in the light (HOT data 1989-2000).

#### *Diversity and vertical distributions of form II RuBisCO genes below the sunlit layer at Station ALOHA*

Fifty-nine *cbbM* gene sequences were obtained from clone libraries of PCR-amplified environmental DNA extracted from samples collected within the mesopelagic zone (200-1000 m) at Station ALOHA. Thirty-two sequences were obtained from 400 m, one sequence from 550 m, 8 sequences from 650 m, and the remaining 18 sequences from 770 m. These *cbbM* sequences clustered with two major clades of  $\gamma$ - and  $\delta$ -proteobacterial form II RuBisCO genes. A majority of the sequences (47) clustered at 99% sequence identity with single amplified genomes found to derive from the SAR324 clade of  $\delta$ -proteobacteria ([Swan et al. 2011](#)). Three of the *cbbM* sequences shared  $\leq 94\%$  sequence identity to *cbbM* genes from SAGs deriving from  $\gamma$ -proteobacterial ribotypes ARCTIC96BD-19 and AGG47 ([Swan et al. 2011](#)). In addition, 9 sequences from the *cbbM* clone library did not share significant sequence identity with SAGs published by Swan et al. (2011) and appear unique to this study (**Figure 2.3**).

#### *Vertical distributions of form II RuBisCO genes*

Two unique sets of qPCR primers (**Table 2.1**) were used to investigate the vertical distributions of distinct groups of  $\gamma$  - and  $\delta$ -proteobacteria *cbbM* gene phylotypes at Station ALOHA in the NPSG. Primers used to enumerate the  $\gamma$  -proteobacterial *cbbM* genes targeted sequence-types that included the ARCTIC96BD-19 and AGG47 clades,

and possibly also the SUP05 clade, while primers used for the  $\delta$ -proteobacteria were specific to *cbbM* genes belonging to the SAR324 clade (Swan et al. 2011). Both of these proteobacterial lineages have previously been reported to occur in the mesopelagic waters of the NPSG (Fuhrman and Davis 1997, DeLong et al. 2006, Brown et al. 2007, Swan et al., 2011). Nucleic acid samples were analyzed from 16 discrete depths between the surface ocean and the deep sea (0-4000 m) on three HOT cruises between 2012 and 2013 (June 2012, June 2013, and October 2013). During the June 2013 cruise, samples were also collected at increased vertical resolution within the mesopelagic waters (175-1000 m) to obtain additional information about the vertical partitioning of these microorganisms.

The resulting qPCR analyses of the  $\gamma$ - and  $\delta$ -proteobacterial *cbbM* phylotypes revealed pronounced depth-dependent patterns in the distributions and *cbbM* gene abundances of these organisms at Station ALOHA. In particular, *cbbM* gene abundances of the  $\gamma$ -proteobacterial phylotype increased sharply through the transition from the base of the euphotic zone into the upper regions of the mesopelagic waters (150-500 m; **Figures 2.4 and 2.5**). Abundances of the  $\gamma$ -proteobacterial phylotype in the well-lit regions of the upper ocean (0-100 m) were low, ranging between  $1.0 \times 10^2$  to  $5.5 \times 10^2$  gene copies L<sup>-1</sup>, increasing to  $1.8 \times 10^2$  to  $2.1 \times 10^3$  copies L<sup>-1</sup> through the dimly-lit regions of the lower euphotic zone (125-150 m). Below the base of the euphotic zone and into the mesopelagic waters, gene abundances increased sharply ( $4.5 \times 10^3$  gene copies L<sup>-1</sup> per m; calculated from 150-500 m on June 2013 cruise), reaching peak gene abundances averaging  $\sim 2.4 \times 10^6$  genes L<sup>-1</sup> (ranging  $2.2 \times 10^6$  to  $2.7 \times 10^6$  genes L<sup>-1</sup>) near 500-700 m depth. Below  $\sim 700$  m, abundances gradually declined with increasing depth, with

abundances in the deep sea ranging between  $4.1 \times 10^4$  and  $1.3 \times 10^6$  gene copies L<sup>-1</sup> in the bathypelagic waters (1000-4000 m; **Figures 2.4 and 2.5**).

Similar to the depth-dependent patterns in gene abundances observed for the  $\gamma$ -proteobacterial phylotype, *cbbM* abundances of the  $\delta$ -proteobacterial phylotype also increased sharply between the lower euphotic zone and the mesopelagic waters (**Figure 2.4 and 2.5**). Throughout the euphotic zone (0-150 m), the  $\delta$ -proteobacterial phylotype was generally below detection (<100 genes L<sup>-1</sup>) by the qPCR assays used in this study. Abundances of this phylotype became detectable below ~200-300 m, where abundances ranged  $9.8 \times 10^2$  to  $3.3 \times 10^4$  gene copies L<sup>-1</sup>. Abundances increased sharply through the mesopelagic waters, peaking at  $1.9 \times 10^5$  to  $9.3 \times 10^5$  copies L<sup>-1</sup> near 500-770 m. Below this depth, *cbbM* gene abundances declined gradually, with abundances ranging  $1.0 \times 10^4$  to  $3.3 \times 10^5$  copies L<sup>-1</sup> in the bathypelagic (1000-4000 m) waters. Notably, *cbbM* gene abundances of the  $\delta$ -proteobacterial phylotype rivaled or occasionally exceeded the abundances of the  $\gamma$ -proteobacterial phylotype through the lower mesopelagic and bathypelagic waters.

#### *Thiosulfate addition incubation experiments*

In addition to evaluating vertical structure of *cbbM* genes during both the June 2012 and October 2013 HOT cruises, incubation experiments where S<sub>2</sub>O<sub>3</sub><sup>2-</sup> was added to otherwise unamended 200 m seawater were conducted to evaluate potential responses by these organisms to additions of this reduced sulfur substrate. For both experiments, seawater was amended with 5  $\mu$ M sulfur (final concentration) as S<sub>2</sub>O<sub>3</sub><sup>2-</sup> and incubated in the dark at *in situ* temperatures over 21 or 24 days (October 2013 and June 2012,

respectively); results from these treatments were compared to unamended controls. To evaluate whether the addition of  $S_2O_3^{2-}$  stimulated microbial production, rates for  $^{14}C$ -bicarbonate assimilation and  $^3H$ -leucine (used as a proxy to estimate rates of bacterial production) were measured at 3-day intervals throughout the incubation period.

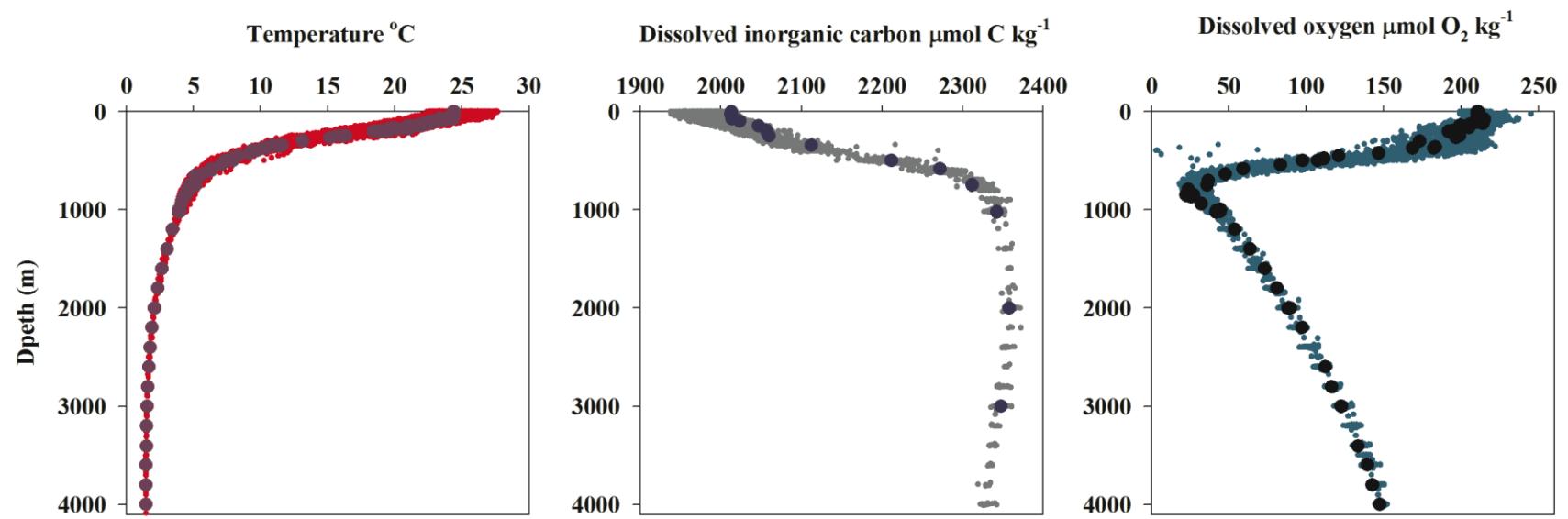
During the June 2012 experiment, over the course of the 24 days of the incubation, rates of both carbon fixation ( $^{14}C$ -bicarbonate assimilation) and bacterial production ( $^3H$ -leucine incorporation) varied in time, but overall the large variability observed among the triplicate carboys resulted in no statistically significant differences in either rates of  $^3H$ -leucine incorporation or  $^{14}C$ -bicarbonate assimilation in the  $S_2O_3^{2-}$  treatments relative to the controls (Two-way ANOVA,  $P > 0.05$ ). In the control carboys, rates of  $^{14}C$ -bicarbonate assimilation varied between 0.9 and 16.3 nmol C L $^{-1}$  day $^{-1}$ , with rates increasing through the initial 6 days of the experiment before declining and remaining relatively unchanged for the remainder of the experiment (**Figure 2.6**). Rates of  $^{14}C$ -carbon fixation in the  $S_2O_3^{2-}$  treatments varied between 0.8 and 88.9 nmol C L $^{-1}$  day $^{-1}$ , with rates greatest 9 to 12 days into the experiment, before returning to rates comparable to those measured at the beginning of the experiment (**Figure 2.6**). The accumulation of inorganic carbon into biomass over the time-course (derived from the time integration of rates of  $^{14}C$ -bicarbonate assimilation over the experiment; **Figure 2.7**) was 529.0 and 201.8 nmol C L $^{-1}$  for the  $S_2O_3^{2-}$  and control treatments, respectively, and not significantly different between treatments (One-way ANOVA,  $P > 0.05$ ). Similar to the time-varying changes observed in rates of  $^{14}C$ -carbon fixation, rates of bacterial production, measured by  $^3H$ -leucine incorporation, were often greater in the  $S_2O_3^{2-}$  treatments than in the controls; however, large variability among the measured rates

resulted in no statistically significant differences between the  $\text{S}_2\text{O}_3^{2-}$  and control carboys (Two-way ANOVA,  $P > 0.05$ ). Rates of bacterial production in the  $\text{S}_2\text{O}_3^{2-}$  treatment ranged 11.8 to 163.6 nmol C L<sup>-1</sup> day<sup>-1</sup>. No statistical significance between treatments was seen in the time-integrated bacterial production (One-way ANOVA,  $P > 0.05$ ) with 878.8 and 1330.3 nmol C L<sup>-1</sup> accumulated in the  $\text{S}_2\text{O}_3^{2-}$  and control treatments, respectively (**Figure 2.7**).

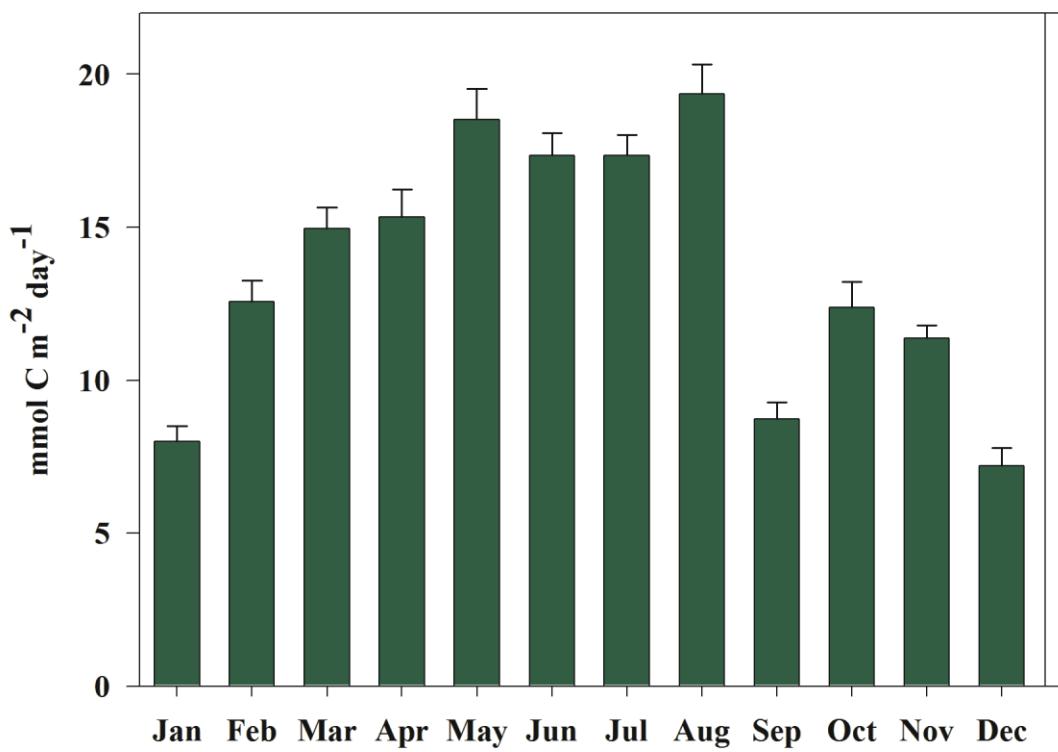
Variation in the abundance of the  $\gamma$ -proteobacterial *cbbM* phylotype throughout the 24-day experiment was also examined. At the onset of the experiment, the  $\gamma$ -proteobacterial *cbbM* phylotype averaged  $1.2 \times 10^3$  gene copies L<sup>-1</sup> in both the  $\text{S}_2\text{O}_3^{2-}$  treatments and controls. Between the 6<sup>th</sup> and 15<sup>th</sup> day of the experiment, the abundance of this phylotype in the  $\text{S}_2\text{O}_3^{2-}$  treatment increased nearly 13-fold, reaching maximum abundances of  $1.9 \times 10^4$  gene copies L<sup>-1</sup>. Throughout the remainder of the experiment, gene abundances averaged  $7.9 \times 10^3$  copies L<sup>-1</sup>, resulting in a ~6-fold increase in abundances between the beginning and end of the experiment. However, gene abundances in the controls also demonstrated large increases (up to 10-fold from those measured at the beginning of the experiment), resulting no significant differences between the  $\text{S}_2\text{O}_3^{2-}$  treatments and the controls (Two-way ANOVA,  $P > 0.05$ ). The abundance of the  $\delta$ -proteobacterial *cbbM* phylotype was consistently below the limits of detection (~100 copies L<sup>-1</sup>) throughout this experiment.

Unlike the results observed in June 2012, over the course of the 21-day experiment conducted in October 2013, the addition of  $\text{S}_2\text{O}_3^{2-}$  significantly stimulated rates of <sup>14</sup>C-bicarbonate assimilation relative to the control (Two-way ANOVA,  $P < 0.001$ ). In the control carboys, rates of <sup>14</sup>C-bicarbonate assimilation were relatively

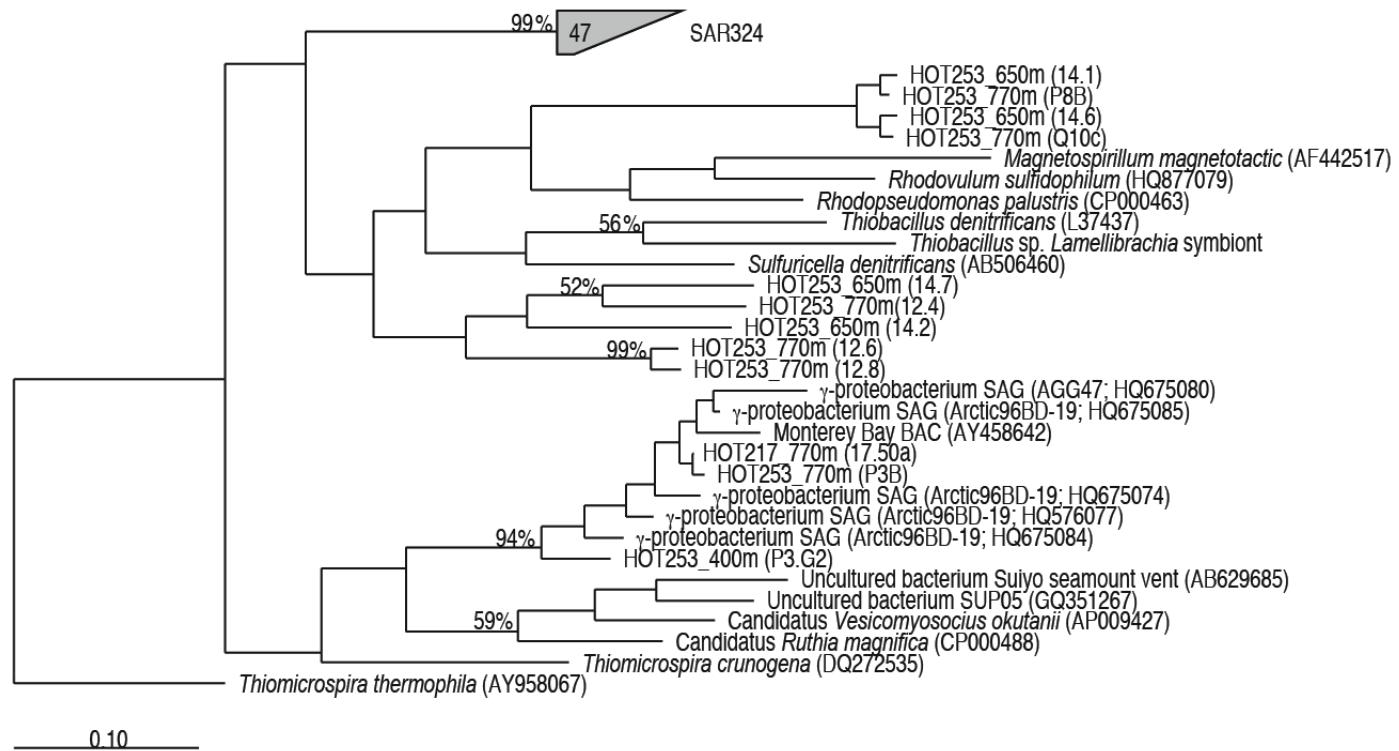
constant for the initial 6 days of the experiment, averaging  $21.7 \text{ nmol C L}^{-1} \text{ day}^{-1}$ , before declining to  $\sim 12.1 \text{ nmol C L}^{-1} \text{ day}^{-1}$  for the remaining experiment (**Figures 2.8 and 2.9**). Rates of  $^{14}\text{C}$ -bicarbonate assimilation in the  $\text{S}_2\text{O}_3^{2-}$  treatments increased nearly 2-fold by the 3<sup>rd</sup> day, declined temporarily, and increased again, eventually maintaining rates similar to those observed at the beginning of the experiment (**Figure 2.8**). The accumulation of inorganic carbon into biomass over the time-course (**Figure 2.9**) was 712.4 and  $415.5 \text{ nmol C L}^{-1}$  for the  $\text{S}_2\text{O}_3^{2-}$  and control treatments, respectively, and were significantly different between treatments (One-way ANOVA,  $P = 0.04$ ). Significant differences in rates of bacterial production between the  $\text{S}_2\text{O}_3^{2-}$  treatment and control were also observed (Two-way ANOVA,  $P < 0.001$ ) and the resulting time-integrated heterotrophic production (One-way ANOVA,  $P = 0.05$ ; **Figure 2.9**) in the  $\text{S}_2\text{O}_3^{2-}$  treatment was significantly greater than the control. Similar to the June 2012 incubation, the *cbbM* genes belonging to the  $\delta$ -proteobacterial phylotype were below the level of detection of the qPCR assay. However, the abundances of the  $\gamma$ -proteobacterial *cbbM* phylotype were detectable throughout the experiment (**Figure 2.8**). Gene abundances of this phylotype increased more than 3.5-fold during the first 3 days in both treatments before leveling off and declining during the latter several days of the experiment. The resulting gene abundances were not significantly different between the treatment and control (Two-way ANOVA,  $P > 0.05$ ).



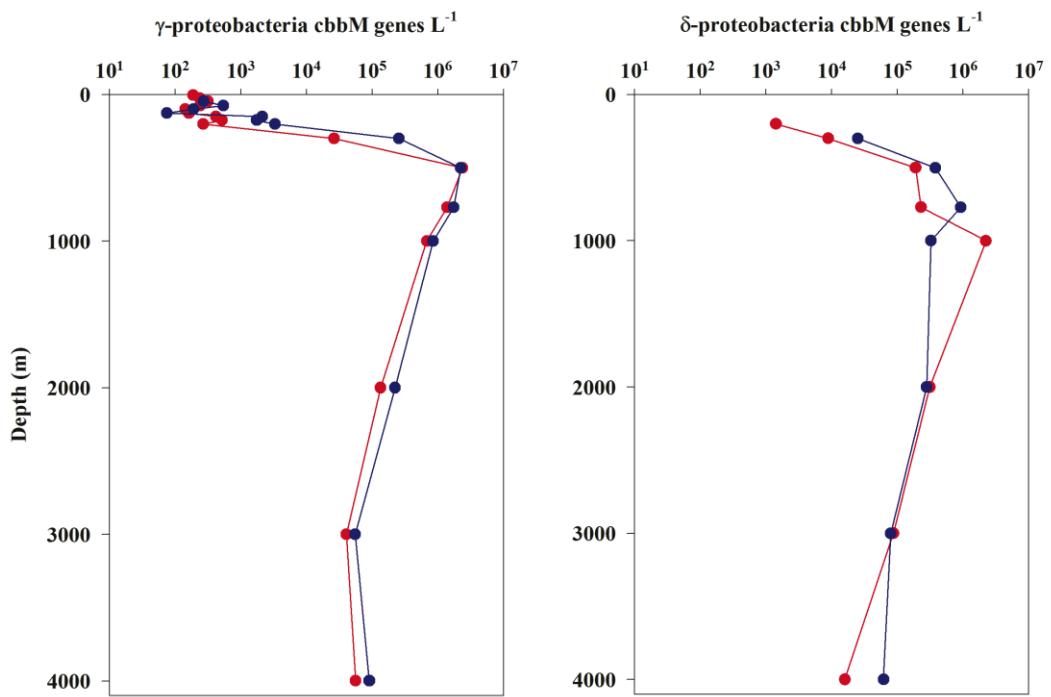
**Figure 2.1.** Time-averaged (1988-2012) vertical profiles of temperature, dissolved inorganic carbon, and dissolved oxygen at Station ALOHA. Measurements from the June 2012 cruise (HOT 243) are plotted in lighter colors over the averages taken from the HOT program.



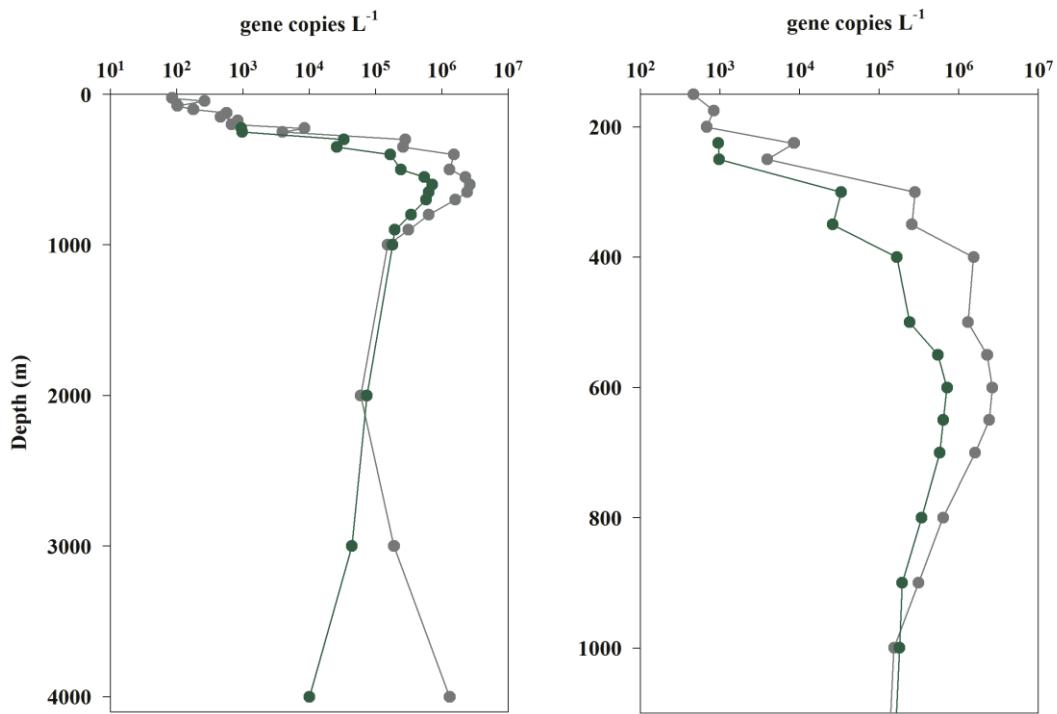
**Figure 2.2.** Monthly variations in particulate carbon flux (150 m) at Station ALOHA measured by the HOT program (1989-2012).



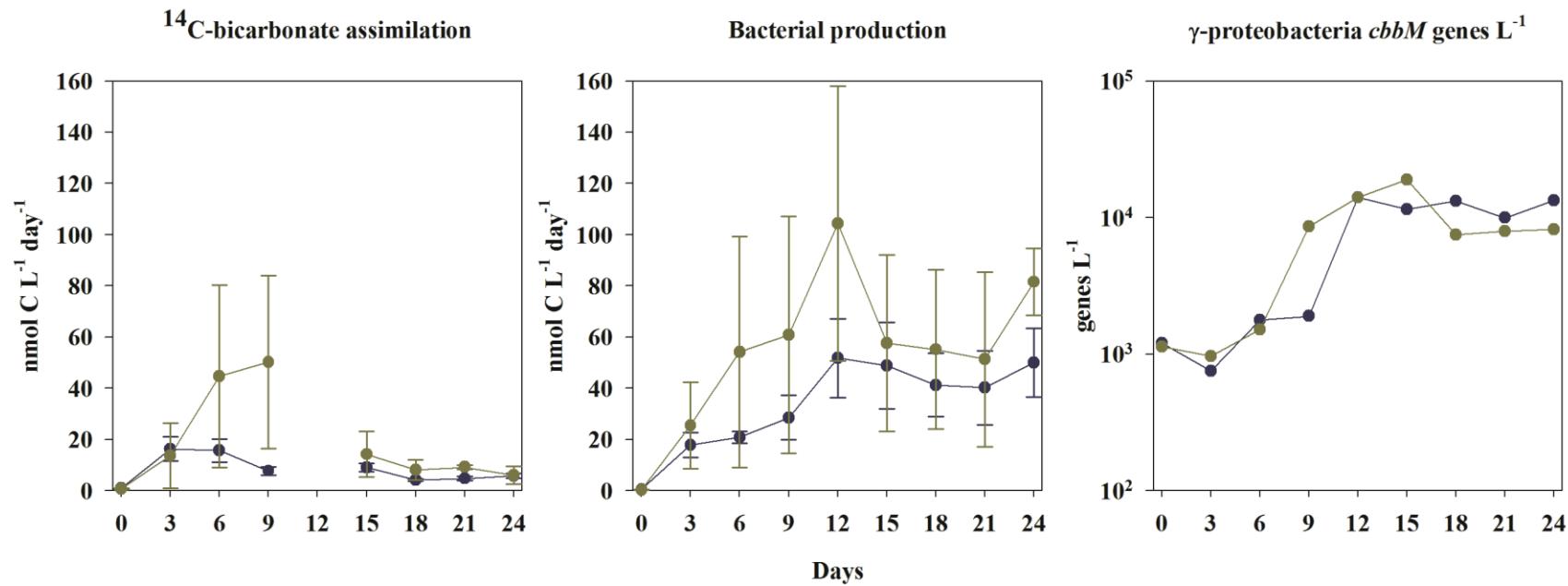
**Figure 2.3.** Neighbor-joining phylogenetic tree of form II RuBisCO (*cbbM*) genes; sequences obtained in this study labeled with prefix “HOT” and the cruise number. The number inside the trapezoid sums clones sequenced and Genbank sequences of the SAR324 cluster.



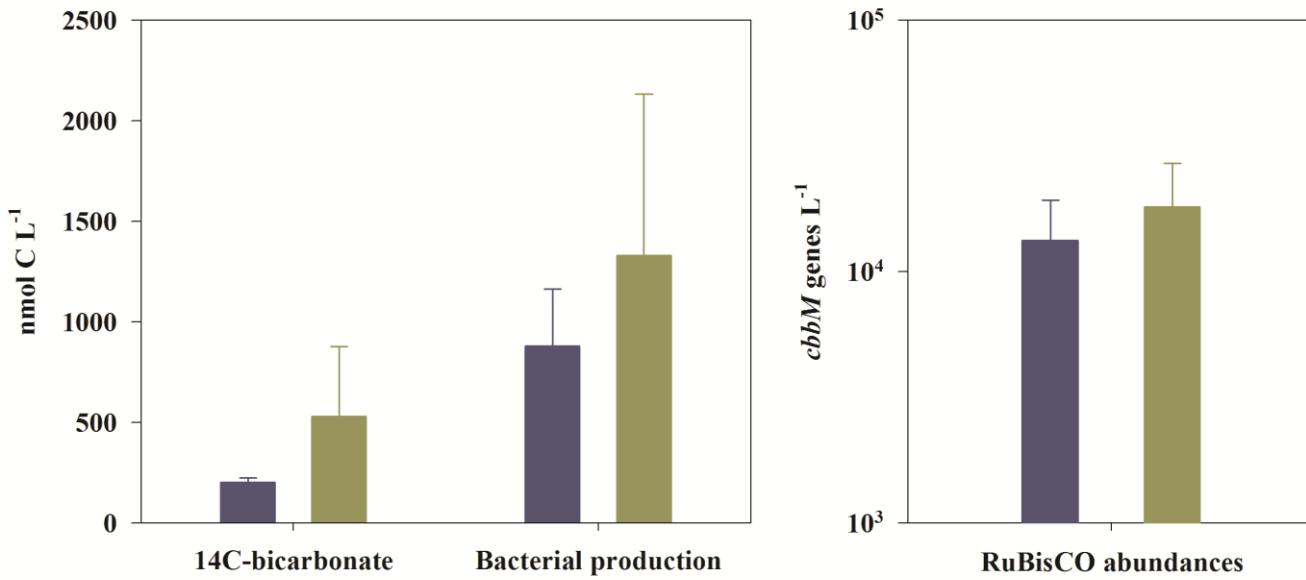
**Figure 2.4.** Vertical distribution of  $cbbM$  genes at Station ALOHA in June 2012 (blue) and October 2013 (red) for the  $\gamma$ -proteobacteria (left) and  $\delta$ -proteobacteria (right) gene phylotypes.



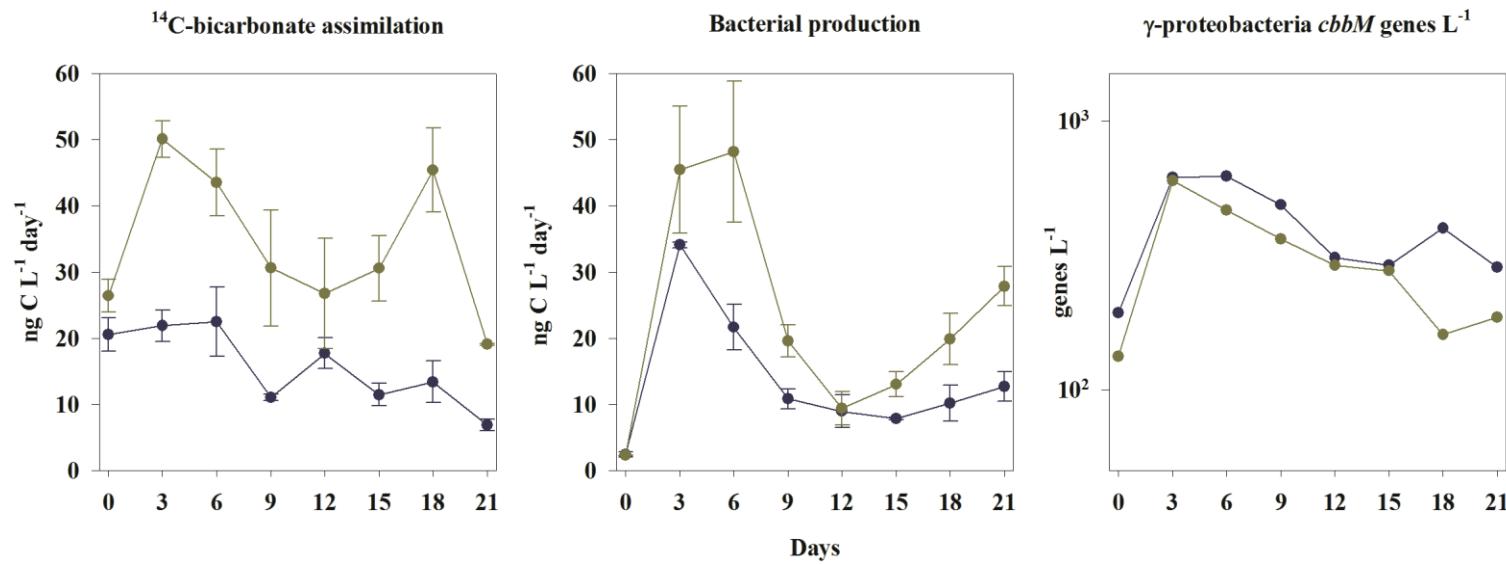
**Figure 2.5.** Vertical distributions of *cbbM* genes at Station ALOHA in June 2013. Right panel depicts higher vertical resolution sampling within the mesopelagic waters (175–1000 m). The  $\delta$ -proteobacterial *cbbM* phylotype in green and the  $\gamma$ -proteobacteria depicted in gray.



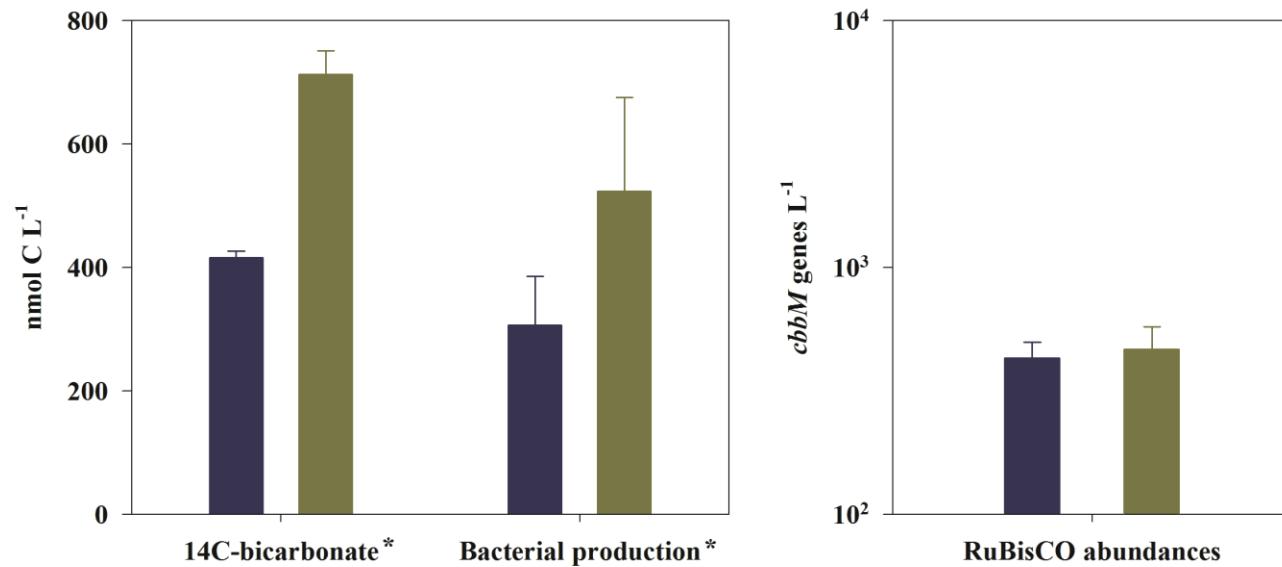
**Figure 2.6.** Results of time course  $S_2O_3^{2-}$  incubation experiment conducted in June 2012. Depicted are rates of  $^{14}C$ -bicarbonate assimilation (in the dark), bacterial production (derived from  $^3H$ -leucine incorporation measurements), and  $\gamma$ -proteobacterial *cbbM* gene abundances. Error bars represent one standard deviation of the means. *cbbM* gene abundances are only reported for  $\gamma$ -proteobacteria because the  $\delta$ -proteobacteria phylotype was below the limit of detection ( $<100$  genes  $L^{-1}$ ) of this qPCR assay. Thiosulfate treatments are shown in yellow and control treatments are shown in blue.



**Figure 2.7.** Time integrated carbon assimilation and bacterial production, and changes in  $\gamma$ -proteobacterial *cbbM* genes during the time course incubation in June 2012. Bars depict carbon accumulated throughout the experiment derived from the rate measurements or maximum changes in gene abundances from qPCR analyses. Error bars represent propagated error. Blue bars represent controls; yellow bars represent thiosulfate additions.



**Figure 2.8.** Results of time course  $S_2O_3^{2-}$  incubation experiment conducted in October 2013. Depicted are rates of  $^{14}C$ -bicarbonate assimilation (in the dark), bacterial production (derived from  $^3H$ -leucine incorporation measurements), and  $\gamma$ -proteobacterial *cbbM* gene abundances. Error bars represent one standard deviation of the means. *cbbM* gene abundances are only reported for  $\gamma$ -proteobacteria because the  $\delta$ -proteobacteria phylotype was below the limit of detection for the qPCR assay. Thiosulfate treatments are shown in yellow and control treatments are shown in blue.



**Figure 2.9.** Time integrated carbon assimilation and bacterial production and changes in  $\gamma$ -proteobacterial *cbbM* genes during the time course incubation in October 2013. Bars depict carbon accumulated throughout the experiment derived from the rate measurements or maximum changes in gene abundances from qPCR analyses. Error bars represent propagated error. Blue bars represent controls; yellow bars represent thiosulfate additions. Asterisks note analyses that resulted in significant differences between treatments.

## Discussion

Microorganisms in the dark ocean represent the largest pool of living biomass in the sea, yet our understanding of their metabolisms remains poor. In this study, I evaluated the diversity, distributions, and activities of specific groups of bacteria that possess form II RuBisCO *cbbM* genes at Station ALOHA in the NPSG. The objectives of my study were to: 1) examine the phylogenetic diversity of bacteria possessing form II *cbbM* genes at Station ALOHA; 2) quantify abundances and evaluate the vertical distributions of these groups of *cbbM* containing  $\gamma$ - and  $\delta$ -proteobacteria; 3) evaluate the responses of these organisms to additions of  $S_2O_3^{2-}$  by measuring inorganic carbon assimilation and heterotrophic production and examining possible changes in the abundances of these proteobacteria phylotypes throughout these experiments.

Sequencing of PCR-amplified *cbbM* genes from Station ALOHA revealed several phylotypes of proteobacteria with the genetic potential for carbon fixation. I found *cbbM* genes previously identified as deriving from cosmopolitan groups of  $\gamma$ - and  $\delta$ -proteobacteria (including phylotypes termed ARCTIC96BD-19/AGG47 and SAR324, respectively). In addition, my sequencing results revealed previously unidentified *cbbM* genes, whose phylogenetic identity is most similar to form II RuBisCO genes from unidentified and/or uncultured groups of prokaryotes. Based on these results, I utilized previously published ([Swan et al., 2011](#)) qPCR assays to evaluate the vertical distributions of the  $\gamma$ - and  $\delta$ -proteobacteria phylotypes.

[Swan et al. \(2011\)](#) showed that a significant portion of single amplified genomes (SAGs), 47% of SAR324 and 25% of  $\gamma$ -proteobacteria, obtained from the mesopelagic of both the Atlantic and Pacific Oceans contained genes belonging to the Calvin-Benson-

Basham cycle, adding to the diversity of potential pathways for carbon fixation in the NPSG. Previous studies have suggested the 3-hydroxypropionate/4-hydroxybutyrate cycle fueled by ammonia oxidation by thaumarchaea dominated dark ocean autotrophy ([Wutcher et al. 2007](#); [Berg et al., 2007](#)). Sampling conducted as part of the Swan et al. (2011) study evaluating bacterial autotrophy focused on two depths, near-surface ocean (25 m) and the mesopelagic (770 m), leaving the vertical distribution of these proteobacterial *cbbM* genes throughout the NPSG water column unresolved.

The relatively few sequences obtained in the current study provided limited information on the diversity of *cbbM* containing organisms. Moreover, the degenerate primers utilized for my study may not amplify the full diversity of *cbbM* genes in this environment. A metagenome reconstruction of SUP05, a  $\gamma$ -proteobacteria cluster closely related to the ARCTIC96BD-19 clade, was found to contain *cbbM* genes ([Marshall and Morris, 2013](#); [Walsh et al., 2009](#)). Yet after successfully isolating a member of the SUP05 lineage from Puget Sound, Marshall and Morris (2013) reported the inability to amplify *cbbM* genes using the same degenerate primers employed by this study. The inability to amplify these genes suggests members of the SUP05 may contain divergent *cbbM* genes or possess alternative forms of RuBisCO (e.g. form IA). As such, the chemoautotrophic  $\gamma$ -proteobacteria inhabiting the oxygenated waters of the mesopelagic at Station ALOHA may be divergent from those commonly retrieved in oxygen deficient waters ([Glaubitz et al., 2013](#); [Marshall and Morris, 2013](#); [Swan et al., 2011](#); [Walsh et al., 2009](#)).

The results from my qPCR based analyses reveal that both the  $\gamma$  - and  $\delta$  - proteobacterial *cbbM* phylotypes increased 3-4 orders of magnitude from the base of the euphotic zone into the mesopelagic waters, with abundances remaining elevated throughout

the bathypelagic zone. Abundances of the  $\gamma$ -proteobacterial phylotypes were maximal ( $\sim 10^6$  genes L $^{-1}$ ) in the mid-mesopelagic zone (500-600 m), while the  $\delta$ -proteobacterial phylotypes reached peak abundances ( $\sim 10^5$  genes L $^{-1}$ ) in the lower mesopelagic waters (600-1000 m). Abundances of both *cbbM* phylotypes in the euphotic zone (<200 m) waters were low, with the  $\delta$ -proteobacteria phylotype consistently below the limits of detection (<100 genes L $^{-1}$ ), while the  $\gamma$ -proteobacteria phylotype was generally detectable at abundances <10 $^3$  genes L $^{-1}$ . The inability to detect  $\delta$ -proteobacteria *cbbM* genes in the upper ocean is consistent with previous reports on the distributions of these organisms, suggesting these organisms are more abundant in the deep sea (Wright et al., 1997; Aristegui et al., 2009).

Karner et al. (2001) reported an overall decrease in bacterial abundance with depth at Station ALOHA, and thus the apparent increases in abundance by these two *cbbM* containing phylotypes with depth suggests that these organisms represent a proportionately greater fraction of the total bacterial abundance in the mesopelagic waters than in the surface waters (Table 2.2). Assuming  $\gamma$  - and  $\delta$  -proteobacteria contain a single *cbbM* gene per cell, I estimate these two proteobacteria phylotypes constitute < 0.01% of the total microbial assemblage in the upper 500 m, but may comprise upwards of ~6% of the total cells in the lower mesopelagic (Table 2.2). This observation is consistent with Swan et al. (2011) who reported increased proportions of both phylotypes to the community at 770 m using SSU rRNA sequence analysis for the identification of these organisms.

My study revealed that the  $\gamma$ -proteobacteria *cbbM* phylotype was found at abundances nearly 10-fold greater than the  $\delta$ -proteobacteria phylotype through the upper mesopelagic (<700 m) waters. Moreover, the  $\gamma$ -proteobacteria were found in low, but quantifiable abundances in the euphotic zone, while the  $\delta$ -proteobacteria was consistently below detection

in the upper ocean. In contrast, Swan et al. (2011) reported that the proportion of ARCTIC96BD-19/AGG47 and SAR324 16S rRNA genes in the SAGs retrieved from 770 m at Station ALOHA had approximately equal representation (17% of total 16S rRNA genes) of both phylotypes. The apparent discrepancy in abundances in the present study relative to the Swan et al. (2011) findings may reflect differences in the forms of RuBisCO utilized by these organisms. Swan et al. (2011) found that form IA RuBisCO genes were more common in SAGs belonging to  $\delta$ -proteobacteria, suggesting these organisms can possess one or both of these phylogenetically distinct forms of the RuBisCO protein. Interestingly, both *cbbM* and *cbbL* genes, form II and form IA RuBisCO, respectively, were reported in SAGs identified as SAR324, whereas only *cbbM* sequences were found in SAGs belonging to  $\gamma$ -proteobacteria (Swan et al. 2011). Of the 29 SAR324 SAGs identified by Swan et al. (2011) only 13% contained *cbbM* sequences. The remaining  $\delta$ -proteobacteria SAGs contain form IA (*cbbL*) RuBisCO genes. Hence, in the current study, the abundance of the  $\delta$ -proteobacteria phylotype was likely underestimated by only examining *cbbM* gene abundances.

Vertical distributions of  $\gamma$ - and  $\delta$ -proteobacteria *cbbM* genes also closely mirror profiles of dissolved oxygen concentrations, with peak abundances occurring near the oxygen minimum (typically 770 m for Station ALOHA). These results suggest that the distributions of these potentially chemoautotrophic organisms may respond to or contribute to observed chemical gradients in this ecosystem. For example, microbial abundances of members belonging to the ARCTIC96BD-19, AGG47, SUP05, and SAR324 clades have all been shown to peak in oxygen minimum zones (Swan et al., 2011; Walsh et al., 2009; Stevens and Ulloa, 2008; Suzuki et al., 2004). However, in contrast to these findings, Suzuki et al. (2004)

described the highest occurrence of ARCTIC96BD-19 from a sample collected in the well-oxygenated waters (80 m) of Monterey Bay.

To examine whether rates of microbial production and growth in mesopelagic waters of the NPSG might be fueled by oxidation of reduced sulfur substrates, I conducted two  $S_2O_3^{2-}$  addition experiments and tracked the activities and abundances these organisms over >20 days. Form II RuBisCO genes belonging to the  $\delta$ -proteobacteria phylotype were undetectable throughout both experiments. Such findings likely reflect the low or undetectable abundances of these organisms in the upper ocean (200 m), or the reliance of these organisms on alternate forms of RuBisCO (specifically form IA) that were not examined in the current study.

Peak abundances of  $\gamma$ -proteobacteria *cbbM* genes observed during the June 2012 experiment were 12- and 17-fold greater than abundances measured at the onset of the experiment in both the control and  $S_2O_3^{2-}$  treatments. During the October 2013 experiment abundances of the  $\gamma$ -proteobacterial phylotype was ~3- and 4.5-fold those measured at the beginning of the experiment. Such changes in *cbbM* abundance did not closely follow patterns of carbon assimilation revealed by the  $^{14}C$ -bicarbonate and bacterial production analyses throughout either incubation. Furthermore, the differences in *cbbM* genes between the two treatments were not statistically significant.

In the same experiments I also investigated how the additions of reduced sulfur influenced microbial growth and productivity in the mesopelagic waters at Station ALOHA. During the June 2012 experiment, the addition of  $S_2O_3^{2-}$  did not result in significant differences between treatments even though rates of carbon assimilation were on average greater in carboys treated with  $S_2O_3^{2-}$  for both the  $^{14}C$ -bicarbonate and  $^3H$ -leucine

measurements. During the experiment conducted in October 2013, the addition of  $\text{S}_2\text{O}_3^{2-}$  resulted in significantly greater rates of  $^{14}\text{C}$ -bicarbonate assimilation and bacterial production relative to unamended controls. During this experiment, rates of  $^{14}\text{C}$ -bicarbonate assimilation were on average 2.3-fold greater in the  $\text{S}_2\text{O}_3^{2-}$  treatment than the control, and rates of  $^3\text{H}$ -leucine also showed a relative increase of ~1.6-fold. In both cases, increased rates of productivity (*i.e.* both “autotrophy” and “heterotrophy”) suggest the addition of  $\text{S}_2\text{O}_3^{2-}$  significantly stimulated microorganism growth.

The  $^{14}\text{C}$ -bicarbonate assimilation and bacterial production results from the June 2012 and October 2013 experiments suggest  $\text{S}_2\text{O}_3^{2-}$  can sometimes stimulate production, but at other times may not influence rates of carbon assimilation. The finding that additions of  $\text{S}_2\text{O}_3^{2-}$  did not lead to significant increases in microbial activity or gene abundances in the June 2012 experiment might be a consequence of the relatively high variability observed among the triplicate measurements, where one replicate of the  $\text{S}_2\text{O}_3^{2-}$  treatment was 2-3 fold higher than the other two for the majority of the time course. Alternatively, the apparent lack of response might be attributable to this experiment being initiated during a time where there were sufficient other sources of reduced substrates present in the seawater allowing other metabolisms to out compete sulfur oxidizing bacteria. If so, then the addition of 5  $\mu\text{M}$  reduced S may not have stimulated growth as much as other more energetically favorable, reduced substrates. The growth efficiency of the sulfur oxidizing bacterium *Thiobacillus neapolitanus* when grown on  $\text{S}_2\text{O}_3^{2-}$  appears very low relative to growth by chemoorganoheterotrophs. Laboratory experiments with *T. neapolitanus* indicate the ratio of sulfur oxidized to carbon produced averages ~1600:1 (mol:mol) (Schlegel 1993), attesting to the very small amount of energy obtained through the oxidation of  $\text{S}_2\text{O}_3^{2-}$ . If this metabolic

efficiency is similar for organisms in the open sea, the addition of 5  $\mu\text{M}$  S to seawater would have represented enough potential energy for sulfur oxidizing bacteria to synthesize  $\sim$ 3 nmol C, equivalent to  $3.6 \times 10^6$  cells produced per liter (assuming a single bacterial cell contains  $10^{-14}$  g C). Given the results of the June 2012 and October 2013 experiments, it seems more likely that the addition of  $\text{S}_2\text{O}_3^{2-}$  stimulated mixotrophic growth by these organisms.

The lack of a significant increase in *cbbM* gene abundance during these incubation experiments is consistent with previous studies; for example, a study in the Arctic Ocean failed to observe changes in the abundances of proteobacterial phylotypes in diluted seawater culture experiments, despite relatively high rates of  $^{14}\text{C}$ -bicarbonate assimilation and  $^3\text{H}$ -leucine incorporation rates ([Alonso-Saez et al., 2010](#)). These authors reported the ARCTIC96BD-16 clade of  $\gamma$ -proteobacteria constituted < 6% of total picoplankton cells but  $\sim$ 40% of these cells actively incorporated  $^3\text{H}$ -leucine, while  $\sim$ 10% of these cells demonstrated  $^{14}\text{C}$ -bicarbonate assimilation. Such results may suggest that inorganic carbon fixation by this phylotype is relatively low, with these organisms relying more heavily on heterotrophic metabolism. As such, the apparent response to the addition of  $\text{S}_2\text{O}_3^{2-}$  in rates of  $^{14}\text{C}$ -bicarbonate observed in the present study may have been mediated by microorganisms whose abundances were not targeted by the qPCR assays used. Alternatively, the lack of measureable increase in *cbbM* gene abundances in the present study could reflect tight coupling in the growth of the chemoautotrophs and removal by their predators or viruses. If so, then active growth of RuBisCO containing proteobacteria and the presumed increases in cell abundance would have been suppressed by active removal processes.

Results from the rate measurements are consistent with other observations in the Atlantic Ocean ([Reinthal et al., 2010; Herndl et al., 2005](#)), the Arctic Ocean ([Kirchman et](#)

al., 2007), and with dilution cultures inoculated with Arctic seawater (Alonso-Saez et al., 2010). Reinthaler et al. (2010) found that the ratio of autotrophic to heterotrophic production (based on  $^{14}\text{C}$ -bicarbonate assimilation relative to carbon-converted  $^3\text{H}$ -leucine incorporation rates) averaged  $1.3 \pm 0.2$ . In the current study, the  $^{14}\text{C}$ -bicarbonate to bacterial production ratios for the  $\text{S}_2\text{O}_3^{2-}$  treatments varied between  $0.25 \pm 0.2$  to  $1.68 \pm 0.8$ , June 2012 and October 2013, respectively. The inferred microbial carbon demand throughout the lower euphotic zone (150-200 m), based on the derived flux attenuation specific to each month, does not appear substantially different between June and October (**Table 2.3**). Measurements from my experiments suggested that in June, rates of microbial production represented <10% of the derived rates of organic matter consumption at the top of the mesopelagic waters (**Table 2.3**), consistent with expected bacterial growth efficiencies of ~10% (Carlson et al., 1999). In contrast, measurements from the October 2013 experiment suggests that the derived rates of microbial production were in excess of the derived organic matter consumption (**Table 2.3**). These latter results suggest that either the measurements of microbial production represent overestimates, or that the derived organic matter consumption was underestimated. Based on historical HOT measurements of PC flux and  $^{14}\text{C}$ -bicarbonate assimilation in the lower euphotic zone, we suspect our rate measurements may have overestimated microbial carbon production.

There are several possible explanations for potential mismatches between microbial carbon supply and demand within the ocean's interior. In the case of  $^{14}\text{C}$ -bicarbonate assimilation, the radiolabeled inorganic carbon may not be utilized exclusively by autotrophic metabolisms. Heterotrophic organisms may utilize  $\text{CO}_2$  without net carbon assimilation by the cell, for example via fatty acid biosynthesis and anaplerotic reactions

(Varela et al., 2011; Alonso-Saez et al., 2010; Reinthlaer et al., 2010; Herndl et al., 2005; Ashworth and Kornberg, 1966; Romanenko, 1964). These reactions serve to replenish carbon intermediates for the tricarboxylic acid (TCA) cycle, and hence ultimately generate cellular energy. Various studies suggest ~1-8% of bacterial carbon uptake derives from anaplerotic reactions (Roslev et al., 2004; Ashworth and Kornberg, 1966; Romanenko, 1964). Reinthaler et al. (2010) and Herndl et al. (2005) examined the contribution of anaplerotic reaction to carbon  $\Delta^{14}\text{C}$  uptake in dark Atlantic waters and concluded measured rates of uptake were derived from autotrophic activity. Moreover, the  $^3\text{H}$ -leucine method has been widely used as a measure of bacterial protein synthesis; however, the carbon conversion factors required for these rates have been shown to vary over an order of magnitude (*e.g.* 0.5 to 3 kg C mol $^{-1}$  leucine incorporated; Simon and Azam, 1989). Uncertainty in isotope dilution further complicates accurate conversion from leucine incorporation rates to carbon production.

With these uncertainties in mind, the results of the  $\text{S}_2\text{O}_3^{2-}$  experiments in this study suggest that rates of chemoautotrophic production may at times rival chemoheterotrophic production in the mesopelagic waters at Station ALOHA (**Table 2.3**). Moreover, the apparent stimulation of both rates by the addition of  $\text{S}_2\text{O}_3^{2-}$  may indicate that the same processes and organisms partly control both rates. To date, it remains unknown if microorganisms taking up inorganic carbon in the dark ocean are exclusively chemoautotrophs (Alonso-Saez et al., 2010) or are simultaneously consuming organic carbon as chemoheterotrophs. Mixotrophy appears prevalent among oceanic picoplankton, and may be a prominent form of metabolism among the sulfur oxidizing chemoautotrophs examined in the current study. Swan et al. (2011) identified amino acid/oligopeptide transporters in the

amplified genomes of the ARCTIC96BD-19 and SAR324 clades. Since these organisms are present in the NPSG and rates of carbon assimilation appear comparable for auto- and heterotrophic processes in my experiments, such results may reflect mixotrophic growth by such organisms.

**Table 2.2.** Proportion of *cbbM* genes to total prokaryotic cells (Karner et al., 2001) with depth.

Depth	Total prokaryotic cells per liter	$\gamma$ - and $\delta$ -proteobacteria genes per liter	% of <i>cbbM</i> genes to total cells
5	$3.39 \times 10^8$	$1.88 \times 10^2$	<0.01%
25	$3.96 \times 10^8$	$1.62 \times 10^2$	<0.01%
45	$3.40 \times 10^8$	$2.86 \times 10^2$	<0.01%
100	$2.65 \times 10^8$	$1.71 \times 10^2$	<0.01%
150	$2.18 \times 10^8$	$1.00 \times 10^3$	<0.01%
250	$8.51 \times 10^7$	$4.91 \times 10^3$	0.01%
300	$4.53 \times 10^7$	$2.11 \times 10^5$	0.47%
500	$5.73 \times 10^7$	$2.25 \times 10^6$	3.9%
1000	$2.19 \times 10^7$	$1.48 \times 10^6$	6.8%
2000	$1.27 \times 10^7$	$3.59 \times 10^5$	2.8%
3000	$9.41 \times 10^6$	$1.66 \times 10^5$	1.8%
4000+	$1.02 \times 10^7$	$5.16 \times 10^5$	5.1%

**Table 2.3.** Measured and derived metabolic parameters for the months June and October using data available from HOT (1989-2012).

$F_{200}$  represents the theoretical upper limit of metabolic activity for the deep sea based on the measured PC flux at 150 m and the Martin curve “b” values derived for the respective months. Measured  $^{14}\text{C}$ -bicarbonate assimilation and  $^3\text{H}$ -leucine incorporation are from the control treatments at T0 for the June 2012 and October 2013 experiments. Inferred microbial carbon demand calculated as the difference between the measured PC flux at 150 m and the derived PC flux at 200 m.

Metabolic parameters	June	October
Measured $F_{150}$ ( $\text{mmol C m}^{-2} \text{ day}^{-1}$ )	$2.73 \pm 0.65$	$2.16 \pm 0.80$
Calculated b term	-0.93	-0.92
Derived $F_{200}$ ( $\text{mmol C m}^{-2} \text{ day}^{-1}$ )	$2.09 \pm 0.48$	$1.66 \pm 0.61$
Inferred microbial carbon demand in lower euphotic zone (150-200 m; $\text{nmol C L}^{-1} \text{ day}^{-1}$ )	$12.79 \pm 3.00$	$10.02 \pm 3.71$
Measured $^{14}\text{C}$ -bicarbonate assimilation (T0 <sub>control</sub> ; $\text{nmol C L}^{-1} \text{ day}^{-1}$ )	$0.94 \pm 0.11$	$20.59 \pm 2.51$
Measured $^3\text{H}$ -leucine incorporation (T0 <sub>control</sub> ; $\text{nmol C L}^{-1} \text{ day}^{-1}$ )	$0.44 \pm 0.01$	$2.45 \pm 0.35$

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