INORGANIC CARBON-ASSIMILATING MICROBIAL COMMUNITIES IN THE PACIFIC NORTHWEST COASTAL MARGIN.

By

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Definition
AAnPB	aerobic anoxygenic photosynthetic bacteria
amoA	ammonia monooxygenase subunit A
anammox	anaerobic ammonium oxidation
AOA	ammonia oxidizing archaea
AOA-A	archaeal ammonia oxidizing Thaumarchaeota Group A
AOA-B	archaeal ammonia oxidizing Thaumarchaeota Group B
AOB	ammonia oxidizing bacteria
CBB	Calvin-Benson-Bassham cycle
CCLME	California Current Large Marine Ecosystem
cDNA	complementary DNA
CR	Columbia River
CTD	conductivity-temperature-depth
DEPC	diethylpyrocarbonate
DIC	dissolved inorganic carbon
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DOC	dissolved organic carbon
EDTA	ethylenediaminetetraacetic acid
ENP	Eastern North Pacific
GF/F	glass microfiber filter
GSO	Gammaproteobacterial sulfur oxidizer
LP	LaPush
MG1	Marine Group 1
N+N	nitrate + nitrite
NH	Newport Hydroline
OMZ	oxygen minimum zone
OTU	operational taxonomic unit
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rTCA	reverse tricarboxylic acid cycle
RT-qPCR	reverse transcription polymerase chain reaction
RuBisCO	Ribulose-1,5-bisphosphate carboxylase oxygenase
SIP	stable isotope probing
SRB	Sulfate-reducing bacteria
TDN	total dissolved nitrogen
TDP	total dissolved phosphorous

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Abstract

The California Current Large Marine Ecosystem (CCLME) spans the coastal waters of Northern Washington through Baja California and is one of five Large Marine Ecosystems in the world that are subject to seasonal upwelling of cold nutrient rich water (Chavez and Messié, 2009). Upwelling promotes areas of intense localized primary productivity, which supports a vast array of fisheries vital to the Pacific Northwest economy. Recent seasonal occurrences of near-shore hypoxia off the coast of Oregon between 44^{o}N and 45^{o}N within the 100m depth contour, and the development of a 3,000 km^{2} dead zone in 2006, are indicators of ecosystem stress. The expansion of oxygen minimum zones (OMZs) in the continental shelf, (Grantham et al., 2004; Karstensen et al., 2008) and blooms fueled by upwelled nutrients are possibly the result of shifts in oceanic climate regimes due to global climate change. Increases in thermocline depth and the intensification of upwelling are predicted responses of eastern boundary current systems to climatic warming, however the role of climate and seasonal changes in the energy flow and population dynamics of microbial species within the CCLME has yet to be elucidated and are essential to identifying changes in ecosystem health (Grantham et al., 2004). The long-term goal of this research is to assess the microbial involvement in the biogeochemical cycling of carbon and nitrogen in relation to hypoxic events in coastal Oregon and Washington. The aim of this project was to identify seasonal variations in active dissolved inorganic carbon (DIC) - assimilating microbial populations in the water column off the coast of Oregon through stable isotope probing (SIP). The identification of active microbial populations promotes a greater understanding of the biogeochemical complexity and processes operating within the Pacific Northwest Coastal Margin while providing a basis for evaluating ecosystem change.

Hypoxic or low oxygen environments (DO < 2.0 mg/L) are generally associated with chemoautotrophic organisms that are able to thrive with little to no oxygen and utilize inorganic carbon. Stable isotope probing (SIP) with ^{13}C-$NaHCO_3$, a technique utilized as the foundation of this dissertation research, was used to examine the diversity of bacteria actively assimilating DIC in the Pacific Northwest coastal margin. SIP results ultimately revealed that that DIC-assimilation is ubiquitous in the Pacific Northwest marine environment, is utilized by both heterotrophic and autotrophic bacteria alike, and may represent a significant metabolic process in transient hypoxic waters. During the phylogenetic analysis of the active DIC-assimilating fractions, two observations were made. First, organisms previously found to be associated with hypoxic and low-oxygen environments all over the world (Canfield et al., 2010; Fuchs et al., 2005; Lavik et al., 2009a; Walsh et al., 2009a), gamma sulfur oxidizing (GSO) clade bacteria SUP05 and ARCTIC96BD-19, were implicated in DIC-assimilation in all SIP samples. Second, sequences for organisms involved in the nitrogen cycling pathways such as ammonia oxidation and heterotrophic denitrification were elevated in the SIP samples from areas that experience hypoxic events. The coupling of ammonia oxidation and low oxygen environments has been observed previously, particularly in the eastern tropical Pacific OMZ where ammonia oxidation was found to account for 6-33% of total NO_2^- production (Lam et al., 2009). While this does not imply that these organisms are absent from oxic environments it suggests that these organisms are potentially more active in these unique environments possibly due to reduced competition for ammonia.

To assess the abundance and distribution of SUP05 and ARCTIC96BD-19 GSO clade bacteria, identified in SIP experiments in relation to environmental variables, we conducted qPCR analysis on 49 samples collected from the Oregon and Washington coasts between 2007 and

2009. QPCR results presented evidence of the widespread occurrence of both SUP05 and ARCTIC96BD-19 in near-shore environment regardless of sampling location or season. SUP05 correlated negatively with dissolved oxygen and was most abundant in hypoxic waters. ARCTIC96BD-19 was much more prominent in inner-shelf samples, and its presence was strongly correlated to the coastal upwelling index, suggesting that wind-driven upwelling events, which pull nutrient-rich OMZ waters onto the continental shelf, influence the presence of GSO bacteria in the coastal margin.

The identification of organisms involved in ammonia oxidation led us to assess *amoA* gene transcription in the Pacific Northwest coastal margin in relation to low oxygen and the influence of freshwater from the Columbia River. Ammonia oxidation was studied by RT-qPCR amplification of *amoA* gene transcripts to assess the levels and distribution of Betaproteobacterial ammonia oxidizing bacteria (AOB), Thaumarchaeota Group A ammonia oxidizing archaea (AOA-A) and Thaumarchaeota Group B ammonia oxidizing archaea (AOA-B) *amoA* gene expression in water collected at two different depths in May and July 2010 from two sampling lines subject to varying influence of freshwater discharge from the Columbia River. Sample sites subject to the greatest Columbia River influence experienced dramatic shifts in DO and nutrient levels possibly linked to the onset of upwelling and decrease river discharge. These samples showed the greatest variability in AOA-A and AOA-B contribution to *amoA* transcription. Our results demonstrate the biogeochemical potential of DIC-assimilating organisms in carbon and nitrogen cycling pathways in the Pacific Northwest coastal margin, influenced by the dynamic environment of the Northeast Pacific coastal zone.

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Chapter 1: Introduction

The California Current, which includes the Pacific Northwest coastal margin, belongs to one of five major eastern boundary currents affiliated with upwelling zones including the Humboldt Current (located off the coast of Chile and Peru), the Canary Current (located off the coast of northwest Africa), the Benguela Current (located off the coast of southwest Africa), and the Somali Current (located in the western Indian Ocean) (Chavez and Messié, 2009). Eastern boundary currents, driven by wind and deflected by landmasses, are home to some of the world's most productive fisheries. The California Current extends from British Columbia through Baja California and is a productive, complex, and dynamic environment (Connolly et al., 2010). Southward wind patterns lead to the offshore transport of surface water, allowing cold, nutrient-laden water to upwell into the photic zone. Upwelling during the summer months stimulates primary production (phytoplankton growth) which, in turn, fuels the productive fisheries characteristic of the Pacific Northwest (Huyer, 1983). Northward wind stress induces downwelling characteristic of winters in the Pacific Northwest coastal margin bringing warm, nutrient–depleted surface water onshore from offshore sources resulting in very low levels of primary production (Huyer, 1983). While fresh water from the Columbia River influences both the Washington and Oregon shelves during summer (Hickey et al., 2006, 2005), nutrient concentrations in the Columbia River plume are typically low compared with upwelled water (Bruland et al., 2008a; Hill and Wheeler, 2002).

The world's largest Oxygen Minimum Zone (OMZ) lies beneath the California Current system (Fig. 1.1) (Gilly et al., 2013). OMZs, oxygen-depleted regions of the ocean often found at the edges of continental margins, form when oxygen demand during the degradation of organic matter exceeds dissolved oxygen (DO) availability in poorly ventilated areas within the water

column (Helm et al., 2011; Karstensen et al., 2008; Lam and Kuypers, 2011; Paulmier and Ruiz-Pino, 2009).

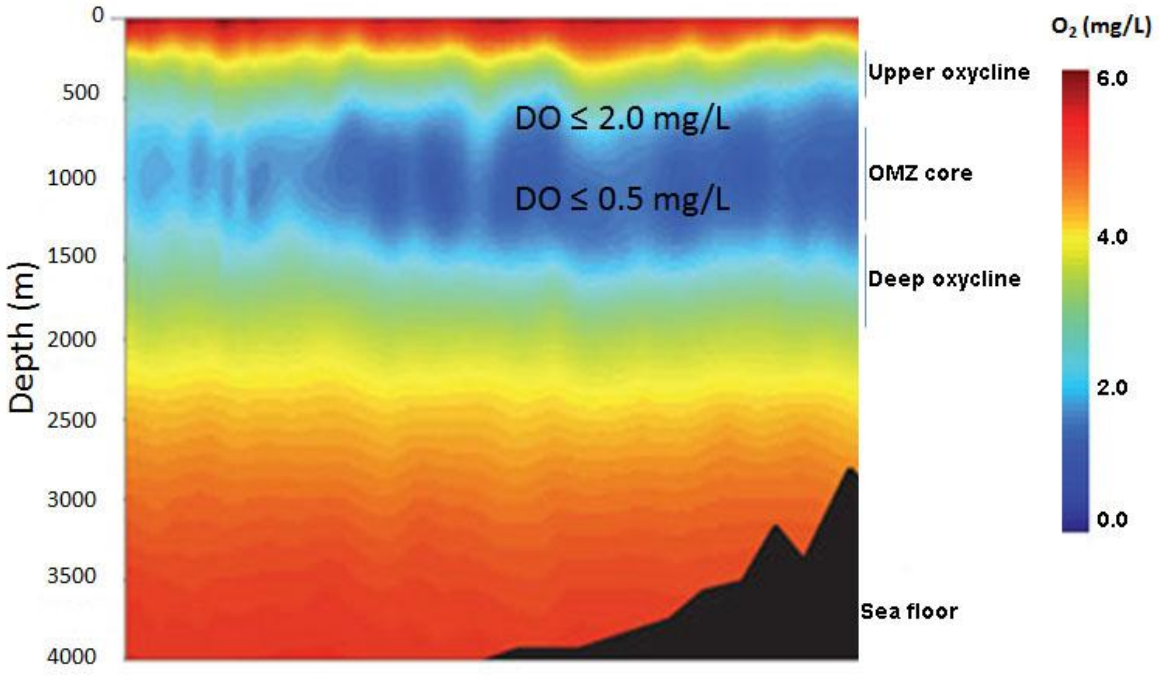

Figure 1.1 Cross-section of the Northeast Pacific oxygen minimum zone (OMZ), showing the O_2 concentration from surface waters to the sea floor. Upper oxycline: transition from surface waters to the OMZ core. Modified from Wright et al., 2012a.

Climate models under global warming scenarios have predicted changes in the balance of factors determining oceanic oxygen levels, leading to an overall decline in oxygen and an expansion of OMZs (Bograd et al., 2008). Models predict a continued decline in DO because oxygen is less soluble in warm water, and because increasing water column stratification results in a reduction in the supply of oxygen-rich water to greater depths within the water column (Helm et al., 2011; Karstensen et al., 2008). Declines of oxygen in the ocean will have potentially serious consequences. Many organisms important to marine fisheries are extremely sensitive to drops in oxygen levels; fluctuations of DO at higher concentrations are tolerated until reaching an intolerable minimum threshold at which life is unsustainable. This threshold varies depending on

the marine taxa, but, in general, fish and crustaceans are the most sensitive to decreases in oxygen (Vaquer-Sunyer and Duarte, 2008).

The northeast Pacific is of particular interest because, as water is brought to the continental shelf via upwelling, a small oxygen decrease in upwelled source water could cause a large expansion of local shelf hypoxia (DO< 2.0 mg/L) (Pierce et al., 2012). Between 1956 and 2006 the DO concentrations in the OMZ in the northeast Pacific Ocean declined by 22%, and the hypoxic boundary layer (DO < 2.0 mg/L) expanded higher into the water column to depths of 300m – 400m from an average of 500m (Bograd et al., 2008; Emerson et al., 2004). In the Northeast Pacific, cold, nutrient-rich OMZ water is pulled into the photic zone by wind driven upwelling events, providing inorganic nutrients that fuel photosynthetic growth. While the process is necessary to sustain the productive Pacific Northwest fisheries, in recent years on the continental shelf, a lack of mixing combined with high DO demand below the surface has led to the depletion of DO often resulting in hypoxic or anoxic environments (Chan et al., 2008; Grantham et al., 2004).

Coastal Oregon has received significant attention in the past decade due to frequent reports of seasonal near-shore hypoxia in shallow areas (<200 meter water column depth) of the continental shelf (Chan et al., 2008). DO is the preferred electron acceptor for the respiration of organic matter in well oxygenated marine environments (Lam and Kuypers, 2011). In hypoxic (and anoxic) environments, however, aerobic respiration is unsustainable (Karstensen et al., 2008; Keeling et al., 2010). Chemoautotrophic organisms that use alternative electron donors and heterotrophic microorganisms that can utilize alternative electron acceptors for respiration (chemoautotrophs), or are capable of harnessing energy from various redox reactions to fix organic carbon, can proliferate under low DO conditions, resulting in the establishment of unique

microbial communities distinct from those of oxic marine environments (Lam and Kuypers, 2011).

The Columbia River, responsible for the second-largest freshwater discharge in the United States, may also have a strong effect on microbial communities in the coastal Northeast Pacific (Fortunato and Crump, 2011; Smith et al., 2010). River discharge fluctuates seasonally with the highest volumes in April through June, due mainly to snowmelts and the lowest volumes in September and October. The river has a profound influence on biogeochemical processes in the coastal ocean as nutrients flushed out with freshwater from the estuary generates a large plume that, during high discharge periods, can extend many kilometers from the river mouth (Bruland et al., 2008a; Hickey et al., 2005), reaching as far south as Newport, Oregon (184 km from the river mouth) in the summer and as far north as La Push (185 km from the river mouth), Washington in the winter. The plume, however, becomes largely relegated to surface waters as distance from the mouth of the river increases. Beneath the plume, particularly off the mouth of the Columbia River, hypoxic environments can persist for long periods of time (Roegner et al., 2011).

1.1 **Marine Microbial Communities and Nutrient Cycling**

Microbial communities are strongly affected by hypoxia and declines in DO. In these environments more production and energy flow through heterotrophic and autotrophic microbial communities rather than through higher trophic levels (Diaz and Rosenberg, 2008; Wright et al., 2012a). Phytoplankton bloom in response to the influx of nutrients carried to the surface waters by wind-driven upwelling of nutrient-rich OMZ water into the inner shelf environment of eastern boundary systems. As the blooms decay and sink, heterotrophic microbial communities respond

by decomposing the organic matter, fueling respiration (Grantham et al., 2004). Heterotrophic microorganisms consume dissolved organic carbon (DOC) using O_2 as the electron acceptor in the final step in aerobic respiration, and respire CO_2 as a byproduct of the citric acid cycle. Continued respiration can further encourage DO deficits, particularly if an abundance of organic carbon sources are available, such as settling carbon from phytoplankton blooms (Chan et al., 2008), and if downwelling events are delayed. The continued depletion of DO through heterotrophic metabolism eventually results in the depletion of O_2, heterotrophic populations become difficult to maintain, and mixotrophic, autotrophic, or facultative autotrophic organisms become favored (Zehnder and Stumm, 1988). If the depletion of O_2 leads to an anoxic environment anaerobic metabolism is then favored.

As OMZs expand and inner shelf hypoxia becomes more persistent, changes in microbial communities and microbe-mediated biogeochemical cycles are hypothesized. When the system becomes anoxic nitrate becomes important in respiration, eventually replacing O_2 as the primary electron acceptor. When nitrate is exhausted, the biogeochemistry becomes dominated by sulfate-reducing microorganisms, which convert sulfate to sulfide (Gilly et al., 2013). The effect of hypoxia on nitrogen cycling has global implications, the extent of which controls the loss of fixed nitrogen in the ocean via denitrification (Keeling et al., 2010). Denitrification, the reduction of nitrate to gaseous products, affects nitrate availability, a limiting nutrient in the world's oceans (Codispoti et al., 2001). DO levels also control the oceanic production of nitrous oxide (N_2O), a powerful greenhouse gas that is produced as a by-product of denitrification and under oxic conditions during nitrification. Increased production of N_2O by nitrification and denitrification will occur with increasingly hypoxic conditions amplifying global warming (Keeling et al., 2010). O_2 also influences the cycling of marine phosphorous (P), with greater

recycling of P from sediments occurring when O_2 levels are low in the overlying water (Wallmann, 2003). In light of this, we hypothesized that on the Oregon and Washington coasts, transient hypoxic and low-oxygen events would select for autotrophic or facultative autotrophic organisms as communities are forced to use alternative electron acceptors when faced with the depletion of DO. Furthermore, we expected to find organisms involved in alternative nitrogen and sulfur cycling pathways to be elevated in areas more frequently subjected to hypoxic conditions.

1.1.1 **Inorganic Carbon Fixation in the Marine Environment**

O_2 is linked to the carbon cycle through photosynthesis and respiration. The oxidation of organic matter in surface waters produces high concentrations of DIC in OMZ waters. Organisms capable of autotrophic metabolism assimilate inorganic carbon into organic carbon. Autotrophic organisms form an integral part of ecosystems by making an otherwise unavailable form of carbon available to other organisms, a central component of the global carbon cycle (Hügler and Sievert, 2011). The assimilation of inorganic carbon is often attributed to primary production carried out by autotrophic organisms (Bassham and Calvin, 1957a). Autotrophs build biomass entirely from inorganic carbon and produce organic carbon used by heterotrophic organisms. Heterotrophs, in turn, complete the carbon cycle by oxidizing the organic carbon back to inorganic carbon. In the ocean, most primary production is carried out in the photic zone by cyanobacteria and eukaryotic phytoplankton. These organisms use the Calvin-Benson-Bassham (CBB) cycle for carbon fixation and oxygenic photosynthesis for energy (Paerl and Pinckney, 1996; Raven, 2009).

1.1.1.1 **The CBB Cycle**

The CBB cycle is the major route in which CO_2 is reduced to form organic carbon. In the first stage, bisphosphate ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) catalyzes CO_2 fixation (Badger and Bek, 2008; Bassham and Calvin, 1957a; Tabita et al., 2007). RuBisCO is considered to be the major global CO_2 fixation catalyst for most autotrophic organisms, including photosynthetic and chemolithoautotrophic bacteria, archaea, eukaryotic algae and plants. While four different types of RuBisCO proteins are known (forms I–IV), only forms I, II, and III catalyze the carboxylation reaction (Tabita et al., 2007). Form IV, known as the RuBisCO-like protein, is considered to be in involved in reactions associated with sulfur metabolism (Badger and Bek, 2008).

Although the CBB cycle is generally considered the dominant pathway for inorganic carbon assimilation in the ocean, recent research has uncovered the extent of bacterial and archaeal DIC assimilation in the ocean beyond oxygenic photosynthesis. Five alternative inorganic carbon fixation pathways are now known in addition to the CBB cycle: the reductive tricarboxylic acid (rTCA) cycle, the reductive acetyl-CoA pathway, the 3-hydroxypropionate (3-HP) cycle, the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle, and the dicarboxylate/4-hydroxybutyrate (DC/4-HB) cycle (Berg et al., 2010; Buchanan and Arnon, 1990; Huber et al., 2008; Ljungdhal, 1986; Zarzycki et al., 2009). Of these alternative fixation pathways, three are known to operate in bacteria: the rTCA cycle, the reductive acetyl-CoA pathway, and the 3-hydroxypropionate (3-HP) cycle (Hügler and Sievert, 2011).

1.1.1.2 **The rTCA Cycle**

The rTCA cycle is essentially a reversal of the oxidative tricarboxylic acid (TCA) cycle used by heterotrophic organisms to oxidize acetyl-CoA to CO_2 and generate energy in the form of

adenosine triphosphate (ATP) (Buchanan and Arnon, 1990). In the rTCA cycle acetyl-CoA is formed from two molecules of CO_2 (Fig. 1.2). The rTCA cycle utilizes enzymes involved in the TCA cycle such as malate dehydrogenase, fumarate hydratase, succinyl-CoA synthetase, isocitrate dehydrogenase, and aconitate hydratase. However, fumarate reductase, 2-oxoglutarate synthase (2-oxoglutarate: ferredoxin oxidoreductase), and the citrate cleaving enzymes are unique to the rTCA cycle (Buchanan and Arnon, 1990). The ATP-dependent cleavage of citrate to acetyl-CoA and oxaloacetate, the central reaction of the rTCA cycle, is catalyzed by ATP citrate lyase or by the combined action of citryl-CoA synthetase and citryl-CoA lyase (Aoshima, 2007).

The rTCA cycle is used by a variety of bacteria, but is generally limited to anaerobic or microaerophilic bacteria due to the oxygen sensitivity of two enzymes involved in the cycle, 2-oxoglutarate synthase and pyruvate synthase (Hügler et al., 2005). Obligate photoautotrophic and anaerobic green sulfur bacteria (Chlorobiales) (Wahlund and Tabita, 1997), members of Aquificales and the Epsilonproteobacteria (Hügler et al., 2005, 2007) nitrite oxidizing bacteria *Nitrospira* (Lücker et al., 2010), and some members of the Alphaproteobacteria (Williams et al., 2006), Gammaproteobacteria (Hügler et al., 2010), and Deltaproteobacteria (Schauder et al., 1987) use the rTCA cycle to assimilate CO_2. In OMZs the rTCA cycle has been identified as active in nitrite oxidizing bacteria among Proteobacteria, *Nitrospira*, and *Nitrospina*, and within the anaerobic green sulfur bacteria, *Chlorobiales* (Hügler and Sievert, 2011).

1.1.1.3 **Reductive Acetyl-CoA Pathway**

The reductive acetyl-CoA pathway combines two molecules of CO_2 to generate acetyl-CoA. The pathway is divided into the methyl and carbonyl branches (Fig. 1.2) (Ljungdhal, 1986). In the methyl branch one molecule CO_2 is reduced to a cofactor-bound methyl residue and in the

carbonyl branch a second molecule of CO_2 is reduced to an enzyme-bound carbonyl residue (Fig. 1.2). CO dehydrogenase/acetyl-CoA synthase catalyzes the reduction of CO_2 to CO as well as the synthesis of acetyl-CoA from the methyl and the carbonyl residues (Pezacka and Wood, 1984; Ragsdale and Wood, 1985).

The reductive acetyl-CoA pathway was first identified in acetogenic bacteria (Ljungdahl and Wood, 1969; Ljungdhal, 1986), most of which are Clostridiales (Drake et al., 2008), as well as Spirochaeta (Leadbetter et al., 1999). Acetogenic bacteria form acetate from H_2 and CO_2, but can also grow heterotrophically (Drake et al., 2008) exhibiting a versatile metabolism. It is also active in autotrophic sulfate-reducing bacteria, archaea and methanogenic archaea (Fuchs, 1995; Jansen et al., 1984; Schauder et al., 1987; Vornolt et al., 1995). In OMZs, however, the reductive acetyl-CoA pathway has been found to be active only in anaerobic ammonium oxidation (anammox) bacteria (Hügler and Sievert, 2011).

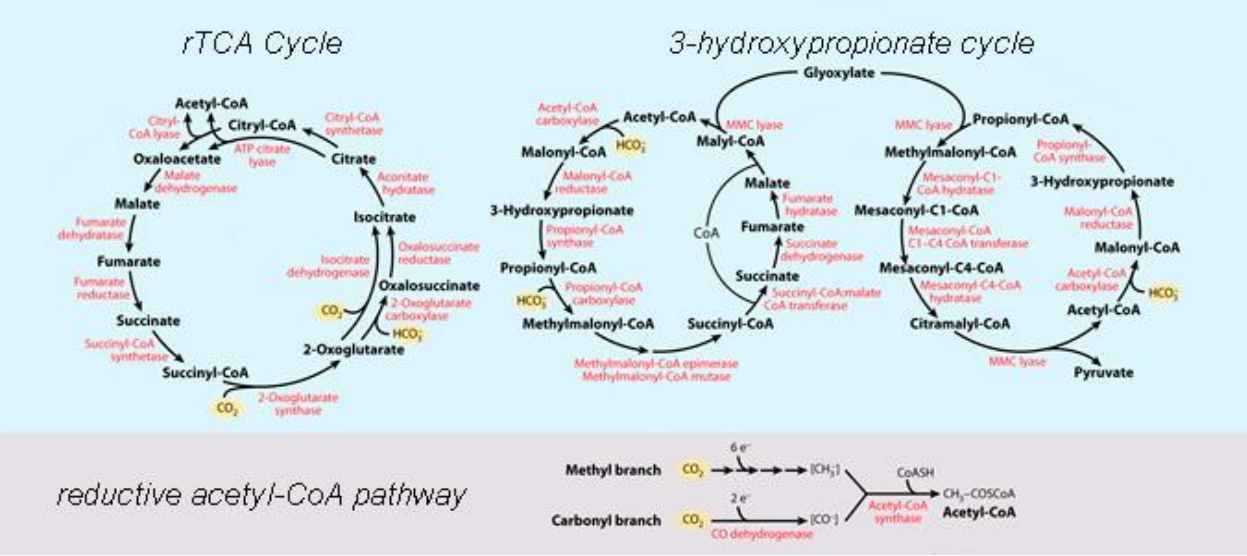

Figure 0.2 Biochemical reactions and enzymes (red) involved in alternative pathways of CO_2 fixation: the reductive tricarboxylic acid cycle (rTCA), 3- hydroxypropionate cycle, and reductive acetyl-CoA pathways. The reductive acetyl-CoA pathway (shaded in pink) is known to operate in both archaea and bacteria. This figure was modified from Hügler and Sievert, 2011.

1.1.1.4 **3-Hydroxypropionate Cycle**

Two cycles are involved in the 3-hydroxypropionate mechanism of carbon assimilation. The first cycle forms glyoxylate as two molecules of bicarbonate are fixed (Fig. 1.2). In the second cycle, glyoxylate and propionyl-CoA are used to produce pyruvate and acetyl-CoA (Zarzycki et al., 2009). The cycle ultimately results in the net fixation of 3 molecules of bicarbonate into 1 molecule of pyruvate, which can then be used to produce cellular building blocks (Fig. 1.2) (Zarzycki et al., 2009).

The 3-hydroxypropionate cycle is used to fix inorganic carbon within green nonsulfur bacteria belonging to the family Chloroflexaceae, and specifically in *Chloroflexus aurantiacus* (Herter et al., 2002; Strauss and Fuchs, 1993; Zarzycki et al., 2009). Some Alphaproteobacteria and Gammaproteobacteria harbor genes encoding enzymes of the 3-hydroxypropionate cycle, but are lacking the full complement of genes (Zarzycki et al., 2009). These organisms likely employ a rudimentary cycle for the mixotrophic assimilation of acetate, 3-hydroxypropionate, and/or propionate under oligotrophic marine conditions (Zarzycki et al., 2009).

These aforementioned alternative carbon fixation pathways are used primarily by chemolithoautotrophic bacteria. However, these pathways are fundamental components of inorganic carbon assimilation for organisms able to carry out mixotrophic metabolism (Moran and Miller, 2007). Evidence of widespread mixtotrophy in the marine environment is well documented (Eiler, 2006; Moran and Miller, 2007). Mixotrophic organisms can use multiple metabolic strategies at the same time to fix carbon, or switch between different metabolic strategies. For example, aerobic anoxygenic photosynthetic bacteria (AAnPB) have been shown to supplement energy needs by using light, making them remarkably efficient in oligotrophic environments (Kolber et al., 2001; Moran and Miller, 2007). Some strains of AAnPB have the

capability to exhibit a mixotrophic carbon metabolism or the ability to grow utilizing light and inorganic carbon (Moran and Miller, 2007; Swingley et al., 2007; Tang et al., 2009). The majority of mixotrophic bacteria utilize CO_2 and HCO_3^- to supplement metabolic pathways that function in the synthesis of nucleotides, amino acids, and fatty acids (Sauer and Eikmanns, 2005). However, DIC assimilation in these organisms depends on several factors including 1) the type of organic substrate utilized for growth (Romanenko, 1964; Sorokin, 1966) and 2) the metabolic state of the organisms (Feisthauer et al., 2008).

1.1.2 **Nitrogen Cycling**

Nitrogen is an essential element of life and its availability limits biological productivity in most marine ecosystems (Francis et al., 2005). Biological nitrogen fixation is the largest source of nitrogen to the ocean while anaerobic microbial processes mediate nitrogen loss (Galloway et al., 2004; Gruber and Sarmiento, 1997). Biological nitrogen fixation and anaerobic nitrogen losses are connected by the microbially mediated process of nitrification. Nitrification is the conversion of ammonium (NH_4^+) to nitrate (NO_3^-) and consists of two steps: the oxidation of ammonium to nitrite, followed by nitrite oxidation to nitrate. Nearly 4×10^{11} kg of nitrogen cycles through the ocean yearly (Galloway et al., 2004), the majority of which is nitrified (Karl, 2002).

In the coastal ocean, nitrification is coupled to denitrification, the microbial reduction of nitrate (NO_3^-) to nitrogen gas (N_2) in the absence of oxygen. In estuaries and continental shelf environments, a portion of anthropogenic nitrogen pollution is removed through this coupled process before it is carried to the open ocean (Francis et al., 2005). In the open ocean, 30-50% of nitrogen loss occurs in oxygen minimum zones (OMZs), but these losses are attributed more to anaerobic ammonium oxidation, or “anammox” rather than denitrification (Kuypers et al., 2005).

During anammox nitrite (NO_2^-) and ammonium (NH_4^+) are converted directly into dinitrogen gas. However, the oxidation of NH_4^+ occurs at the expense of NO_2^- produced through heterotrophic NO_3^- reduction or aerobic ammonia oxidation, the first step of nitrification (Francis et al., 2005).

Differences in the sensitivity of nitrogen cycling processes to temperature, light, oxygen, pH, and other environmental factors allow for hypoxic events to potentially alter the nitrogen cycle. Competition for inorganic nitrogen is intense in marine environments, particularly in the photic zone where nitrifying microbial populations compete with photosynthetic organisms and facultative lithotrophs (Zehr and Ward, 2002).

Ammonia oxidation, the first rate-limiting step in nitrification, is performed by a few species of bacteria among the Beta- and Gammaproteobacteria and by members of the archaeal ammonia oxidizing Thaumarchaeota Group A (AOA-A) and archaeal ammonia oxidizing Thaumarchaeota Group B (AOA-B). Ammonia oxidizing archaea (AOA) are considered to be autotrophic (Hatzenpichler et al., 2008; Könneke et al., 2005; De La Torre et al., 2008) as are AOB (Soriano and Walker, 1968). AOB assimilation of carbon dioxide is initiated by a type I RuBisCO as part of the CBB cycle (Chain et al., 2003; Schramm et al., 1998; Utåker et al., 2002) whereas AOA fix DIC through the 3-hydroxypropionate cycle (Hallam et al., 2006; Pratscher et al., 2011).

Nitrate is the first preferred alternative electron acceptor to oxygen and therefore an important energy source in low-oxygen and transient hypoxic environments. In low oxygen environments of the Eastern Tropical South Pacific, nitrification and CO_2 fixation were identified in the water column - in particular, ammonia oxidation rates were highest in the oxycline and within the OMZ (Ward et al., 1989). More recent studies have identified active nitrification activity (Füssel et al., 2012; Kalvelage et al., 2011) and the expression of ammonia oxidation (*amoA*) genes

(Lam et al., 2009) at very low oxygen concentrations. These studies demonstrate the potential for aerobic metabolism in persistently low oxygen environments. Ulloa et al., 2012, suggest that the process of ammonia oxidation may be dormant until activated by recurrent oxygen injection events such as downwelling events.

The significance of AOA vs. AOB activity has been a topic of interest over the past decade. Previous studies in marine environments have shown that AOA were two to three orders of magnitude more abundant than AOB (Mincer et al., 2007; Wuchter et al., 2006), and research conducted in the Humboldt system off the coast of northern Chile observed AOA transcripts and genes involved in ammonia oxidation in the oxycline (Stewart et al., 2012), suggesting the importance of AOA within the oxic-anoxic transition zone. In general, AOA are considered to be the dominant ammonia oxidizers in marine environments, while AOBs occupy different aquatic niches, e.g. freshwater systems (Bouskill et al., 2012; Mincer et al., 2007). Niche differentiation may be due to the high ammonium affinity (Km = 133 nM NH_4^+) and low thresholds (≤10 nM) of AOA to its substrate (Martens-Habbena et al., 2009), making AOA uniquely adapted to thrive in oligotrophic, or low nutrient environments. In contrast, the AOB have a much lower affinity for ammonium compared to AOA (the lowest recorded Km = 30-80 µmol NH_4^+ in *N. oligotropha*).

1.1.3 **Sulfur Cycling**

Sulfate reduction, where sulfate is reduced to sulfide, is a process most commonly found in marine sediments (Jørgensen, 1977) and within the water column of anoxic basins (Hastings and Emerson, 1988), but recent research has detected this process within the water column of hypoxic and anoxic environments. Sulfate-reducing bacteria (SRB) in low oxygen waters were detected in the OMZ of the Somali-Arabian Sea upwelling system (Fuchs et al., 2005), the

Benguela upwelling system (Lavik et al., 2009b), and Humboldt upwelling system (Canfield et al., 2010). Sulfide is toxic to many marine organisms, yet Canfield et al., 2010 found that the sulfide was rapidly oxidized by sulfur oxidizing bacteria (SOB) in the Humboldt system, limiting the accumulation of sulfide in the water column.

The Gammaproteobacterial sulfur oxidizers (GSOs), SUP05 and ARCTIC96BD-19, are now synonymous with hypoxic and anoxic environments having been found in oxygen-deficient marine environments around the world (Fuchs et al., 2005; Lavik et al., 2009b; Stevens and Ulloa, 2008; Walsh et al., 2009a). SUP05 and ARCTIC96BD-19 have non-identical, overlapping distribution patterns. SUP05, which derives energy from the oxidation of reduced sulfur compounds with nitrate as a terminal acceptor, is more abundant in slightly to moderately sulfidic conditions at the base of the sulfide-nitrate transition zone (Lavik et al., 2009b; Walsh et al., 2009a). ARCTIC96BD-19 is most abundant in hypoxic to suboxic waters and derives energy from reduced sulfur compounds with oxygen as a terminal electron acceptor (Swan et al., 2011; Walsh and Hallam, 2011). Both SUP05 and ARCTIC96BD-19 bacteria have the potential to fix inorganic carbon via RuBisCO and to obtain energy from the oxidation of reduced sulfur compounds (Walsh and Hallam, 2011). SUP05 and ARCTIC96BD-19 GSO clade bacteria have been identified in the seasonally hypoxic coastal waters of Oregon and Washington (Fortunato et al., 2013).

1.2 **Linking Microbial Community Structure and Function**

Microbial community diversity in the environment is governed by ecological niche diversity (Neufeld et al., 2007). Individual populations within microbial communities respond to particular carbon sources and energy sources. As a result, the types and availability of substrates regulate community composition. This is referred to as bottom up control, where microorganisms are

limited by organic and inorganic nutrients, as opposed to top down control where microorganisms are limited by the number of bacterial predators (Solic et al., 2009). Techniques to evaluate the metabolic roles of microorganisms in the environment focus on the enrichment and isolation of organisms on defined media containing specific nutrient sources. This approach has been effective in retrieving and characterizing individual organisms that metabolize specific substrates. Cultivation-dependent methods, however, are limited by the large proportion of microorganisms that are not readily cultivated (Amann et al., 1995) because conditions present in the environment are often different from what the laboratory is able to provide.

The introduction of cultivation-independent molecular methods in the 1980's and 1990's has exponentially increased our understanding of microbial community structure in natural ecosystems (Amann et al., 1995). The 16S rRNA gene is now an integral part of microbial diversity studies and has been widely used to examine differences in community structure. Molecular techniques such as cloning or pyrosequencing of 16S rDNA are excellent tools for the evaluation of uncultivated organisms in the environment. However, 16S rRNA gene sequences alone provide very little information about an organism's role within the microbial community and the environment, particularly if the sequences identified belong to uncultured organisms with unknown functions. DNA can be used to determine which organisms are present, and what functional genes have the potential to be expressed, but not which organisms are active. Some genes within an organism's DNA may not be expressed; furthermore DNA can persist in the environment for a relatively long period of time therefore identifying some organisms that may not be present in a viable state.

DNA-based stable-isotope probing (SIP) is a technique used in microbial ecology to link the identity of microorganisms to their function (Neufeld et al., 2007; Radajewski et al., 2000).

Labeled substrates such as ^{13}C or ^{15}N enriched compounds are added to environmental samples and incubated (Radajewski et al., 2000). The labeled substrate is incorporated into the biomass of organisms that have ability to utilize it; the stable isotope is integrated into the DNA. The ^{13}C or ^{15}N renders the DNA of the active substrate-utilizing organisms slightly heavier than those that are dead, or simply unable to metabolize the substrate. To briefly summarize the process, DNA from the sample incubation is extracted and added to a cesium chloride gradient with ethidium bromide (EtBr) to visualize DNA that migrates to different densities within the gradient. The sample is then centrifuged at speeds greater than 33,000 rpm for a minimum of 24 hours. The bands separate along the cesium chloride gradient during centrifugation with the heavy band, consisting of DNA that incorporated the isotope, migrating down and away from the lighter band of DNA bearing no heavy isotope. DNA bands are then recovered and subjected to further processing and analysis. In the case of our work in the Pacific Northwest coastal margin, the recovered DNA was purified and PCR amplified with primers specific for bacterial 16S rDNA. The PCR fragments of 16S rDNA gene sequences were cloned and subject to sequence analysis. The separation and molecular analysis of the labeled DNA revealed phylogenetic and functional information about the microorganisms responsible for the active metabolism of the added, isotopically labeled substrate. To limit the influence of the cross-feeding and the potential "bottle effect," in which microorganisms in batch-wise incubations (confined environments) are subject to influences of grazing/bacteriovory, changes in viability and activity parameters, changes in cultivability, and changes in population composition (Hammes et al., 2010), we limited all SIP incubations to 3hrs.

1.3 Assessing Inorganic Carbon Assimilating Communities in the Pacific Northwest Coastal Margin

Numerous surveys of microbial diversity within the Pacific Northwest Coastal Margin have provided considerable insight into the structure of microbial assemblages residing within various depths of the water column (Bertagnolli et al., 2011; Fortunato and Crump, 2011; Fortunato et al., 2013; Smith et al., 2010, 2013). Tag-encoded pyrosequencing of the 16S rRNA gene found that bacterioplankton communities in the Columbia River plume, epipelagic ocean, shelf bottom (depth<150 m), and slope bottom (depth>300 m) formed distinct assemblages (Fortunato and Crump, 2011). However, it was also found that epipelagic communities can be altered in response to local physical, chemical, and biotic conditions, suggesting that distinct assemblages of bacterioplankton communities can shift in microbial composition as a result of local environmental perturbations (Morris et al., 2006).

Recurrent seasonal hypoxia and anoxia in the Pacific Northwest Coastal Margin, particularly within the inner shelf environment, in concert with the suspected OMZ expansion below the California Current, highlights the need to understand the microbial structure and dynamics within this complex environment. Predictions regarding OMZ expansion and increased near-shore hypoxia favor the diversion of energy flow into lower trophic levels, particularly autotrophic bacteria, impacting the carbon, nitrogen and sulfur cycles (Wright et al., 2012a). A framing hypothesis for this thesis was that the transient hypoxic environment would select for autotrophic organisms able to utilize alternative electron acceptors, e.g. NO_3^-. Thus, we used SIP with ^{13}C-$NaHCO_3$ as the foundation to assess DIC-assimilation, a requirement for autotrophic growth.

The primary questions addressed were:

1) What bacteria actively assimilate DIC in the Pacific Northwest Coastal Margin?
2) Are any of the bacteria identified through SIP associated with low-oxygen or hypoxic environments?
3) What are the influences of coastal upwelling of OMZ water and the Columbia River on processes associated with nitrogen cycling in the coastal environment?

In Chapter 2, DNA-based SIP analysis with ^{13}C-$NaHCO_3$ combined with 16S rDNA sequence analysis was employed to examine the active DIC-assimilating microbial community in bottom water samples at three locations within the near-shore, inner shelf environment. Samples were collected from surface (2m) and 5m from bottom depth at 3 different sites off the Oregon and Washington coasts in May 2010, and one site off the Oregon Coast in September 2008 and March 2009 and incubated in the absence of light to minimize the stimulation of photoautotrophy. Surprisingly, results revealed that DIC-assimilation is not limited to autotrophic or even mixotrophic bacteria. In fact, a large diversity of marine bacteria, including many bacteria considered to be heterotrophic, was able to actively assimilate DIC.

Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes contributed the largest proportion to the active DIC-assimilating communities. 16S rRNA gene sequences related to the GSO clade ARCTIC96BD-19 were observed in all active DIC-assimilating populations. Although not a dominant contributor to our active clone libraries, Betaproteobacteria, when identified, were predominantly comprised of Burkholderia, in particular organisms potentially involved in ammonia oxidation, with the vast majority of clones found on the Columbia River Line sampling location in May 2010. An analysis of DIC-assimilating bacterial communities was also conducted at one site off the Oregon Coast that may be subject to seasonal variations in river

particulate discharge as well as hypoxia. These data are presented in Appendix A. The findings of Chapter 2 and Appendix A lay the foundation for Chapter 3 and Chapter 4.

Chapter 3 details a study that was initiated by the finding that GSO clade bacteria were identified as actively assimilating DIC in all SIP clone libraries. Given the association between SUP05 and ARCTIC96BD-19 and hypoxic and anoxic environments (Fuchs et al., 2005; Lavik et al., 2009b; Stevens and Ulloa, 2008; Walsh et al., 2009a) and the assertion that SUP05 may serve as a sentinel for hypoxia and OMZ expansion (Walsh et al., 2009a) our goal was to evaluate the distribution and occurrence of GSO clade bacteria in the Pacific Northwest Coastal Margin. Focusing on the same sampling lines used for SIP analysis, DNA from 49 individual samples collected between August 2007 and September 2009 were analyzed for the presence of SUP05 and ARCTIC96BD-19 bacteria using qPCR. Samples were collected 5m from the benthic boundary layer, with varying depth along 3 coastal ocean transects, as well as from within the OMZ. Our results indicate that both SUP05 and ARCTIC96BD-19 have a consistent presence within the inner continental shelf of the Northeast Pacific Ocean. ARCTIC96BD-19, however, was much more abundant, particularly in inner-shelf samples, and strongly correlated to the coastal upwelling index suggesting that wind-driven upwelling events influence the abundance of ARCTIC96BD-19 in the coastal margin.

In Chapter 4, I quantified *amoA* gene transcript levels using RT-qPCR amplification to assess the abundance and distribution of AOB, AOA-A, and AOA-B *amoA*. Samples were characterized by varying degrees of influence from the Columbia River plume during periods of high flow, pre-freshet (May 2010) and low flow, post-freshet (July 2010). In general, AOA-A *amoA* gene transcript copy numbers/ml were higher than AOB copy numbers in Newport Hydroline surface and bottom samples in all seasons. In the Columbia River line samples, however, AOB *amoA*

gene transcript copy numbers/ml were higher than AOA-A transcript copy numbers/ml in surface and bottom samples during high flow conditions. Ratios of AOA-A and AOA-B *amoA* gene transcripts to marine group 1 Crenarchaea 16S rRNA gene transcripts suggest shifts in AOA-A and AOA-B contributions to archaeal ammonia oxidation at bottom samples on the Columbia River Line. AOA-B increased ammonia oxidation activity concurrent with a decline in AOA-A activity from May to July. The sample collected closest to the mouth of the Columbia River, subject to the greatest influence from freshwater discharge experienced dramatic shifts in DO and nutrient levels possibly linked to the onset of upwelling and concurrent decreases in river flow. Columbia River Line sites experienced the greatest variability in AOA-A and AOA-B contribution to *amoA* transcription compared to the Newport Hydroline samples 184 km south. Our results suggest that freshwater discharge of the Columbia River, as well as coastal upwelling events may have a profound influence on microbial mediated ammonia oxidation in Northeast Pacific coastal zone.

Understanding how hypoxia in the marine environment is changing is important, not just because of the impact on fisheries, but because measured changes in DO, together with changes in nutrients, are a useful diagnostic of changing biogeochemistry (Keeling et al., 2010). Many biogeochemical cycles are controlled by microorganisms. We sought to assess the microbial community and its biogeochemical potential in carbon and nitrogen cycling pathways in the Pacific Northwest coastal margin in response to seasonal hypoxia. We uncovered a dynamic environment that may be adapted to the influx of upwelled OMZ waters characterized by low DO, high nutrients, and high DIC. This environment could be further influenced by the freshwater input of the Columbia River. The results of this dissertation work in concert with a growing body of evidence that the Pacific Northwest coastal margin is a complex ecosystem that

plays an integral role in marine nutrient cycling (i.e. Bertagnolli et al., 2011; Fortunato and Crump, 2011; Fortunato et al., 2011, 2013; Smith et al., 2010, 2013), serving as a foundation for which the measurements of ecosystem change can be assessed.

Chapter 2: Ubiquitous dissolved inorganic carbon assimilation by marine bacteria in the Pacific Northwest coastal ocean as determined by stable isotope probing

2.1 Abstract

In order to identify bacteria that assimilate dissolved inorganic carbon (DIC) in the northeast Pacific Ocean, stable isotope probing (SIP) experiments were conducted on water collected from 3 different sites off the Oregon and Washington coasts in May 2010, and one site off the Oregon Coast in September 2008 and March 2009. Samples were incubated in the dark with 2 mM ^{13}C-$NaHCO_3$, doubling the average concentration of DIC typically found in the ocean. Our results revealed a surprising diversity of marine bacteria actively assimilating DIC in the dark within the Pacific Northwest coastal waters, indicating that DIC fixation is relevant for the metabolism of different marine bacterial lineages, including putatively heterotrophic taxa. Furthermore, dark DIC-assimilating assemblages were widespread among diverse bacterial classes. Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes dominated the active DIC-assimilating communities across the samples. Actinobacteria, Betaproteobacteria, Deltaproteobacteria, Planctomycetes, and Verrucomicrobia were also implicated in DIC-assimilation. *Alteromonadales* and *Oceanospirillales* contributed significantly to the DIC-assimilating Gammaproteobacteria within May 2010 clone libraries. 16S rRNA gene sequences related to the sulfur-oxidizing symbionts ARCTIC96BD-19 were observed in all active DIC-assimilating clone libraries. Among the Alphaproteobacteria, clones related to the ubiquitous SAR11 clade were found actively assimilating DIC in all samples. Although not a dominant contributor to our active clone libraries, Betaproteobacteria, when identified, were predominantly comprised of *Burkholderia*. DIC-assimilating bacteria among *Deltaproteobacteria* included members of the SAR324 cluster. Our research suggests that DIC-assimilation is ubiquitous

among many bacterial groups in the coastal waters of the Pacific Northwest marine environment and may represent a significant metabolic process.

2.2 **Introduction**

Inorganic carbon assimilation in the euphotic zone is most often attributed to oxygenic photosynthesis. Photosynthetic organisms fix DIC via the Calvin-Benson-Bassham (CBB) cycle, utilizing the CO_2 fixing enzyme ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBisCO) (Bassham and Calvin, 1957b). However, recent research has uncovered widespread bacterial DIC-assimilation by mixotrophic organisms and alternative carbon fixation pathways (Eiler, 2006), blurring the lines between strict autotrophic and heterotrophic behavior.

Ubiquitous in the euphotic zone, strains of aerobic anoxygenic photosynthetic bacteria, particularly members of the *Roseobacter* clade (Kolber et al., 2001) have been shown to use a mixotrophic carbon metabolism (Moran and Miller, 2007; Swingley et al., 2007; Tang et al., 2009). Additionally, it has been demonstrated that related strains, including *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*, can fix CO_2 independently of RuBisCO (Wang et al., 1993). Furthermore, photoheterotrophic members of the Gammaproteobacteria group NOR5/OM60, such as strain HTCC2080, may even be capable of mixotrophy via the 2-hydroxypropionate cycle (Thrash et al., 2010).

Previous research has demonstrated the importance of CO_2 assimilation in heterotrophic bacteria, which depends on factors including 1) the type of organic substrate utilized for growth (Feisthauer et al., 2008; Romanenko, 1964; Sorokin, 1966) and 2) the metabolic state of the organisms (Feisthauer et al., 2008). For example, Feisthauer and co-workers noted a high-level, growth phase-independent, labeling of the oxaloacetate-derived amino acids when grown on glucose as the sole carbon source, indicating the occurrence of heterotrophic CO_2 fixation

in *Pseudomonas knackmussii* and the Alphaproteobacterium *Rhodococcus opacus*. Examples of facultative autotrophy and mixotrophy are also present among other Alphaproteobacteria including members of the genera *Rhodopseudomonas, Bradyrhizobium, Nitrobacter, Xanthobacter, Paracoccus, Rhodobacter, Sinorhizobium*; Betaproteobacteria including members of the genera *Hydrogenophaga, Rubrivivax, Cupriavidus, Burkholderia, Xanthanomonas, Thiomonas* (formerly known as *Thiobacillus*) and *Alcaligenes*; the Gammaproteobacterium *Methylococcus capsulatus*; the Firmicute *Halarsenatibacter silvermanii* and the Actinobacterium *Nocardia opaca* (Badger and Bek, 2008; Blum et al., 2009; Kuenen and Beudeker, 1982; Moreira and Amils, 1997; Schäferjohann et al., 1993; Shively et al., 1998). These cultured strains usually contain the type IC (or IA) subgroup of red-like RuBisCO genes (Badger and Bek, 2008), and a distinct diversity of the type IC genes have been found in organic rich environments such as soils (Selesi et al., 2005, 2007; Videmsek et al., 2009). The genes were found to be abundant in soils (Selesi et al., 2007) and highly active (Yuan et al., 2012).

Alternative CO_2 fixation strategies may also play a role in heterotrophic or mixotrophic DIC-assimilation. For example studies of the RuBisCO-lacking Flavobacterium *Polaribacter* sp. MED152 showed light-stimulated CO_2 incorporation suggesting that DIC uptake might play an important role in the life strategy of this marine heterotroph (Gonzalez et al., 2008). It is unclear how important many of the alternative CO_2 fixation strategies are for *Polaribacter* and other heterotrophic species in marine waters, although Alonso-Sáez et al. demonstrated that heterotrophic CO_2 assimilation was active within the nutrient-depleted stationary phase conditions in Arctic sea water cultures (Alonso-Sáez et al., 2008).

The majority of marine bacteria remain uncultured and the processes governing DIC-assimilation are difficult to elucidate. However, Swan and co-workers reported that RuBisCO is encoded in the genomic DNA of uncultured marine bacteria identified through singled-cell sorting and whole-genome amplification (Swan et al., 2011). The authors conclude that with the presence of RuBisCO in the genomic DNA the potential for chemolithoautotrophy exists among Gammaproteobacteria clusters ARCTIC96BD-19 and Agg47 and some uncultured *Oceanospirillales*. RuBisCO has also been detected in the Alphaproteobacteria *Aurantimonas manganoxydans* (Caspi et al., 1996), which has been found in the Pacific Northwest coastal margin (Anderson et al., 2009) and is present among many other Alphaproteobacteria that may be able to grow mixotrophically or chemoautotrophically under certain conditions (Swingley et al., 2007). Overall, a growing body of evidence suggests that DIC-assimilation in the complex marine environment does not fit squarely in the canonical dichotomy of autotrophy versus heterotrophy.

Here we employ stable isotope probing (SIP) with ^{13}C-$NaHCO_3$ incubations to examine the contribution of various classes of bacteria to active DIC-assimilation in the Pacific Northwest coastal waters at three geographically distinct sites. The Newport Hydroline was sampled 10 km offshore (NH-10) at 80 m in September 2008, March 2009, and May 2010 to examine temporal patterns among DIC-assimilating organisms. In May 2010 NH-10, the Columbia River Line 20 km from the mouth of the Columbia River (CR-20), and La Push Line 6 km offshore (LP-6) were sampled to examine spatial variations among DIC-assimilating communities (Fig. S2.1). The goal of this study was to determine if DIC-assimilation is widespread within the heterotroph-dominated marine microbial community and to identify dominant organisms among DIC-assimilating communities in the coastal environment.

2.3 **Methods**

2.3.1 **Shipboard**

Water samples were collected from three geographically distinct coastal ocean transects off the Oregon and Washington coasts during three separate CMOP (Center for Coastal Margin Observation and Prediction) cruises aboard the *R/V Wecoma* (Fig. 2.1). NH-10 (124.296^{o}W, 44.652°N) was sampled 5 meters from bottom depth during cruises on September 18, 2008 (75.5 m, dissolved oxygen (DO) 2.1 mg/L), March 19, 2009 (75 m, DO 2.9 mg/L), and May 21, 2010 (75.5 m, DO 2.9 mg/L). CR-20 (124.453°W, 46.166°N) and LP-6 (124.793°W, 47.917°N) were sampled at 125 m (DO 1.9 mg/L) and 50 m (DO 5.5 mg/L) respectively on the May 26, 2010 and May 24, 2010. Water samples were collected with 10-L Niskin sampling bottles attached to a SeaBird CTD (conductivity-depth-temperature) rosette equipped with an O_2 sensor.

Samples were dispensed under low light conditions into 3 L amber Nalgene bottles, amended with 2 mM unlabeled $NaHCO_3$, 2 mM ^{13}C-$NaHCO_3$, or no additional carbon source, and gassed with N_2 to in situ oxygen levels as recorded with the CTD O_2 sensor. Bottles were immediately incubated in the dark at 7°C for 3 hours. Immediately following incubation, water was filtered through a 0.2-µm pore-size Sterivex filter (PES, ESTAR, Millipore) using a peristaltic pump, then fixed with 2 mL RNAlater (Ambion) to preserve DNA and frozen at −80°C.

2.3.2 **Laboratory Analysis**

(Fig. 2.2)

DNA Extraction. DNA was extracted using a phenol chloroform method previously described by Herfort et al., 2011.

Stable Isotope Probing.

Stable isotope probing employing the archaea *Halobacterium salinarum* (strain *ATCC* 29341/DSM 671/R1) DNA as a ^{13}C carrier was performed as previously described by Gallagher et al., 2005. The *H. salinarum* used for visualization of ^{13}C-DNA was grown in a ^{13}C-labeled ISOGRO powder growth medium (Isotec, Miamisburg, OH). The stable isotope enriched medium was prepared for Van Niel's media amended with 25% NaCl. *H. salinarum* was grown aerobically at 25°C for approximately 20 days before the cells were harvested. DNA was extracted as described above.

Approximately 300 ng of environmental sample DNA and 300 ng of ^{13}C carrier DNA were added to a 500-µl CsCl density gradient (1 g/mL) containing 20 µg ethidium bromide. The ^{12}C and ^{13}C fractions were resolved by centrifugation in a TLA 100 rotor on a Beckman ultracentrifuge (Palo Alto, CA) at 225,000×*g* for 24 hours. The bands were then visualized by UV light and were withdrawn from the gradient by first removing the ^{12}C-DNA band, changing the pipette tip, releasing a small air bubble above the height of the ^{12}C-DNA band (to pass through the band without catching ^{12}C-DNA in the tip), and subsequently removing the ^{13}C-DNA band from the gradient. The DNA was then suspended in 200 µl of 10 mM Tris-HCl, 0.5 mM EDTA and cleaned using Millipore Montage PCR Filter Units (Millipore Corp, USA) as per manufacturer's protocol.

Cloning and Sequencing of 16S rRNA genes.

Cleaned DNA was PCR amplified in triplicate using universal bacterial 16S rRNA gene primers, 27f and 1492r (Lane, 1991). The cycling conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, with a final extension at 72°C for 10 min. Triplicate 16S rRNA gene PCR products were the pooled and ligated with TOPO vector (pCR

2.1, Invitrogen) and introduced by transformation into One Shot Top 10 electrocompetent *E. coli* cells from the TA cloning kit (Invitrogen). Transformants were inoculated on LB agar supplemented with 40 µg/mL XGAL (bromo-chloro-indolyl-galactopyranoside) and 50 µg/mL ampicillin and grown overnight at 37°C. Positive (white) colonies were picked randomly and transferred into 96-well plates containing 150 µl of 2× Yeast Extract/Tryptone amended with 50 µg/mL ampicillin and incubated overnight at 37°C with shaking at 50 rpm. Following the addition of 30 µl of 50% glycerol, the 96-well plates were then stored at −80°C and sent either to the Genome Sequencing Center at Washington University in St. Louis (September 2008 and March 2009 samples) or Beckman Coulter Genomics (May 2010 samples) for sequencing with primers M13f, M13r, 704f, and 926r to obtain full length 16S rRNA gene sequences. Both facilities employ capillary sequencing using the Big Dye protocol (Applied Biosystems).

2.3.2.1 **Control Samples**

To examine differences between the ^{12}C and ^{13}C DNA fractions extracted from ^{13}C-$NaHCO_3^-$, bacterial 16S rRNA gene clone libraries were constructed for both fractions from water collected in September 2008 and March 2009 on the Oregon coast at NH-10. For both of these samples, large differences in bacterial composition at both class and species levels could be discerned between ^{12}C and ^{13}C labeled DNA fractions, and the Bray-Curtis indices of similarity between the ^{12}C and ^{13}C fractions varied from 10.37 to 14.5 out of 100 for all samples analyzed. These population differences allowed us to compare the total microbial community of the water samples with actively DIC-assimilating communities and to rule out significant band cross-contamination between the ^{12}C and ^{13}C fractions.

^{12}C and ^{13}C fractions recovered from the SIP environmental samples amended with 2 mM unlabeled $NaHCO_3$, 2mM ^{13}C-$NaHCO_3$, or no additional carbon source for each season and/or

location were subjected to qPCR with universal bacterial 16S rRNA gene primers, 27f and 519r (Lane, 1991). The amplification protocol consisted of an initial denaturation of 94°C for 3 min, 40 cycles of amplification at 94°C for 30 s, 52°C for 30 s, and 70°C for 30 s followed by a terminal extension step of 72°C for 5 min. Bacterial 16S rRNA gene quantities were standardized using almost full-length amplicons of the ARCTIC96BD-19 16S rRNA gene obtained from cloning of environmental DNA obtained in this study.

2.3.2.2 **Sequence Analysis**

Trimmed sequences were assembled into full length 16S rRNA contigs using Geneious Pro 5.3.4 (Drummond et al., 2010). Sequence contigs were then aligned using the greengenes.lbl.gov (DeSantis et al., 2006) Nearest Alignment Space Termination (NAST) program. Chimeras were identified using the online program Bellerophon (Huber et al., 2004) and subsequently removed from analysis. A phylogenetic tree of 16S rRNA sequences was constructed with the remaining sequence contigs against the SILVA database (Pruesse et al., 2007) in ARB (Ludwig et al., 2004). Sequences identified as eukaryotic chloroplasts were removed from the clone libraries. In ARB, nearest neighbors were identified and their sequences were acquired from NCBI: BLAST. The sequence alignments were transferred back into Geneious Pro 5.3.4 for phylogenetic analysis of active bacterial classes. Over all seasons 2,880 bacterial clones were screened resulting in 1,780 quality contigs. During analysis 430 clones were identified as eukaryotic and removed from the dataset (Table 2.1). Bray Curtis indices of similarity (Bray and Curtis, 1957) and Shannon indices (Shannon, 1948) were calculated by season and location using the Plymouth Routines in Multivariate Ecological Research (PRIMER) software version 6 (PRIMER/E Ltd, UK) program (Table 2.2).

2.4 **Results**

2.4.1 **Comparison of Clone Libraries from ^{12}C and ^{13}C Bands in NH-10 September 2008 and March 2009 Reveals a Distinct Bacterial Population Actively Assimilating DIC**

In September 2008, Alphaproteobacteria and Bacteroidetes dominated the bacterial assemblage in both ^{12}C and ^{13}C-labeled fractions, but their distribution between ^{12}C and ^{13}C bands increased from 30% to 38% for Alphaproteobacteria and from 25% to 33%, for Bacteroidetes, respectively (Fig. 2.3). Alphaproteobacteria and Bacteroidetes taxa represented a greater percentage of the actively DIC-assimilating community versus the total assemblage in the coastal ocean in September. In contrast, the distribution of Gammaproteobacteria in the two fractions dropped from 24% in the ^{12}C band to only 14% of the active DIC-assimilating community.

Ribosomal Database Project (RDP) naïve Bayesian classifier, a statistical test that assumes that the presence (or absence) of a particular feature of a class is unrelated to the presence (or absence) of any other feature, identified the overall Proteobacteria shift between the ^{12}C and ^{13}C bands as significant at 2.8^{-4}, and within the Gammaproteobacteria at 6.8^{-4} at a 95% confidence threshold for both Proteobacteria and Gammaproteobacteria (Table 2.2). When grouped by order, representative Gammaproteobacteria species among the ^{12}C band were largely populated by *Oceanospirillales* comprising 21% of all species, but only 8% of the active DIC-assimilating community at that time. Among the Alphaproteobacteria *Rhizobiales* constitute a significant fraction of the active DIC-assimilating community at 25%, but only 9% of species identified in the ^{12}C band. *Flavobacteriales* make up a significant portion of both fractions with 28% of the ^{12}C band and 25% of the ^{13}C band. Shifts between the distributions of the total bacterial population versus the active DIC-assimilating community were observed among less dominant

groups, such as the Betaproteobacteria and Verrucomicrobia. Similar shifts were observed between active and inactive fractions among orders within the Bacteroidetes in September 2008 and March 2009 (Fig. 2.3).

The differences observed between the clone libraries derived from the ^{12}C and ^{13}C fractions collected from each sample, in terms of taxonomic class and family abundance, suggests that cross-contamination was unlikely. Shifts between the percentage of bacterial communities in the ^{12}C vs. ^{13}C clone libraries indicate that numerically dominant organisms may not be the most metabolically active in terms of DIC-assimilation, supporting observations by Musat et al., 2008 who determined that inconspicuous microbes can play a significant role in environmental nitrogen and carbon cycles. For example, while *Rhodobacteraceae* are relatively abundant in both September 2008 and March 2009. *Flavobacteriaceae* appear to be more active in DIC-assimilation during these periods (Fig. 2.4).

Cyanobacteria represented primarily by *Synechoccocus sp.* 16S rRNA gene sequences, were active in DIC-assimilation only in March 2009 at NH-10 and in May 2010 at LP-6 contributing no more than 9% of 16S rRNA gene sequences identified.

2.4.2 **Spatial and Temporal Variations in DIC-assimilating Bacteria**

A variety of taxa were implicated in active DIC-assimilation in the northeast Pacific Ocean. Bacterial 16S rRNA gene sequences for DIC-assimilating organisms from the Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes were abundant at NH-10 in September 2008, March 2009, and at all locations in May 2010 (Fig. 2.5). Sequences that affiliated with Actinobacteria, Verrucomicrobia, Planctomycetes, and Delta- and Betaproteobacteria were also uncovered from the ^{13}C fraction in samples regardless of location,

depth, or season, although to a lesser degree than the aforementioned three classes. However the phylogenetic composition within taxonomic groups showed variation at finer taxonomic resolution.

2.4.2.1 **Alphaproteobacteria.**

At NH-10 Alphaproteobacteria were the dominant active DIC-assimilating population in both September 2008 and March 2009, representing 38% and 39%, respectively, of the total assemblage detected in the ^{13}C fraction, but formed a smaller portion in May 2010 at 13% (Fig. 2.5). The reduction in active Alphaproteobacteria 16S rRNA gene sequences observed in the ^{13}C fraction was also observed at the other two sites; 25% and 15% of the ^{13}C fractions were found at CR-20 and LP-6, respectively.

Members of the SAR11 clade comprised a large portion of the active Alphaproteobacteria assemblage in all samples. Sequences related to the cosmopolitan SAR11 species, *Pelagibacter ubique HTCC1062* were recovered from the ^{13}C fractions in the September 2008 and March 2009 samples from NH-10 and in the ^{13}C-DNA fractions from all of the May 2010 samples (Fig. 2.6 and Fig. 2.7).

Analysis of 16S RNA gene clone libraries constructed from ^{13}C-labeled fractions of the NH-10 samples collected in September 2008, March 2009, and May 2010 implicated the *Roseobacter* clade in active DIC-assimilation. Among the organisms identified *Roseovarius mucosus* were found only in May 2010 NH-10 and CR-20 samples. This cluster forms 100% of the contribution of the *Roseobacter* clade to DIC-assimilation at NH-10 in May and 25% of the overall Alphaproteobacterial assimilation. Members of the *Roseobacter* clade active in DIC-assimilation at LP-6 were closely related to *Octadecabacter antarcticus* and *Loktanella rosea*.

Rhizobiales constituted a significant portion of sequences from the ^{13}C-DNA fraction of the CR-20 sample. The most notable sequences within this order were related to *Aurantimonas manganoxydans* and were exclusive to CR-20. *Bradyrhizobium* sequences related to *Oligotrophicus carboxidovorans* were detected in the active fraction at NH-10 in May 2010 and March 2009. *Paracoccus sp.* was found to be active in DIC-assimilation among the *Rhodobacterales* in March 2009 (Fig. 2.7).

2.4.2.2 **Gammaproteobacteria.**

At NH-10 the Gammaproteobacteria played a small role in DIC-assimilation in September 2008 and March 2009, comprising only 14% and 11% of the active bacterial assemblage, but became the dominant class of DIC-assimilating bacteria (36% - 47%) in all May 2010 clone libraries.

In all samples, two orders of bacteria, *Alteromonadales* and *Oceanospirillales,* were most prevalent among the heterotrophic DIC-assimilating community (Figs. 2.8 and 2.9). Uncultured organisms related to *Alteromonadales* showed the most even distribution among the three sampling sites. Sequences from these organisms were also found in the March 2009 ^{13}C-DNA fraction, but were more dominant among the DIC-assimilating organisms in May 2010. Organisms related to unclassified *Oceanospirillales* were identified among the sequences within the ^{13}C-labeled fraction in both September and May samples. Similar to SAR11, this group showed temporal variability, being dominant in the May 2010 sample but representing only 7% of the clones within the group in September 2008 (Fig. 2.8). Sequences of the unclassified *Oceanospirillales* members were the majority at NH-10 and CR-20 in May 2010 with a portion of clones grouping with sulfur oxidizing symbionts, in particular SUP05 and ARCTIC96BD-10 (Fig. 2.9).

In the active fraction of May 2010 samples organisms related to *Acinetobacter* and organisms within the Agg47 cluster were only identified at CR-20 and NH-10, although the *Acinetobacter* cluster was more abundant at CR-20 (Fig. 2.9). Also identified in DIC-assimilation at CR-20 were species closely related to *Pseudomonas aeruginosa;* sequences related to those of *Colwellia piezophila* were identified at NH-10 in September 2008 (Fig. 2.8).

2.4.2.3 **Bacteroidetes.**

Sequences representing Bacteroidetes were identified in the ^{13}C-fractions of DNA all NH-10 samples (Fig. 2.5). Bacteroidetes comprised the third largest class at NH-10 (12%) and LP-6 (14%), and the second largest class at CR-20 (21%) active in DIC-assimilation in May 2010 (Fig. 2.2). Analysis of the DIC-assimilating Bacteroidetes assemblage at the species level identified members of the *Flavobacteria*. Sequences specifying *Cloacibaterium normanense* were identified at NH-10 only in May 2010, as well as clones related to *Fluviicola sp.* (also identified in March 2009) (Figs. 2.10 and 2.11). Members of the *Polaribacter* clade were active in DIC-assimilation at NH-10 in September 2008 and May 2010 and at CR-20 in May 2010. Organisms of the order Sphingobacteriales, related to *Lewinella nigricans* and *Saprospira grandis* were identified in the analysis of the ^{13}C-DNA fraction of DNA samples collected at NH-10 and CR-20 in May 2010. Sphingobacteriales, however, were not found to be active in any other samples and were only found in the ^{12}C clone libraries from September 2008 and March 2009 (data not shown).

2.4.2.4 **Deltaproteobacteria.**

Deltaproteobacteria contributed to DIC-assimilation in the Pacific Northwest Coastal Margin, based on analysis of the ^{13}C fraction, but abundance varied across sampling sites and seasons (Fig. 2.12). Deltaproteobacteria contributed only 2% and 6% respectively of clone libraries

derived from the ^{13}C fraction of September 2008 and March 2009 (Fig. 2.11). SAR324 bacteria were not identified in the March and September clone libraries when the majority of Deltaproteobacteria sequences were related to uncultured species. At NH-10 and CR-20 in May 2010 16S rRNA gene sequences in the active fractions were affiliated with the SAR324 cluster and also *Nitrospina sp.* (Fig. 2.13). *Nitrospina spp.*, however, were active in the March 2009 sample.

2.4.2.5 **Betaproteobacteria.**

Betaproteobacteria represented a small fraction of the active DIC-assimilating community overall (Fig. 2.5). At NH-10 in March 2009 the Betaproteobacteria were predominantly affiliated with *Acidovorax sp.* and *Aquabacterium sp.* of Burkholderiales (Fig. 2.12). In May 2010 16S rRNA gene sequences identified at NH-10 were related to *Dechloromonas sp.* A large number of clones identified at CR-20 in May 2010 were related to *Dechloromonas sp.*, *Diaphorobacter nitroreducens*, *Acidivorax temperans*, and *Aquabacterium sp.* (Fig. 2.13). Betaproteobacteria were not detected in the active fraction at LP-6.

2.4.2.6 **Actinobacteria.**

16S rRNA gene sequences representing Actinobacteria were identified in the ^{13}C fraction of all samples (Fig. 2.5). The majority of sequences were affiliated with uncultured environmental clones. Clones in the active fraction of the NH-10 May 2010 samples were associated with an uncultured *Actinobacterium* clone distantly related to *Candidatus Microthrix parvicella* (Fig. 2.12). However, a fraction of the active species identified at NH-10 and CR-20 in May 2010 was closely related to *Ilumatobacter fluminis* (Fig. 2.13). The contribution of these species to DIC-assimilation appears to be more prominent at CR-20 in May 2010 as *Ilumatobacter sp.* accounted for 33% of active Actinobacteria species identified in that clone library.

2.4.2.7 **Planctomycetes.**

Planctomycete species were identified in all active fractions with a number of clones associated with the traditionally heterotrophic genera *Pirellula* and *Rhodopirellula*. Sequences related to those of *Rhodopirellula baltica* were identified within all NH-10 samples (Fig. 2.12). A few clones also grouped with *Planctomyces maris* in the May 2010 NH-10 samples. Both *Rhodopirellula baltica* and *Planctomyces maris* were identified in the active fraction at LP-6 in May 2010, yet these organisms were not found in the CR-20 clone library (Fig. 2.13).

2.4.2.8 **Verrucomicrobia.**

Verrucomicrobia were identified in the active fractions of all samples (Fig. 2.5). A number of these 16S rRNA gene sequences were associated with the order *Verrucomicrobiales*. A small percentage of the sequences from the NH-10 May 2010 and March 2009 clone libraries aligned closely with *Persicirhabdus sediminis* (Fig. 2.12). 16S rRNA gene sequences related to *Lentisphaera araneosa* and *Rubritalea tangerina* were also identified in the active DIC-assimilating fraction at NH-10 and CR-20 in May 2010 (Fig. 2.13).

2.5 **Discussion**

2.5.1 **DIC-assimilation- General Considerations**

Bacterial 16S rRNA gene sequence data from SIP experiments conducted on water collected from the Oregon and Washington coasts demonstrate that Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes species are the bacterial classes most active in assimilating DIC into their DNA. Their predominance among DIC-assimilating communities is not entirely surprising, because the three classes have been identified as dominant in marine waters (Brown et al., 2009; Gómez-Pereira et al., 2010). Several other bacterial classes such as

Betaproteobacteria, Deltaproteobacteria, Verrucomicrobia, Actinomycetes, and Planctomycetes were also observed to participate in DIC-assimilation (Fig. 2.5). Distinguishing between heterotrophic and autotrophic metabolism is difficult due to the fact that many of the organisms identified within our data sets are uncultured or returned matches to sequences in GenBank from non-marine sources, such as soil and sediment. Our data suggest that prokaryotic DIC-assimilation, whether heterotrophic, chemoautotrophic, or mixotrophic, is a more significant process in the coastal environment than previously thought and is, in fact, widespread among marine bacterial classes.

2.5.2 **Methodological Considerations**

It is important to note that we cannot entirely rule out the possibility of cross-feeding of ^{13}C-labeled substrates between organisms. One of the major caveats to many, if not all, SIP protocols is the possibility of detecting secondary consumption of stable isotope-labeled metabolites produced by other organisms. In our case, the uptake of ^{13}C-labeled HCO_3^- during autotrophic carbon fixation could lead to the production of ^{13}C-labeled organic compounds. Although carbon fixation can occur within minutes (Stange et al., 1960), the production of sufficient amounts of organic carbon able to be incorporated into the DNA at a distinguishable level could take hours. In an effort to limit cross-feeding we chose a short incubation time of 3 hours, which is the minimum time required to achieve successful labeling of DNA with ^{13}C. While previous studies in coastal sediments were able to discern labeled DNA within 1 hour of incubation with the addition of the *Halobacterium salinarum* carrier DNA to the CsCl gradient (Gallagher et al., 2005), we found it difficult to consistently obtain sufficient amounts of labeled DNA for 16S rRNA gene amplification and cloning from incubations under 3 hours. To the best of our knowledge, our incubation time is the shortest reported for a stable isotope experiment in marine

waters. In fact recent SIP publications in similar environments report incubation times ranging from 24 hours (Frias-Lopez et al., 2009) to 72 hours (Glaubitz et al., 2010).

While a short incubation time mitigates the effects of cross-feeding, the possibility that our ^{13}C amplification data is merely the result of the buoyant density of DNA affected by G+C content must be directly addressed. Previous research suggests that ^{12}C-DNA with high G+C content may co-migrate with ^{13}C-labeled DNA, resulting in ^{13}C fractions containing isotopically labeled DNA, as well as unlabeled DNA from organisms with a high genome G+C content (Buckley et al., 2007; Radajewski et al., 2000). In our study we found little evidence of bacterial DNA contamination in the ^{13}C band in the unamended environmental samples as well as the environmental samples incubated with 2 mM ^{12}C-$NaHCO_3$. Q-PCR samples showed only minor, late stage amplification at 36 cycles (out of 40) in the ^{13}C fraction of control groups when compared to the experimental fraction (data not shown). This amplification is likely the effect of primer dimers forming late in the amplification process. Efforts to clone 16S rRNA genes from the ^{13}C control fractions failed. Furthermore, average G+C content of the 16S rDNA sequences identified in the ^{12}C versus ^{13}C fractions were nearly identical in the September 2008 and March 2009 samples, varying by only 1% between the fractions (data not shown). Therefore, in good agreement with the results of Gallagher et al., (2005) our controls suggest that the variable G+C content in environmental samples did not affect our SIP study that utilized carrier DNA.

2.5.3 **Mechanisms of DIC-assimilation in Marine Bacteria**

2.5.3.1 **Obligatory and facultative use of RuBisCO.**

Studies of Alonso-Sáez et al., (2008) highlighted the potential for high bicarbonate assimilation among Arctic heterotrophic bacteria. Swan et al., (2011) combined single-cell sorting with

whole-genome amplification to identify RuBisCO and sulfur oxidation genes in a number of Gammaproteobacteria, including uncultured ARCTIC96BD-19 and *Oceanospirillales* as well as the potential for C-1 metabolism among Deltaproteobacteria within the SAR324 cluster. The authors suggest the potential for widespread chemolithoautotrophy among uncultured Proteobacteria lineages in the dark ocean.

Supporting the findings by previous studies (Alonso-Sáez et al., 2008; Swan et al., 2011), sequence data from our 16S rRNA gene clone libraries confirmed that the Gammaproteobacteria were actively incorporating DIC in the coastal environment, but their contribution to total DIC-assimilation may vary spatially or temporally. A large assemblage of DIC-assimilating Gammaproteobacteria dominated by ARCTIC96BD-19 and uncultured *Oceanospirillales* was found in May. Furthermore, the identification of the SAR324 cluster bacteria in the clone libraries of active fractions suggests the potential for DIC-assimilation involved in C-1 (compounds containing only one carbon atom, i.e. CO or CO_2) metabolism in the Pacific Northwest Coastal Margin.

Among the Deltaproteobacteria, 16S rRNA gene sequences associated with the chemoautotrophic species *Nitrospina gracilis*, which uses CO_2 as its sole source of carbon, was identified (Fig. 2.13). The majority of remaining Deltaproteobacteria were related to organisms associated with sulfur metabolism such as *Pelobacter sp.*, which have shown the ability to utilize CO_2 during fermentation processes (Schink et al., 1987). This class of bacteria was not active in DIC-assimilation at LP-6, possibly due to the location and shallow depth (50 m compared to NH-10 and CR-20, see *Methods*) of the sampling site which was considerably more oxygenated that the other locations (5.5mg/L compared to <2.5mg/L in the other samples).

Further potential for DIC-assimilation exists among Alphaproteobacteria clones found within the ^{13}C-fractions, such as nitrogen-fixing, CO-oxidizing heterotrophs among the *Bradyrhizobia, Oligotropha carboxidovorans*, and *Bradyrhizobium elkanii* are known to contain RuBisCO (Tolli and King, 2005) (Fig. 2.6 and Fig. 2.7). RuBisCO has been identified in *Aurantimonas manganoxydans* and may be capable of CO_2 fixation under the proper conditions (Dick et al., 2008).

The detection of Betaproteobacteria *Diaphorobacter sp.*, *Acidovorax sp.*, and *Dechloromonas sp.* suggests that CO_2 assimilation, possibly through RuBisCO, associated with nitrogen cycling may have been occurring among members of this class (Fig. 2.13). Within some *Burkholderia* species, such as the facultative mixotroph *Burkholderia xenovorans*, genes encoding the enzymes of the Calvin Cycle have been identified; furthermore the potential for CO_2 assimilation associated with methylotrophic bacteria was also identified within this class (Figs. 2.12 and 2.13) (Badger and Bek, 2008; Tolli and King, 2005).

A number of *Mycobacterium* species of Actinobacteria have demonstrated growth through C-1 metabolism. Some species such as *Mycobacterium smegmatis* and *Mycobacterium gordonae* contain genes encoding the enzymes of the Calvin–Benson–Bassham pathway, while *Mycobacterium gastri* has exhibited RuBisCO activity as well (Park et al., 2008). The recent discovery of autotrophic methanotrophy among Verrucomicrobia by *Methylacidiphilum fumariolicum Sol V,* further suggests that some methanotrophs may be able to fix CO_2 through the Calvin Cycle, presumably using CH_4 mainly as an energy source (Khadem et al., 2011). A growing body of research suggests the occurrence of alternate CO_2 fixation pathways across numerous classes and clades leading us to believe that there is still much to be discovered about the metabolism of diverse oceanic bacteria.

2.5.3.2 **Proteorhodopsin and Bacteriochlorophyll.**

Proteorhodopsin and bacteriochlorophyll, identified in a number of marine bacteria, interact with light, converting it into energy for growth and survival. Light-stimulated uptake of CO_2 has been reported for *Polaribacter* MED152 (Gonzalez et al., 2008) and light stimulated growth of *Dokdonia sp.* MED134 was observed in media with minimal organic matter (Gomez-Consarnau et al., 2007). Both studies suggest that light may play an important role in the life and resilience of these organisms. It is also well known that the cosmopolitan SAR11 bacterium *Pelagibacter ubique* expresses proteorhodopsin proteins (Steindler et al., 2011). It has been suggested that CO_2 uptake stimulated by proteorhodopsin may fuel such processes as anaplerotic carbon fixation in some marine heterotrophic bacteria (Gonzalez et al., 2008), although these reactions are often balanced by carboxylation reactions resulting in no net gain of carbon. As of yet, the benefit of these light-dependent proton pumps for many marine bacteria remains unknown.

Many of the clones within the ^{13}C fractions implicated in heterotrophic DIC-assimilation, including a number related to *Polaribacter sp.* (Fig. 2.10 and Fig. 2.11), are known to contain proteorhodopsin, for example members of the SAR86 clade of the Gammaproteobacteria (Fig. 2.8 and Fig. 2.9). Others contain bacteriochlorophyll, a light harvesting protein found in a number of *Roseobacter* species. Clones identified within the active fractions, among bacteriochlorophyll-containing clades, were related to *Roseovarius mucosus,* the species *Loktanella rosea,* and *Thalassobacter oligotrophus* (Fig. 2.6 and Fig. 2.7). Organisms containing proteorhodopsin or bacteriochlorophyll cannot grow autotrophically, but many can grow mixotrophically. The presence of proteorhodopsin and bacteriochlorophyll among clades identified within the active ^{13}C fractions allows for the potential energy to fix assimilated CO_2.

Unfortunately the mechanism of CO_2 assimilation that may be supported by proteorhodopsin and bacteriochlorophyll among these organisms is difficult to ascertain.

2.5.3.3 **Facultative methylotrophy.**

A few clones within the ^{13}C fractions grouped with clades containing facultative methylotrophs. Among the Alphaproteobacteria, sequences specifying *Hyphomicrobium sp.* and *Thalassobaculum litoreum* were observed (Figs. 2.6 and 2.7). Facultative methylotrophy has been reported among Bacteroidetes, including *Flavobacterium glycines* (Madhaiyan et al., 2010), and occurrences among the Gammaproteobacteria are well documented. Doronia and Trosenko (1985) found that *Pseudomonas* sp. M4 assimilated 2.1% of cellular biomass from CO_2 when grown on glucose, but 47.1% when grown on methylamine. Ro et al. (1997) found that RuBisCO was active in *Acinetobacter* sp. strain JC1 DSM 3803 cultures when grown on methanol. *Corynebacterium spp.* have also been implicated in facultative methylotrophy (Bastide et al., 1989; Laget et al., 1987).

2.5.3.4 **Acetyl-coA Pathway.**

Among the Planctomycetes the potential for CO_2 assimilation is evident among members of the anammox bacteria, which can fix inorganic carbon via the acetyl-coA pathway (Kuenen, 2008). While Planctomycetes were identified in the active fraction of all samples, anammox bacteria were not detected. The mechanism of DIC-assimilation among the organisms potentially implicated in DIC-assimilation such as *Planctomycetes maris* remains unknown (Fig. 2.12 and Fig. 2.13).

Although it is difficult to ascertain the mechanisms of DIC-assimilation for each organism or class identified in our 16S rRNA clone libraries, this research provides a foundation for further inquiry regarding widespread DIC-assimilation by bacteria in the marine environment.

2.5.4 **Spatial Distribution of SAR11 and ARCTIC96BD-19**

To gain insight into the spatial distribution of DIC-fixing organisms found within our active clone libraries, a handful of sequences were added to a denoised tag pyrosequence database developed by Fortunato et al. (2011). The OTUs were re-clustered with samples from 329 different water samples taken from the Pacific Northwest coastal margin from 2007 through 2008. Approximately 40% of the bacterial community in ocean samples was represented by two OTUs related to *Pelagibacter ubique* HTCC1062 and the chemoautotrophic organism ARCTIC96BD-19. Clone Mar09NH10_186h10, related most closely to *Pelagibacter ubique* HTCC1062 (Fig. 2.6), accounted for nearly 21% of the total bacterial population identified in the tag pyrosequence database, while clone May10LP6_231F03, representing ARCTIC96BD-19, accounted for 18%. In samples taken from bottom depths, however, ARCTIC96BD-19 bacteria almost always exceeded the percentage of SAR11 bacteria observed (data not shown).

The third most abundant OTU found in the database was May10NH10_237G09, which resembles unclassified *Oceanospirillales*. These organisms, representing nearly 20% of the active DIC-assimilating *Gammaproteobacteria*, contributed significantly to DIC-assimilation in the May 2010 samples but account for only 3.2% of the total bacterial population in the tag pyrosequence database. The organisms appeared in all bottom samples along the coastal shelf regardless of season or location, yet do not appear to participate significantly in DIC-assimilation in September 2008, and were absent from the March 2009 clone libraries (Fig. 2.8). The Tag-

encoded pyrosequencing database results suggest that the major DIC-assimilating participants identified in the ^{13}C fractions of our SIP clone libraries are found throughout the Pacific Northwest Coastal Margin. However, in the case of the uncultured *Oceansospirillales* clone, DIC-assimilating ability does not necessarily correlate with the numerical abundance of an organism in the sample.

2.6 **Conclusion**

As a survival strategy many marine bacteria are able to undergo shifts in metabolism to adapt to a changing nutritional environment (Roszak and Colwell, 1987) causing some heterotrophic bacteria to utilize alternative pathways of carbon uptake that are characteristic of mixotrophic life styles including facultative chemoautotrophy, mixotrophy, methylotrophy, and other forms of inorganic carbon fixation and anaplerotic reactions. Recent research suggests the potential for DIC fixation among *Oceanospirillales*, and a number of other bacterial families that reside in the mesopelagic ocean (Swan et al., 2011). While the actual contribution of these processes to the oceanic carbon cycle is unclear, our results show that bacterial DIC-assimilation operates in Oregon coastal waters among a wide variety of microorganisms. The rapidity of DIC incorporation into DNA suggests that this process may involve a variety of assimilation pathways, chemoautotrophic or mixotrophic lifestyles.

2.7 **Acknowledgments**

This work was carried out within the context of the Science and Technology Center for Coastal Margin Observation and Prediction (CMOP), supported by NSF grant number OCE/0424602. Special thanks to Dr. Lee Kerkhof from the Institute of Marine and Coastal Sciences, Rutgers University, for help with our SIP protocol development. We would also like to express a sincere thank you to Captain Rick Verlini and the crew of the R/V Wecoma.

2.8 **Figures**

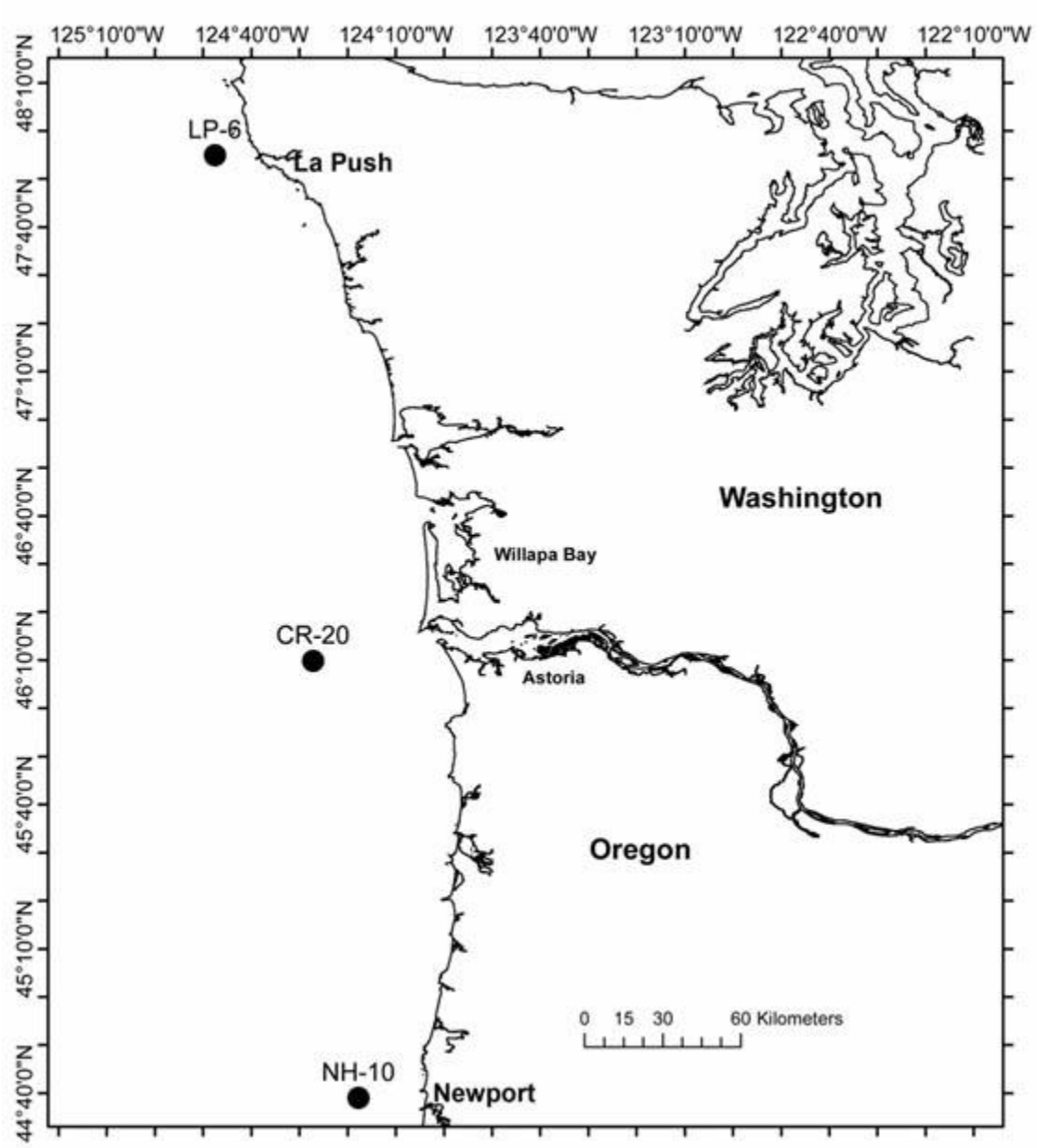

Figure 2.1 Map of sampling locations along the Pacific Northwest Coastal Margin.

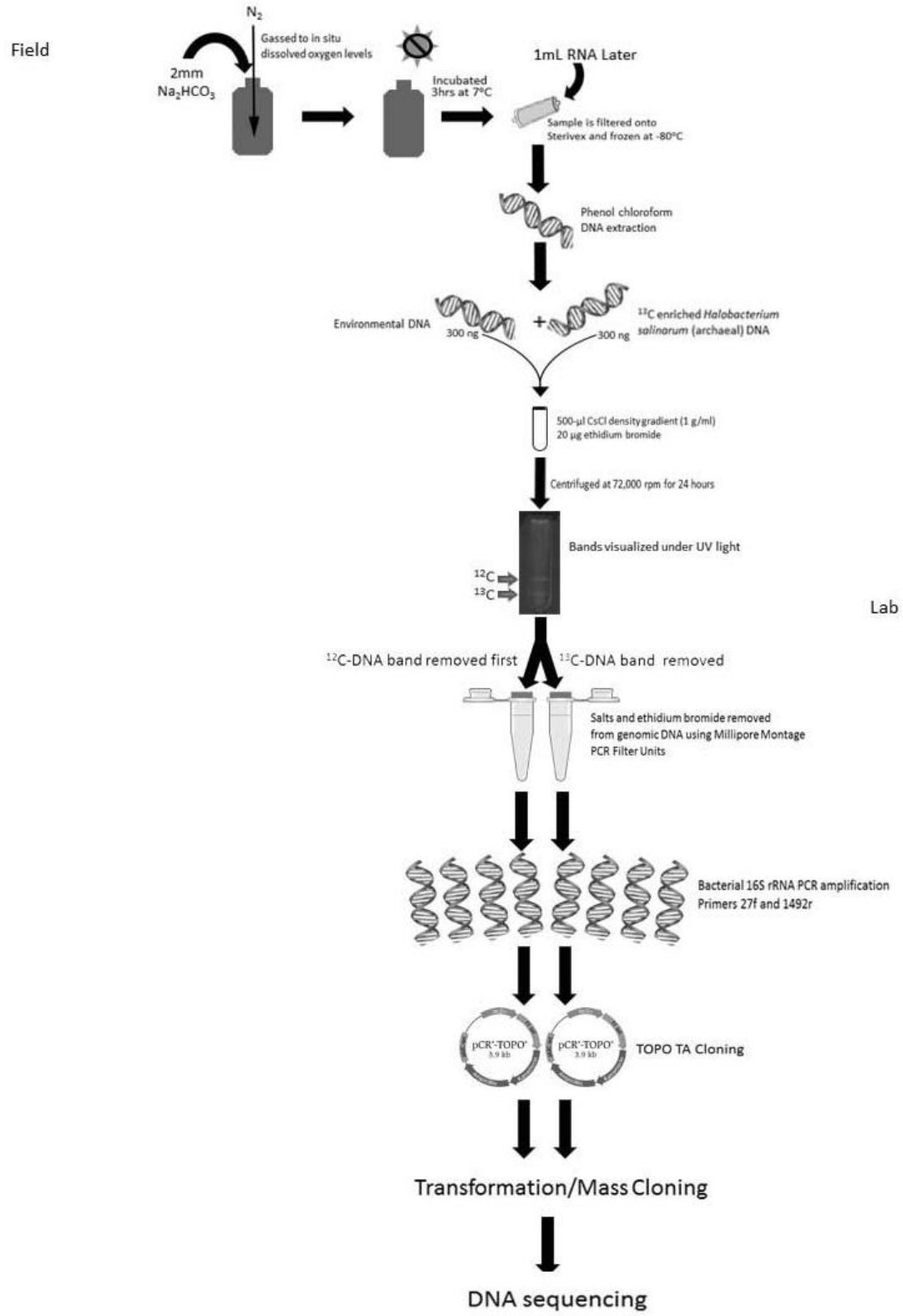

Figure 2.2 Diagram of experimental methods from field sampling through DNA sequencing.

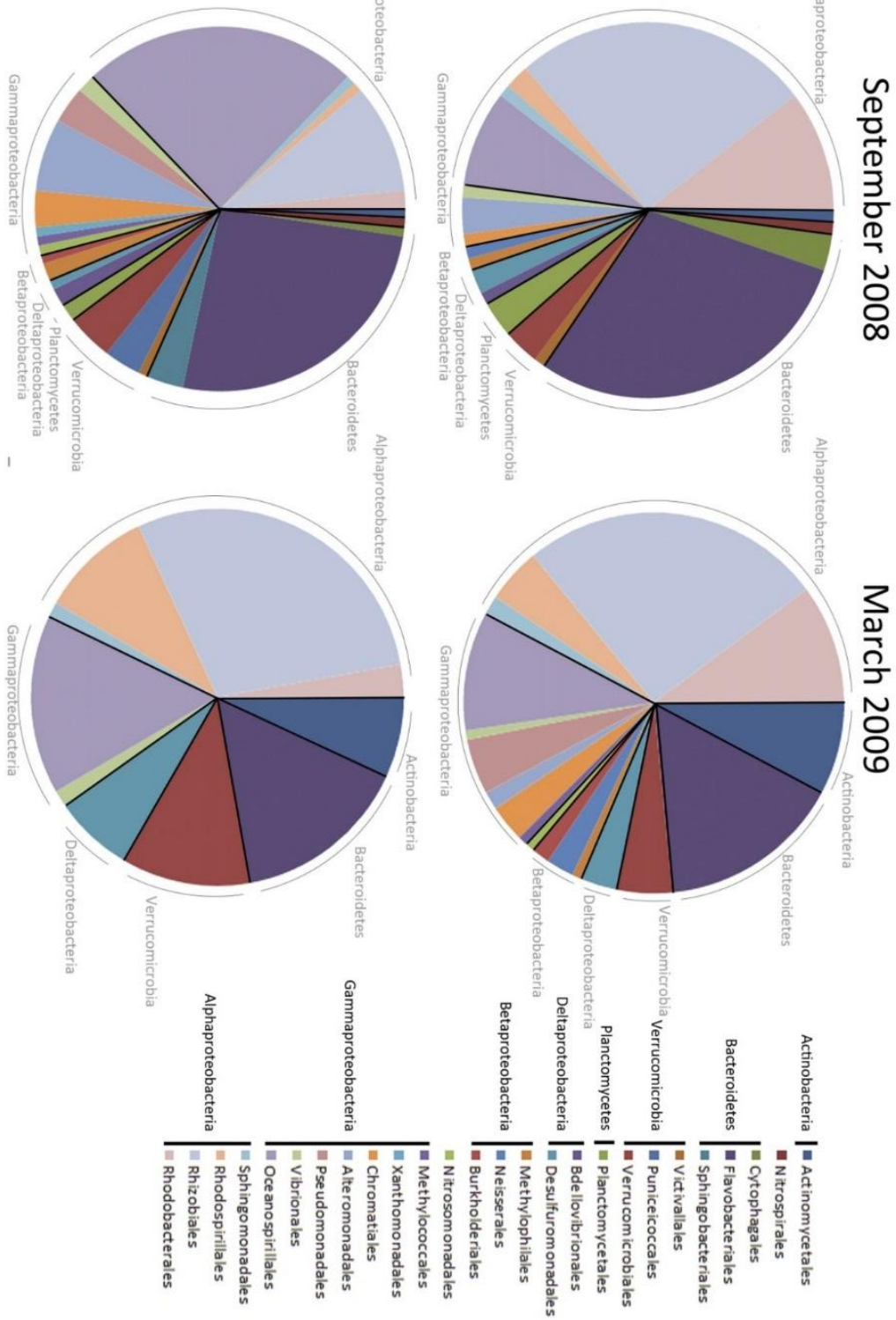

Figure 2.3 Distribution of bacterial orders observed in 16S rRNA gene clone libraries of ^{12}C vs. ^{13}C fractions from ^{13}C-$NaHCO_3$ incubations at NH-10 in September 2008 and March 2009.

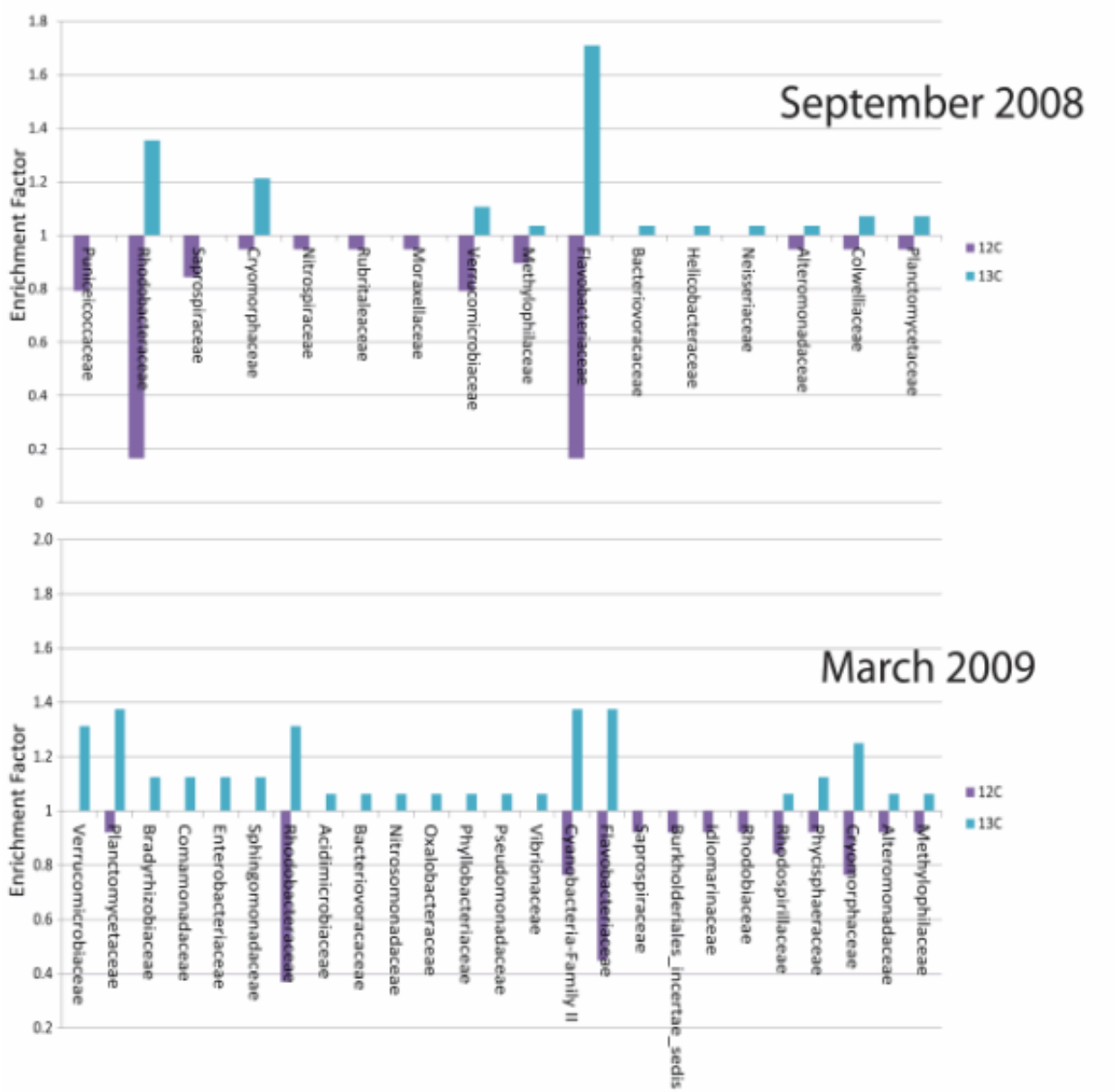

Figure 2.4 Relative changes in the contribution of individual clones to the September 2008 and March 2009 the clone libraries are represented as dimensionless enrichment factors.

The enrichment factor was calculated by dividing the relative abundance of a clone by the relative abundance of the total respective clones in the clone library. Values less than 1 (purple) indicate depletion and values greater than 1 (blue) indicate enrichment of the particular group in the heavier fraction.

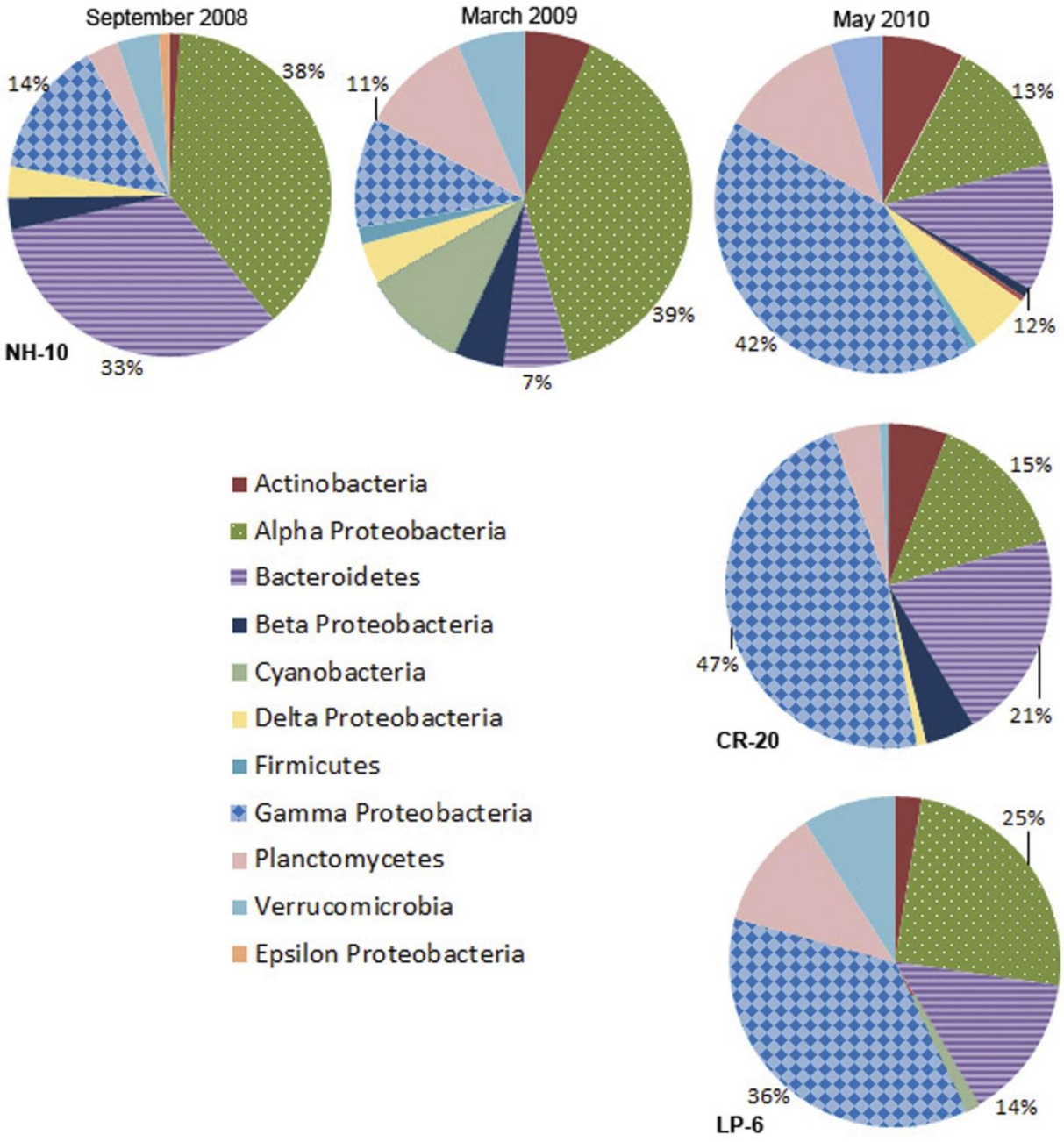

Figure 2.5 Class distributions of 16S rRNA gene clone libraries of ^{13}C fractions from all ^{13}C-$NaHCO_3$ incubations at NH-10 in September 2008, March 2009, and May 2010, as well as at LP-6 and CR-20 in May 2010.

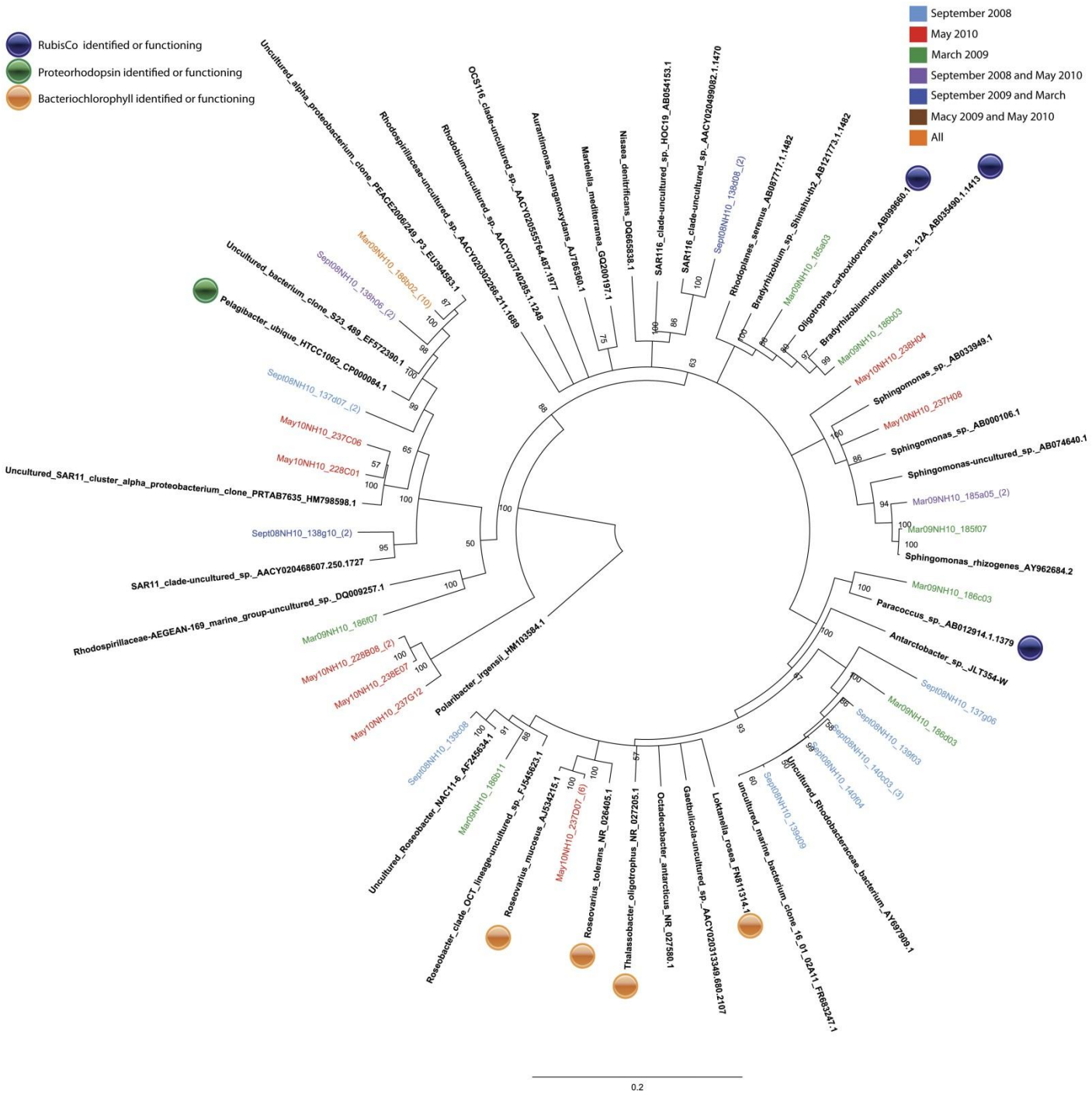

Figure 2.6 Rooted neighbor-joining phylogenetic tree of Alphaproteobacteria species actively involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of 13C fractions from SIP experiments conducted at NH-10 in September 2008, March 2009, and May 2010.

Clones representing highly similar OTUs were collapsed for clarity.

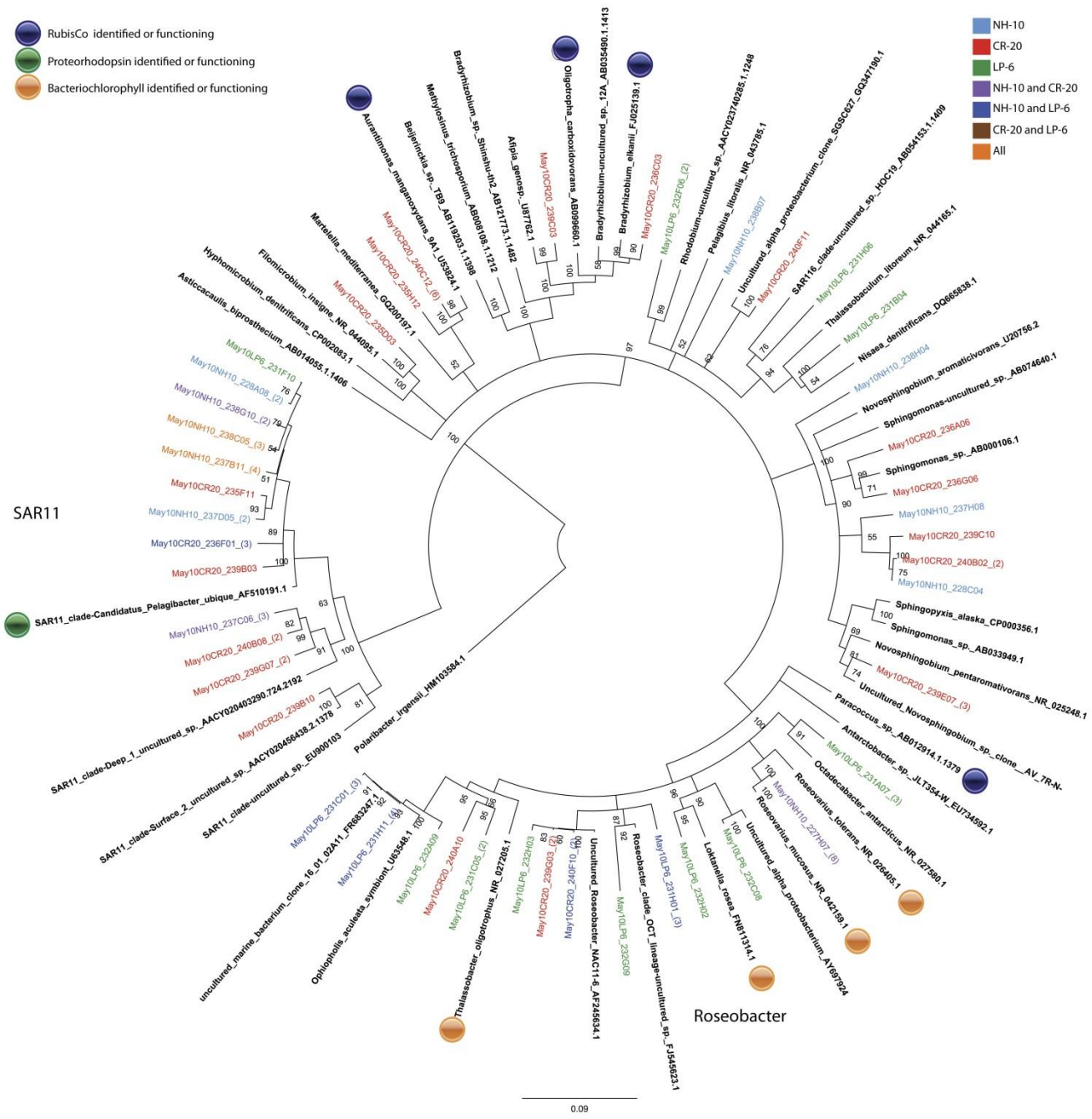

Figure 2.7 Rooted neighbor-joining phylogenetic tree of Alphaproteobacteria involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of ^{13}C fractions from SIP experiments conducted at NH-10, CR-20, and LP-6 in May 2010.

Clones representing highly similar OTUs were collapsed for clarity.

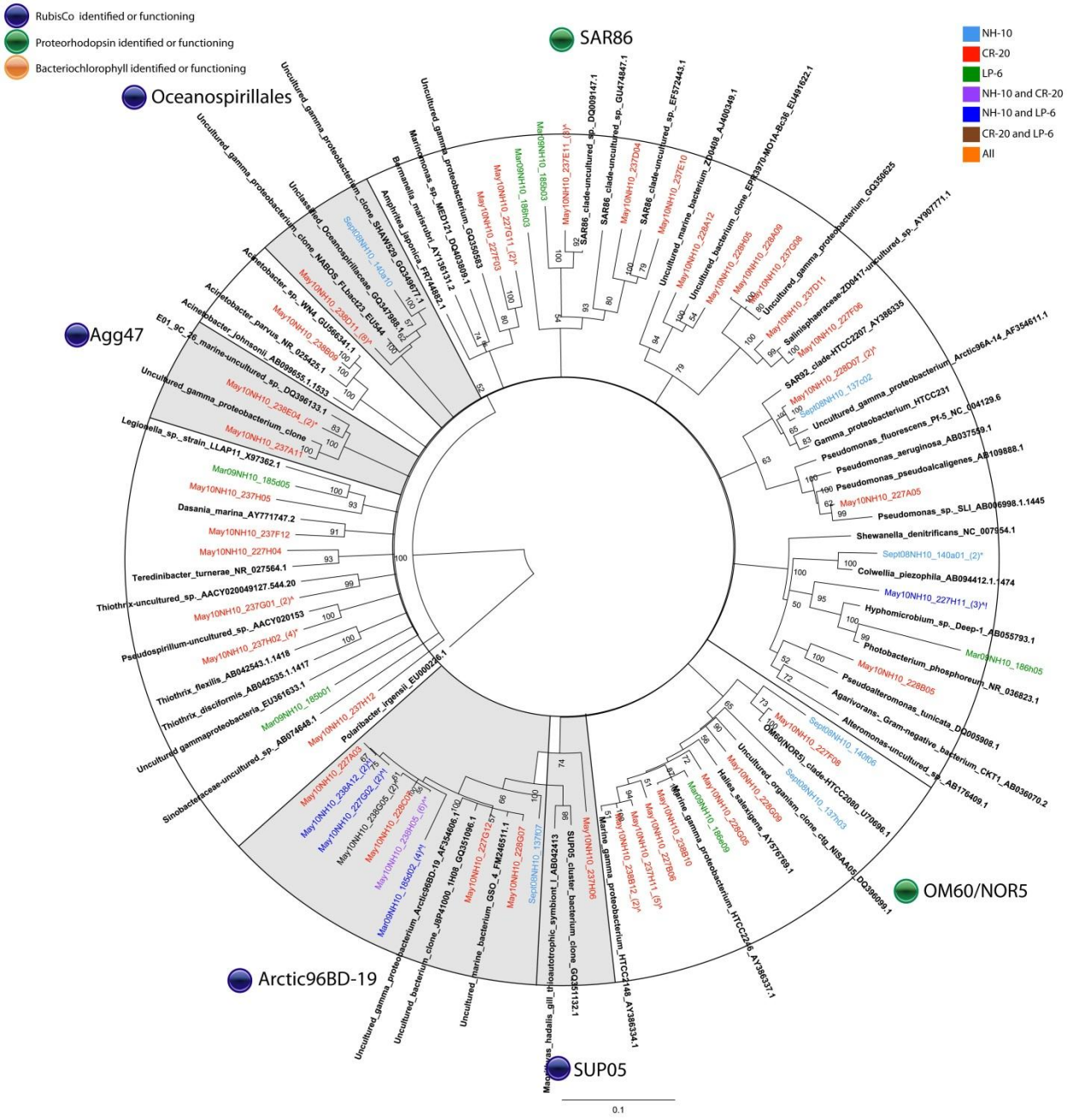

Figure 2.8 Rooted neighbor-joining phylogenetic tree of Gammaproteobacteria involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of ^{13}C fractions from SIP experiments conducted at NH-10 in September 2008, March 2009, and May 2010.

Clones representing highly similar OTUS were collapsed for clarity. Shaded clades represent those identified by Swan et al., 2012 to contain RuBisCO and possibly participate in dark carbon fixation.

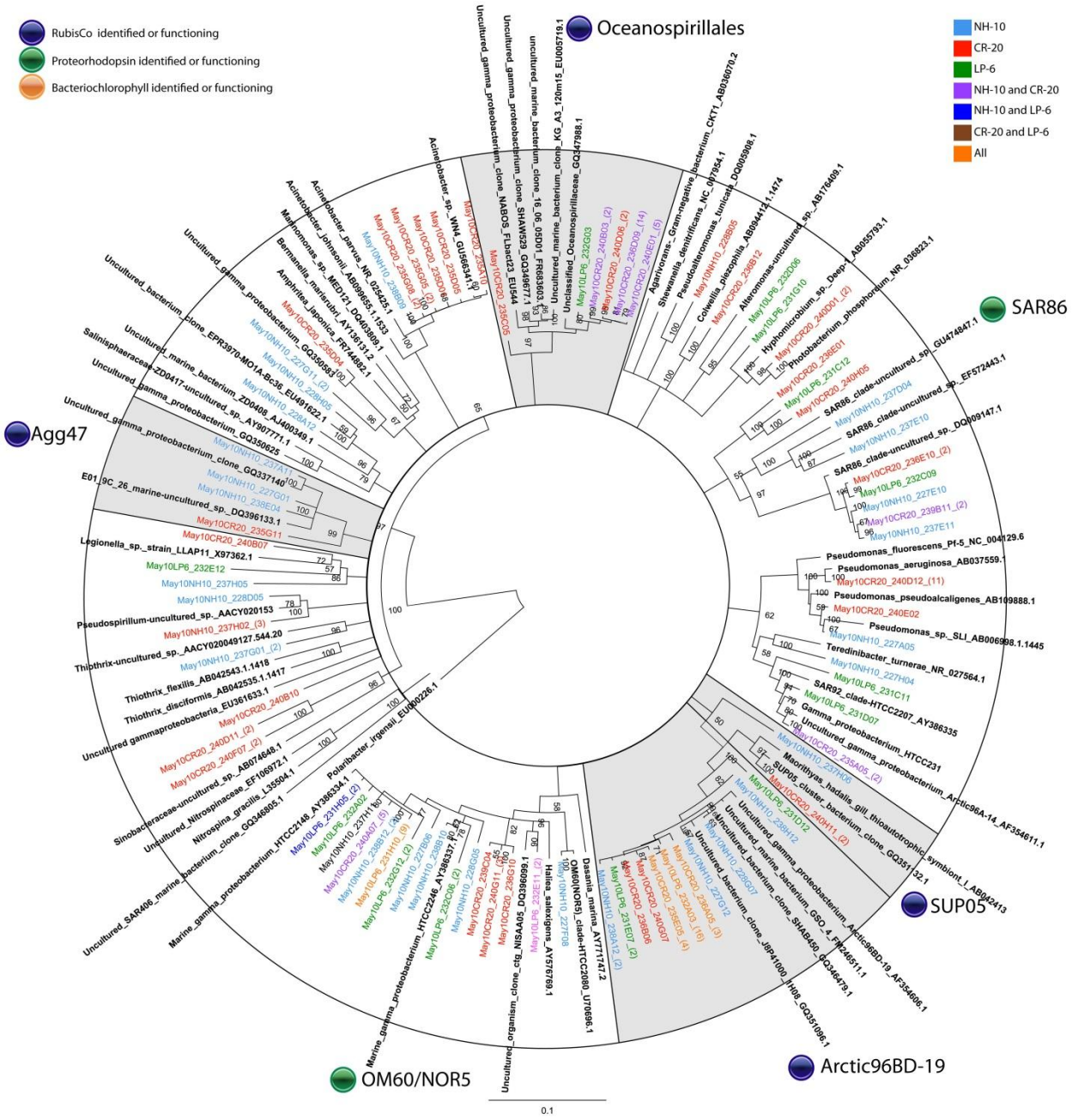

Figure 2.9 Rooted neighbor-joining phylogenetic tree of Gammaproteobacteria involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of 13C fractions from SIP experiments conducted at NH-10, CR-20, and LP-6 in May 2010.

Clones representing highly similar OTUS were collapsed for clarity. Shaded clades represent those identified by Swan et al., 2012 to contain RuBisCO and possibly participate in dark carbon fixation.

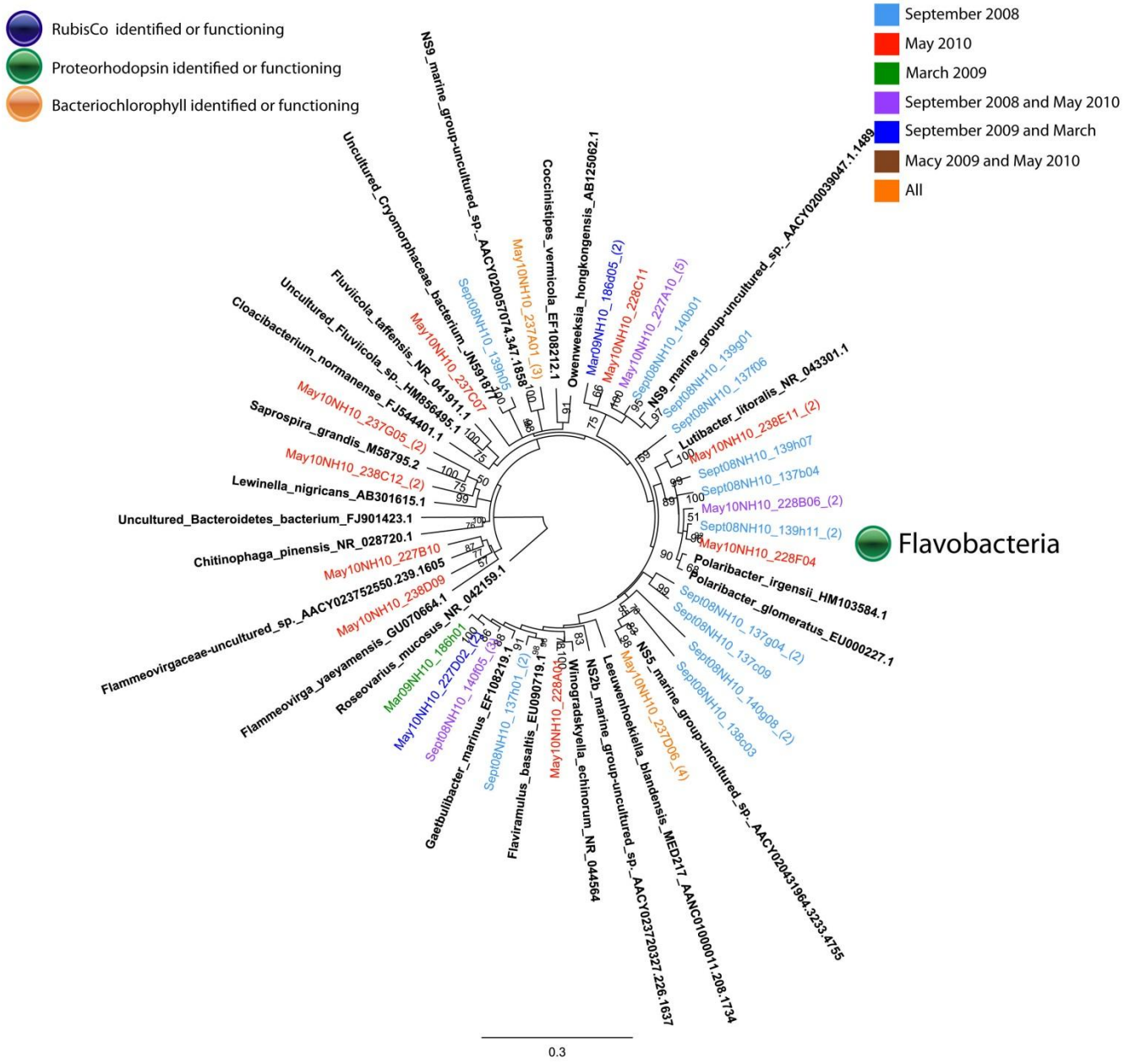

Figure 2.10 Rooted neighbor-joining phylogenetic tree of Bacteroidetes involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of 13C fractions from SIP experiments conducted at NH-10 in September 2008, March 2009, and May 2010.

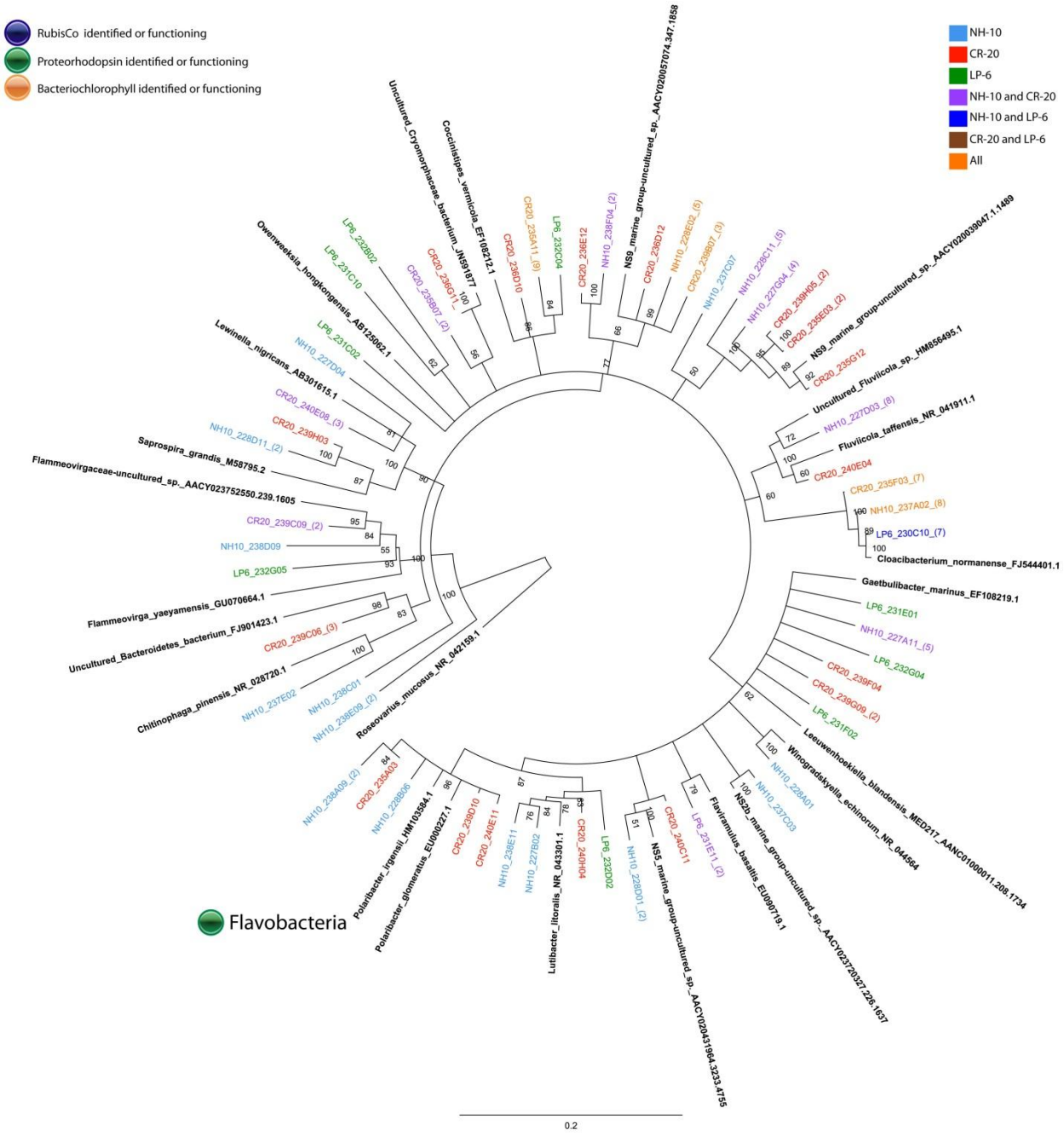

Figure 2.11 Rooted neighbor-joining phylogenetic tree of Bacteroidetes involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of ^{13}C fractions from SIP experiments conducted at NH-10, CR-20, and LP-6 in May 2010.

Highly similar OTUS were collapsed for clarity.

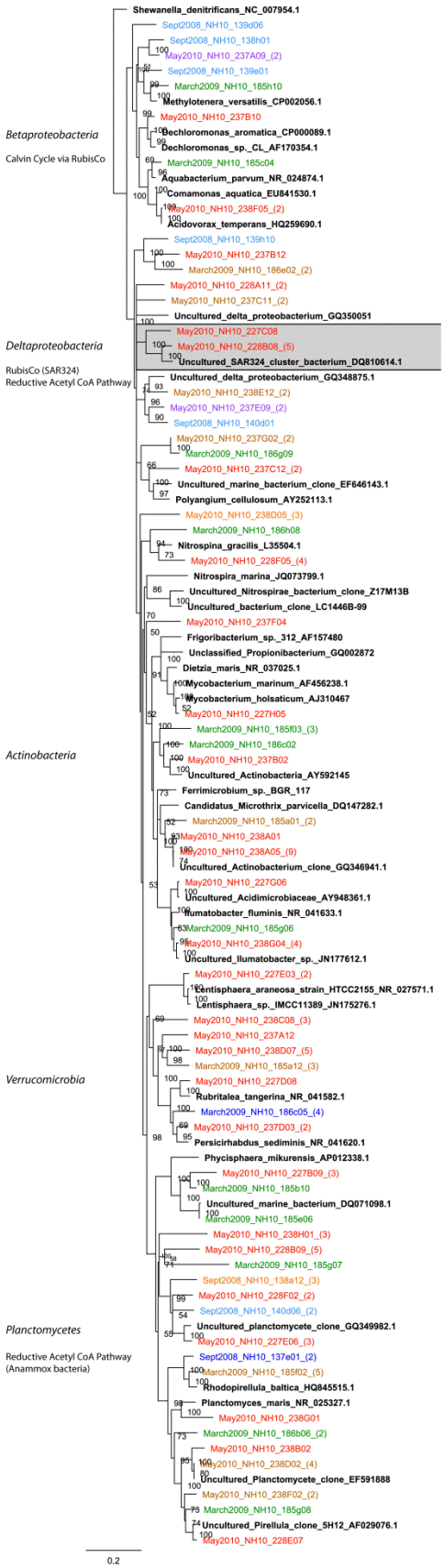

Figure 2.12 Rooted neighbor-joining phylogenetic tree of Deltaproteobacteria, Betaproteobacteria, Verrucomicrobia, Actinobacteria, and Planctomycetes involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of ^{13}C fractions from SIP experiments conducted at NH-10, CR-20, and LP-6 in May 2010.

Highly similar OTUs were collapsed for clarity. Shaded clades represent those identified by Swan et al., 2012 to possibly participate in C-1 metabolism or dark carbon fixation.

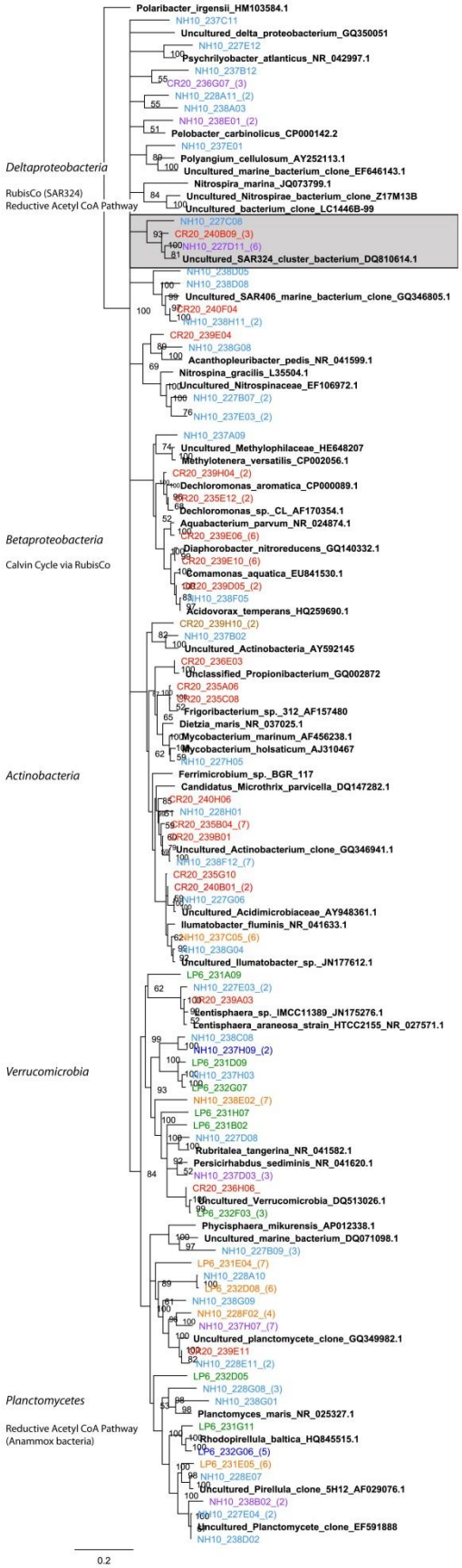

Figure 2.13 Rooted neighbor-joining phylogenetic tree of Deltaproteobacteria, Betaproteobacteria, Verrucomicrobia, Actinobacteria, and Planctomycetes involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of ^{13}C fractions from SIP experiments conducted at NH-10, CR-20 and LP-6 is May 2010.

Highly similar OTUs were collapsed for clarity. Shaded clades represent those identified by Swan et al., 2012 to possibly participate in C-1 metabolism or dark carbon fixation.

2.9 **Tables**

Table 2.1 Total number of clones screened, number of contigs acquired, eukaryotic contigs removed from analysis and the final number bacterial 16S rRNA sequences analyzed are listed by location and season.

Sample	Number of 96-Well Plates	Clones Screened	16S rRNA Contigs Acquired	Eukaryotic Contigs Removed	Bacterial 16S rDNA Analyzed
^{12}C Band NH-10 September 2008	8	768	456	158	255
^{13}C Band NH-10 September 2008	8	768	301	105	176
^{12}C Band NH-10 March 2009	2	192	137	34	103
^{13}C Band NH-10 March 2009	2	192	161	36	125
^{13}C Band NH-10 May 2010	4	384	285	62	210
^{13}C Band CR-20 May 2010	4	384	294	9	259
^{13}C Band LP-6 May 2010	2	192	146	26	94

Table 2.2 Bray-Curtis Similarity Index calculated for Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes in September 2008, March 2009, and May 2010 samples at NH-10 and NH-10, CR-20, and LP-6 in May 2010.

	Alphaproteobacteria				Gammaproteobacteria				Bacteroidetes			
	Shannon	Bray-Curtis Similarity Index			Shannon	Bray-Curtis Similarity Index			Shannon	Bray-Curtis Similarity Index		
	Index	Sep-08	Mar-09	May-10	Index	Sep-08	Mar-09	May-10	Index	Sep-08	Mar-09	May-10
Sep-08	1.98				1.79				1.98			
Mar-09	2.14	25.18			1.95	0.00			2.14	11.59		
May-10	1.44	20.90	26.79		2.72	11.32	3.91		3.05	21.26	11.74	
		NH-10	CR-20	LP-6		NH-10	CR-20	LP-6		NH-10	CR-20	LP-6
NH-10	1.44				2.72				3.05			
CR-20	2.28	44.68			2.53	35.84			2.99	30.15		
LP-6	2.34	10.35	18.33		2.32	33.24	35.45		2.69	4.30	9.99	

Chapter 3: Widespread Occurrence of SUP05 and ARCTIC96BD-19 in the Pacific Northwest Coastal Margin

3.1 Abstract

The Gammaproteobacterial sulfur oxidizers (GSOs), SUP05 and ARCTIC96BD-19, have been found in oxygen-deficient marine environments around the world. However, knowledge regarding their abundance, distribution, and ecological role is just now emerging. In this study, quantitative polymerase chain reaction (qPCR) was used to analyze the abundance and distribution of SUP05 and ARCTIC96BD-19 16S rRNA gene sequences in 49 samples in the Pacific Northwest Coastal Margin collected between August 2007 and September 2009. SUP05 and ARCTIC96BD-19 were detected to varying degrees in all samples analyzed. SUP05 abundance ranged from 5.6 x 10^3 16S rDNA gene copies/mL (depth 49m) to 1.5 × 10^7 16S rDNA gene copies/mL (depth 781m). SUP05 16S rDNA copies/mL correlated negatively with temperature (p=0.0094; *rho*= -0.3676; n= 49) and oxygen (p=0.0132; *rho*= -0.3518; n= 49). ARCTIC96BD-19 represented a varying, but at times significant proportion of the total bacterial population ranging from less than 0.1% to 14.2%. ARCTIC96BD-19 16S rDNA copies/mL was positively correlated with coastal upwelling index at both 45^{o}N (p=<0.0001; *rho*= 0.5681; n= 48) and 48^{o}N (p=0.0056; *rho*= 0.3936; n= 48). While these organisms are known to co-occur, our data supports previous research suggesting that SUP05 and ARCTIC96BD-19 thrive in similar but distinct ecological niches. Overall, while SUP05 is consistently detected in the inner shelf region in the Pacific Northwest, ARCTIC96BD-19-related organisms were more abundant and may be more significant contributors to marine biogeochemistry in shallow bottom waters.

3.2 **Introduction**

Areas of hypoxia, where dissolved oxygen concentrations in the water column fall below 2 mg/L, occur naturally worldwide in many marine environments, including coastal upwelling zones, inland seas with restricted water exchanges, periodically stratified fjords, and oxygen minimum zones (OMZs) (Wright et al., 2012b). OMZs, large regions of the global ocean in which dissolved oxygen in the water column is reduced or nearly absent due to poor circulation combined with a high demand for oxygen (Wright, et al., 2013), are prominent in the eastern tropical and subarctic Pacific and the northern Indian Oceans. Oxygen-deficient and nitrate-rich water define the major OMZs of the world, which support diverse microbial communities that influence the nitrogen budget of the oceans and contribute to major losses of fixed nitrogen as dinitrogen (N_2) and nitrous oxide (N_2O) (Molina et al., 2010) through coupled energy-yielding processes in nitrogen cycling (nitrification, denitrification, and anammox). OMZs are also characterized by sulfur cycle processes, sulfide oxidation, sulfate reduction (Jørgensen et al., 1991; Molina et al., 2010). Both of these cycles fuel light-independent carbon dioxide fixation at depths below the photic zone (Jost et al., 2008; Taylor et al., 2001).

Recent work has demonstrated the widespread distribution of SUP05 and ARCTIC96BD-19, two closely related, co-occurring, thiotrophic Gammaproteobacteria belonging to the Gammaproteobacterial sulfur oxidizer (GSO) cluster in a number of low oxygen environments. These include sulfidic and non-sulfidic OMZs (Dubilier et al., 2008; Glaubitz et al., 2010; Walsh et al., 2009b), and highly productive upwelling regions such as the Eastern South Pacific (ESP) and the Eastern North Pacific (ENP) (DeLorenzo et al., 2012; Lavik et al., 2009a; Stevens and Ulloa, 2008). SUP05 has also been identified in seasonally anoxic fjords with changing sulfide conditions (Walsh et al., 2009b; Zaikova et al., 2010), with abundance found to be as high as

30% of the total bacteria and in the Black Sea (Glaubitz et al., 2013), where SUP05 ranged from 10% to 30% of the total prokaryotic community.

Recent seasonal occurrences of near-shore hypoxia off the coast of Oregon between 44^{o}N and 45^{o}N within the 100m-depth contour, and the development of a 3,000 km^{2} dead zone in 2006, are indicators of ecosystem stress. The expansion of OMZs in the continental shelf, (Grantham et al., 2004; Karstensen et al., 2008) and blooms fueled by upwelled nutrients are possibly the result of shifts in oceanic climate regimes due to global climate change. In the coastal ENP, seasonal hypoxic conditions are induced by upwelling of O_2-deficient waters from coastal OMZs, bringing cold, nutrient rich water onto the inner shelf region resulting in variable biogeochemical conditions. This coastal upwelling fuels productive fisheries, but over the past two decades, shelf intrusions of O_2-deficient water have become more frequent and pronounced as OMZ expansion has been observed throughout the world (Stramma et al., 2008a). Not surprisingly, ARCTIC96BD-19 was identified as a dominant species of the benthic boundary layer of the shallow inner shelf region of coastal Oregon (Bertagnolli et al., 2011), and both SUP05 and ARCTIC96BD-19 were implicated in DIC assimilation through DNA-based stable isotope probing analyses in the bottom waters of the inner shelf region of the Pacific Northwest (DeLorenzo et al., 2012).

The presence and activity of ARCTIC96BD-19 and SUP05 GSO populations in the nonsulfidic waters of the ENP suggests operation of alternative modes of microbial energy generation through activity related to sulfur and nitrogen cycling. This is supported by the detection of putative gene products in SUP05 that function in oxidation of reduced sulfur compounds (e.g., the *sox*-encoded multienzyme complex), the reduction of oxidized nitrogen (e.g., respiratory nitrate reductase, *nar*), and CO_2 fixation (*cbbM*-encoded ribulose bisphosphate

carboxylase/oxygenase (RuBisCO) (Walsh et al., 2009). Therefore, members of the GSO may contribute substantially to major biogeochemical cycles in oxygen-depleted habitats (Jost et al., 2008; Newton et al., 2007).

The potential importance of ARCTIC96BD-19 and SUP05 to carbon, nitrogen, and sulfur cycling in OMZs and hypoxic waters, supports the need for further study to establish a deeper understanding of the breadth and distribution of these lineages in an effort to constrain their roles in eastern boundary upwelling systems, and to obtain additional information regarding their respective ecological and biogeochemical roles. It has been suggested that the occurrence of SUP05 species in non-sulfidic OMZs may serve as a biomarker for changing ecosystem dynamics and periodic or persistent anoxia (Zaikova et al., 2010). Specifically SUP05 can serve as an indicator of a changing water column if found in an environment where it previously did not exist. The major objective of the present study was to gain insights into the abundance and distribution of SUP05 and ARCTIC96BD-19 in the Pacific Northwest Coastal Margin, examine the occurrence of these taxa of emerging interest in relation to coastal upwelling and hypoxia, and evaluate the potential use of these organisms as indicators for ecosystem change.

3.3 **Methods**

Water samples were collected in the Pacific Northwest coastal margin (latitude 44.652 to 47.917, longitude −123.874 to −125.929) (Fig. 3.1) as part of the NSF-funded Center for Coastal Margin Observation and Prediction (CMOP). Samples were collected between 2007 and 2009 on 14 cruises aboard the *R/V Wecoma*. Water samples were collected from and three coastal lines Columbia River (CR) line, Newport Hydroline (NH), La Push (LP) line. The NH Line was sampled in August, and November of 2007; April, June, July, and September of 2008 aboard

the *R/V Wecoma*; and September 2009 aboard the *R/V New Horizon*. The CR Line was sampled in August, and November of 2007; April, June, July, and September of 2008 aboard the *R/V Wecoma* In 2009. The LP Line was sampled in August, and November of 2007 and July 2008 aboard the *R/V Wecoma*. Water samples were collected with 10-L Niskin sampling bottles attached to a SeaBird CTD (conductivity-depth-temperature) rosette equipped with an O_2 sensor.

3.3.1 **Environmental Parameters**

Water was also collected and analyzed for environmental parameters including nutrients, pigments, and rate of bacterial production as described below. For collection of chlorophyll *a*, 0.5–2 L of water was filtered through 25 mm GF/F (Whatman) filters and frozen in liquid nitrogen. Concentrations were determined using high performance liquid chromatography (HPLC) (Wright et al., 1991).

Samples for the analysis of dissolved organic carbon (DOC), total dissolved nitrogen (TDN) and phosphorus (TDP), and nutrients were filtered through ashed 25mm GF/F (Whatman) filters. DOC, TDN, and TDP were then analyzed as previously described in (Fortunato and Crump, 2011) by Horn Point Laboratory (HPL) analytical service. For DOC analysis, 20 mL of filtrate were stored at −20°C in polypropylene vials and analyzed by Horn Point Laboratory (HPL) analytical services (Sugimura and Suzuki, 1988; Valderrama, 1981). 25 mL of filtrate was collected in acid-washed polyethylene vials, and stored at −20°C for dissolved inorganic nutrient measurements. Nutrients were analyzed using an autoanalyzer for ammonium, nitrate + nitrite, nitrite, dissolved silica, and soluble reactive phosphorus at Oregon Health & Science University by the Needoba lab (Gordon et al., 1993). Samples collected prior to 2008 were run at Oregon State University by the Jennings lab.

Bacterial production was measured as the rate of incorporation of L-[^{3}H] leucine (20 nM final concentration) as describe previously by (Fortunato and Crump, 2011). Bacterial carbon production (BP) was calculated from leucine incorporation with a ratio of cellular carbon to protein of 0.86, a fraction of leucine in protein of 0.073, and an intracellular leucine isotope dilution of 2 (Kirchman et al., 1993).

250–1000 mL of water were filtered onto 25 mm diameter ashed (4.5 h at 500 °C) GF/F filters (Whatman) for particulate organic carbon (POC) and particulate nitrogen (PN) analysis and stored at −20 °C. POC and PN content of the suspended particulate matter on acid-fumed filters (Hedges and Stern, 1984) were determined at the University of California, Davis (Verardo et al., 1990). Upwelling index at latitudes 45°N and 48°N (m^3 s^{-1} per 100 m) (Schwing and Mendelssohn, 1997) were reported by the US National Oceanographic and Atmospheric Administration (NOAA, http://www.pfel.noaa.gov/products/PFEL/modeled/indices/PFELindices.html).

3.3.2 **DNA Extractions**

Water for DNA samples (2-6 L per sample) was filtered through a 0.2-µm pore-size Sterivex filter (PES, ESTAR, Millipore) using a peristaltic pump and frozen at −80°C. DNA was extracted using a phenol chloroform method previously described by (Herfort et al., 2011).

3.3.3 **Quantitative Polymerase Chain Reaction**

Total bacterial 16S rRNA gene copy numbers were determined by qPCR using bacterial-specific 533F (5′-GTGYCAGCMGCCGCGGTAA) (Weisburg et al., 1991) and 684R (5′-TCTACSSATTTYACYSCTAC) (Bandi et al., 1994). SUP05 was quantified using a bacteria-

specific forward primer Ba519F, (5′-CAGCMGCCGCGGTAANWC-3′) and a group-specific reverse primer 1048R_SUP05, (5′-CCATCTCTGGAAAGTTCCGTCT-3′) (Zaikova et al., 2010). ARCTIC96BD-19 was quantified using Ba519F and a group-specific primer, 1048R_Arctic (5′-CTATTTCTAGAAAGTTCGCAGG-3′) (Walsh and Hallam, 2011). Each 25 µl reaction contained 12.5 µl DyNAmo Flash SYBR Green (Thermo Scientific), 300 nM final concentration of each primer, 1 µl of template (20-30 ng/µl of DNA) and 10 µl of sterile, nuclease-free water. The reactions were carried out in 96-well qPCR plates (Bio-Rad). Reactions were run on a StepOnePlus Real-Time PCR detection system (Invitrogen) under the following PCR conditions: initial denaturation at 95°C for 3 min, followed by 45 cycles of 95°C for 15 s, primer annealing at 55°C (total bacteria), 63°C (SUP05), 59^{o}C for ARCTIC96BD-19 for 30 s, extension at 72°C for 30 s; following these 45 cycles, a melting curve from 55°C to 95°C, held at each 0.5°C increment for 1 s was performed to check for specificity of the reaction. Real-time data were analyzed with StepOne™ Software (Invitrogen).

Plasmids containing cloned inserts of the target sequence (TOPO pCR 2.1, Invitrogen) obtained from previous studies in the region were used as standards. DNA was extracted and cloned as previously described (DeLorenzo et al., 2012). Plasmids were recovered using the FastPlasmid^{TM} Mini-Prep Kit (5Prime) according to manufacturer's instructions. The concentrated plasmid DNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific). A 10-fold dilution series for each standard ranging from 1.2×10^4 to 1.2×10^9, 1.4×10^4 to $1.4 \times 10^9$and from 1.7×10^4 to 1.7×10^9 copies per µl for bacteria, ARCTIC96BD-19, and SUP05, respectively, was used in analysis. R^2 values for standard curves were 0.97 or better for all runs.

3.3.4 **Statistical Analysis**

Nonparametric Spearman Correlation analysis was conducted using GraphPad Prism Version 6 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3.4 **Results**

A total of 49 individual samples were collected 5-10m from the ocean floor of the Pacific Northwest Coastal Margin with the exception of samples taken off the continental shelf, which were most often collected within the oxygen minimum zone (500-1000m). Nucleic acid extracted from the samples was analyzed for SUP05, ARCTIC96BD-19, and total prokaryotic 16S rDNA by qPCR. The samples covered three different geographic sampling lines (Fig 3.1) over a period of 2 years, with a primary focus on the coastal zone at Newport, OR which experienced periods of anoxia in 2006 (Chan et al., 2008). In all of the samples investigated, ARCTIC96BD-19 and SUP05 rDNA were detected at varying levels.

3.4.1 **SUP05**

QPCR data from near-shore shallow samples from the NH line ranged from $7.8x10^3$ in September 2008 to $1.5x10^6$ 16S rRNA gene copies/mL at NH-5 to $6.3x10^3$ to $3.0x10^6$ 16S rDNA gene copies/mL at NH-10 (Table 3.1). Deeper samples ranged from $1.9x10^4$ in September 2008 to $6.4x10^6$ in July 2008 at NH-20. The deepest location, NH-55 was difficult to compare between sample sets due to inconsistency of sampling depth. Within OMZ range (600m to 1000m; DO <0.5), SUP05 averaged $2.2x10^5$, ranging from $4.3x10^4$ to $6.1x10^5$ copies/ml. In NH-55 samples collected 5-10m above the seafloor in July 2008 and September 2008 (>2000m), SUP05 16S rDNA abundance was $3.0x10^5$ and $2.4x10^6$ copies/m (Table 3.1). DO at this depth was slightly higher averaging 1.0 mg/L (Table 3.2).

Maximum SUP05 copies of 16S rRNA genes/mL reached highest levels in samples collected farthest from the shoreline at depths ranging from 500-1000m where oxygen concentrations were

at a minimum (OMZ waters) compared to other locations on the sampling line. SUP05 abundance reached a maximum of 1.2×10^7 16S rRNA gene copies/mL at depth of 781m at LP-32 in July 2008 where DO reached a low at 0.31 mg/L (Table 3.1; Fig. 3.2a and 3.3). In contrast SUP05 reached 2.9 x 10^6 16S rDNA gene copies/mL in July at a depth of 1565m further off the continental shelf at LP-52 where DO measured 1.19 mg/L (Table 3.1 and Figure S1c). Similar patterns were observed for other deep samples below the OMZ collected in July 2008 at NH-55 on the Newport Hydroline and September 2009 again at LP-52. SUP05 was consistently detected in shallow, near-shore waters, regardless of season. The lowest value recorded for near-shore locations was 5.6 x 10^3 16S rDNA gene copies/mL at CR-7 in November 2007 compared to the highest recorded value at the sample location in July 2008 of 1.6 x 10^6 16S rRNA gene copies/mL (Table 3.1; Fig 3.2b and 3.3).

When normalized to bacterial 16S rDNA gene copy number/mL, however, values suggest that SUP05 often represents a small portion of the total bacterial population. In fact on the CR-Line SUP05 never reached beyond 0.7% of the population, this maximum was observed at CR-40 in July 2008. Normalized values at the LP-32 represented the largest proportion of the prokaryotic population of this sample set, 4.2% of the total population in July 2008. Locations where oxygen concentrations were at a minimum (OMZ waters) often had the highest percentage of SUP05 16S rDNA copy number relative to shallower sites on the same sampling line. All samples taken together, samples below 600m averaged 2.3% where DO averaged 0.5 mg/L, while samples taken above 600m averaged 0.6% when the average DO was 2.2 mg/L suggesting that SUP05 may not be as abundant a contributor to microbial carbon and sulfur cycling on the inner shelf region in the Pacific Northwest compared to other locations where it accounted for up to 10% -

30% of the prokaryotic population (Glaubitz et al., 2013; Lavik et al., 2009a; Stevens and Ulloa, 2008).

3.4.2 **ARCTIC96BD-19**

ARCTIC96BD-19 showed a more variable distribution compared to SUP05. At the near shore NH-5 location, ARCTIC96BD-19 ranged from $7.8x10^3$ to $1.5x10^6$ 16S rRNA gene copies/mL in August 2007 and July 2008, respectively and at NH-10 $2.0x10^5$ and $3.0x10^6$ 16S rRNA gene copies/mL in April 2008 and July 2008, respectively (Table 3.5 and Figure 3.2a). ARCTIC96BD-19 reached a minimum of $4.4x10^4$ 16S rDNA gene copies/mL at NH-55 in April 2008 and a maximum of $7.4x10^7$ 16S rDNA gene copies/mL at NH-20 in July 2008 (Table 3.5). July 2008 samples exhibited the highest abundance of ARCTIC96BD-19 overall on the NH Line (Fig 3.2a).

In general, ARCTIC96BD-19 copies of 16S rDNA gene copy number/mL were highest on the CR Line and peaked at CR-15, $9.1x10^7$ copies/mL in July 2008 with a minimum at the same location in April 2008, $3.3x10^5$ copies/mL. All data taken together, the CR Line had the highest average copy #/mL, 2.9×10^7 16S rDNA gene copies/mL, of ARCTIC96BD-19 16S rDNA when compared to NH and LP sampling lines (Figure 3.4). Bacterial 16S rDNA gene copy number/mL on average at CR was nearly 1 order of magnitude higher than the other lines.

When normalized to bacterial 16S rDNA gene copy number/mL, however, values suggest that ARCTIC96BD-19 often represents a variable but at times significant proportion of the total bacterial population. Samples ranged from less than 0.1% in September 2008 at NH-5, depth 48m to 14.2% in April 2008 at the same location on the NH Line. CR line samples ranged from 0.1% at CR-15 in September 2009 depth 109m to 6.3% at CR-25 in July 2008, depth 318m with an average of 1.6%. LP line samples ranged from less than 0.1% in August 2007 at LP-32 (depth

775m) to about 14% in August 2007 and the near shore location, LP-6 (depth 50m). All samples taken together, samples below 600m averaged 1.0% when normalized while samples taken above 600m averaged 1.7% of the total bacterial population when normalized. On average, normalized ARCTIC96BD-19 values suggest these organisms are a more dominant proportion of the population than SUP05 in the inner shelf region in the Pacific Northwest.

3.4.3 **Statistical analyses**

Nonparametric Spearman rank correlation analyses of the SUP05 data (*n* = 49) were performed on 23 environmental variables collected during the CMOP sampling cruises. Environmental parameters used for Nonparametric Spearman rank correlation analysis are provided in Tables 3.2, 3.3, and 3.4. There was a positive correlation of SUP05 16S rDNA gene copy number/mL with salinity (p=0.0438; *rho*= 0.2895; n= 49). SUP05 16S rDNA gene copy number/mL correlated negatively with temperature (p=0.0094; *rho*= -0.3676; n= 49), oxygen (p=0.0132; *rho*= -0.3518; n= 49), conductivity, pigments such as phaeophytin and chlorophyll, DOC, POC, PON, $PO^{13}C$, bacterial production (BP) (p=0.0407; *rho*=-0.344; n=49), and BP Leucine (p=0.0485; *rho*=-0.3481; n=49) (Table 3.6). Perhaps surprisingly, SUP05 copy #/mL showed no significant correlation to sampling depth. No correlation was found between SUP05 and PO_4^{3-}, N+N, silicate, NO_2^-, NH_4^+, DOC, TDN, TDP, $PO^{15}N$, wind speed, wind direction, or coastal upwelling index.

There was a highly significant positive correlation between ARCTIC96BD-19 16S rDNA gene copy number/ml abundance and coastal upwelling index at both 45^oN (p=<0.0001; *rho*= 0.5681; n= 48) and 48^oN (p=<0.0056; *rho*= 0.3936; n= 48) (Table 3.6 and Fig. 3.4) and a negative correlation to POC (p=0.0489; *rho*= -0.3023; n= 48) (Table 3.6). No significant correlation between other nutrient and physicochemical parameters could be discerned for ARCTIC96BD-

19, however SUP05 and ARCTIC96BD-19 16S rDNA gene copy number/mL abundance were positively correlated with each other ($P < 0.05$) (data not shown).

3.5 **Discussion**

This study provides evidence that the co-occurring, GSO bacterial taxa SUP05 and ARCTIC96BD-19 are present throughout bottom waters of the inner shelf region and OMZ waters of the ENP irrespective of season, location, or bottom depth. Measurements of SUP05 16S rDNA gene copy number/mL suggests a negative correlation with dissolved oxygen, temperature, and bacterial production (Table 3.3). This is consistent with recent research by Fortunato and co-workers who also uncovered negative correlations between relative abundances of SUP05 and temperature, DO, and bacterial production rates (Fortunato et al., 2013). The authors found, particularly in bottom environments, that sequences taxonomically identified as SUP05 contributed up to 38% of the community in the ENP coastal margin. In this study, however, normalized values suggest SUP05 peaked at only 4% of the total prokaryotic population, but ARTIC96BD-19 16S rDNA gene copy number/mL contributed up to 14% of the community. The discrepancy between these values may be due to DNA extraction efficiencies as well as inherent bias in results between qPCR analysis conducted in this study, and pyrosequencing conducted by Fortunato et al. (2013).

Overall, we found it difficult to characterize patterns of ARCTIC96BD-19 abundance and distribution with the metadata collected. Of the 23 metadata parameters evaluated, significant correlations were found only with POC and upwelling indices at 45^{o}N and 48^{o}N (Table 3.3 and Fig. 3.4). This supports a link between wind-driven upwelling events and abundance of GSO clade bacteria in the coastal margin, where cold, nutrient rich, oxygen depleted water can be

delivered onto the near shore shelf from the off-shelf OMZ (Chan et al., 2008; Lavik et al., 2009a; Stevens and Ulloa, 2008) .

In a study off Monterey Bay, Suzuki et al. (2004) found the ARCTIC96BD-19 clade to be the most abundant Gammaproteobacteria group in all investigated depths. The authors suggest that ARCTIC96BD-19 bacteria significantly contribute to the bacterial community only under specific ecological conditions, which could be the spatial and temporal dynamics of coastal regions. However, ARCTIC96BD-19 was not detected in the Wadden Sea, another dynamic coastal system subject to seasonal upwelling (Stevens et al., 2005) suggesting that upwelling events alone are not sufficient to sustain ARCTIC96BD-19 populations, and it is likely specific ecological conditions are required. This further implies that not all coastal systems have the environments necessary to sustain ARCTIC96BD-19 populations. Stevens et al. (2008) suggest the specific ecological conditions responsible for the presence of the ARCTIC96BD-19 clade could be attributed to gradients in oxygen and/or nitrite, while others found that ARCTIC96BD-19 derives energy from reduced sulfur compounds with oxygen as a terminal electron acceptor (Swan et al., 2011 and Walsh and Hallam, 2011). Given the strong correlation observed between ARCTIC96BD-19 and the upwelling index along the Oregon and Washington coasts, such gradients may be generated by the mixing of OMZ waters with shallow shelf waters, or may be supplied as shearing occurs along the benthic boundary, both of which occur through the influence of wind-driven upwelling events. Considering sulfur compounds were not evaluated among the 23 metadata parameters used in this study, the occurrence of ARTIC96BD-19 in the coastal margin should be further evaluated in the context of sulfur compound concentrations to gain further insight into the ecological niches that these organisms inhabit.

Unexpectedly, dissolved oxygen concentrations were significantly correlated with the levels of SUP05 and *not* ARCTIC96BD-19 rDNA copy number/ml. However, a lack of correlation between ARCTIC96BD-19 and DO levels had been observed previously in Oregon coastal waters (Bertagnolli et al., 2011). Yet, in other studies ARCTICBD-19 has been found to be consistently most abundant in dysoxic and suboxic waters (Swan et al., 2011; Walsh and Hallam, 2011). Both SUP05 and ARCTIC96BD-19 members have the potential to use the energy gained from the oxidation of reduced sulfur compounds to fix inorganic carbon via RuBisCO. Furthermore, Fortunato et al. (2013) suggest that the prevalence of taxonomic groups such as SUP05 and ARCTIC96BD-19 in the ENP coastal margin implies that chemolithoautotrophic processes are important in the carbon cycle of these low oxygen environments. In fact, both SUP05 and ARCTIC96BD-19 have been found to actively utilize inorganic carbon at NH-10, CR-20 (5km west of CR-15), and LP-6 in the Pacific Northwest Coastal Margin under both oxic and suboxic conditions, therefore establishing a definitive role in the local carbon cycle (DeLorenzo et al., 2012).

The presence of pelagic SUP05 within H_2S and NO_3^- gradients (Anderson et al., 2013; Glaubitz et al., 2013), and chemolithoautotrophic metabolism based on oxidation of reduced sulfur compounds with NO_3^- through multiple and highly regulated bioenergetic routes (Walsh et al., 2009b), suggests that sulfide is likely present in the system, at least transiently, despite the ENP being classified as non-sulfidic. Together with the presence of the terminal electron receptor, NO_3^-, sulfide concentration may be elevated by suspension (possibly associated with upwelling) from the benthos into the water column on the continental shelf potentially sustaining SUP05 and ARCTIC96BD-19 populations, although it is possible that ARCTIC96BD-19 and SUP05 populations may undergo resuspension from the benthos as well. Investigations of volatile sulfur

compound production during respiration of algal biomass under anaerobic conditions have shown that hydrogen sulfide and other reduced compounds can be produced in large quantities (Emeis et al., 2004; Zubkov et al., 2002). Hydrogen sulfide fluxes from the benthos have been observed in other productive coastal environments such as the South Atlantic and off the coast of Namibia (Emeis et al., 2004). However, information regarding sulfur speciation and abundance is lacking in the Pacific Northwest coastal margin and further research is warranted.

Previous research suggested that SUP05 species may serve as biomarkers for changing ecosystem dynamics in non-sulfidic OMZs and associated coastal upwelling zones. Given the ubiquity of SUP05 observed in this study these GSO organisms may not be optimal sentinels for the potential onsets of near-shore hypoxic events in the coastal margin. SUP05, however, may still serve as a marker for ecosystem change in areas such as estuaries and bays that are not consistently impacted by OMZ waters. This study provides a baseline for SUP05 and ARCTIC96BD-19 abundance and distribution in the Pacific Northwest coastal margin and may serve as a benchmark to evaluate how the abundance and occurrence of these organisms change in relation to future perturbations in the coastal environment.

It is difficult to determine if the consistent detection of these organisms in the shallow near shore region of the coastal margin is the result of regular upwelling events, OMZ expansion, or exchange processes with the benthic boundary layer. However, the ubiquity and abundance of SUP05 and ARCTIC96BD-19 clade bacteria throughout the Pacific Northwest coastal margin is apparent. Considering the recent, well documented association of GSO bacteria with OMZs, it has been surmised that as habitat range increases with OMZ expansion and intensification, the role of organisms such as SUP05 will become more significant in contributing to microbial community responses to the changing marine environment (Zaikova et al., 2010). As such, this

paper provides a foundation for the understanding of distribution, significance, and relevance of the GSO clades SUP05 and ARCTIC96BD-19 in the region.

3.6 **Tables**

Table 3.1 Mean copy number/mL of SUP05 16S rDNA for samples collected on the Newport Hydroline, Columbia River Line and LaPush Line from August 2007 through September 2009.

(-) denotes no sample was available.

	Mean Copy #/ml					
Sample Site	August-07	November-07	April-08	July-08	September-08	September-09
NH-5	1.6E+03	-	2.4E+05	8.4E+05	2.9E+03	4.3E+04
NH-10	4.0E+03	2.9E+04	3.9E+04	9.4E+05	5.1E+03	3.2E+04
NH-20	7.1E+04	2.8E+04	1.7E+05	2.6E+06	7.8E+03	5.9E+04
NH-55	3.7E+04	1.6E+04	4.3E+04	7.3E+02	1.3E+04	1.3E+05
CR-7	3.1E+03	3.7E+03	3.2E+05	6.3E+05	-	-
CR-15	4.4E+03	7.1E+03	8.7E+03	1.2E+05	6.4E+05	-
CR-30	-	3.6E+04	-	4.0E+05	-	-
CR-40	7.2E+04	2.0E+05	4.5E+04	1.0E+06	7.1E+06	-
LP6	1.3E+05	1.1E+04	-	-	-	-
LP17	3.5E+07	1.8E+04	-	-	-	-
LP32	1.2E+07	4.5E+04	-	4.9E+07	-	-
LP52	4.3E+06	1.0E+04	-	3.8E+04	-	-

Table 3.2 Metadata for NH samples collected from August 2007 to September 2009

	Sample Site																							
	Aug-07				Nov-07				Apr-08				Jul-08				Sep-08				Sep-09			
Environmental Parameter	NH-5	NH-10	NH-20	NH-55	NH-5	NH-10	NH-20	NH-55	NH-5	NH-10	NH-20	NH-55	NH-5	NH-10	NH-20	NH-55	NH-5	NH-10	NH-20	NH-55	NH-5	NH-10	NH-20	NH-55
Depth (m)	42	75	135	665	55	75	315	860	40	75	139	1000	49	83	144	2867	44	76	136	830	44	76	136	830
Salinity (psu)	33.7	33.9	33.9	34.2	33.7	33.9	34.0	34.4	33.6	33.8	34.0	34.4	34.0	33.9	34.0	34.7	33.7	33.9	33.9	34.3	33.7	33.9	33.9	34.3
Temperature (°C)	8.0	8.1	7.4	4.8	8.2	7.4	6.5	4.0	8.2	7.8	7.2	3.6	7.0	7.1	6.9	1.7	7.9	7.7	7.6	4.2	7.9	7.7	7.6	4.2
Dissolved Oxygen (mg/L)	2.1	3.0	1.2	0.5	2.9	1.8	1.7	0.4	3.3	2.9	2.3	0.4	1.8	1.9	1.8	1.0	3.2	2.1	2.3	0.3	3.2	2.1	2.3	0.3
PO^4 (µM)	2.9	3.1	2.9	3.2	2.5	2.7	2.7	2.3	0.2	2.1	2.3	2.7	2.7	1.7	2.3	2.9	1.6	1.9	2.3	2.6	1.6	1.9	2.3	2.6
Nitrate + Nitrite (µM)	32.0	34.9	34.9	43.0	29.1	33.8	34.8	27.3	0.5	23.2	28.8	35.0	34.6	19.4	30.6	39.1	19.6	22.6	27.9	29.1	19.6	22.6	27.9	29.1
Silicate (µM)	50.7	64.9	60.1	96.2	48.7	54.1	58.6	70.1	2.4	34.2	47.5	103.2	59.8	30.3	48.0	180.3	23.2	36.4	43.9	74.5	23.2	36.4	43.9	74.5
NO^2 (µM)	0.31	0.26	0.27	0.04	0.34	0.13	0.09	0.13	0.06	0.15	0.16	0.04	0.18	0.06	0.15	0.00	0.10	0.14	0.14	0.03	0.10	0.14	0.14	0.03
NH_4 (µM)	1.40	0.44	0.86	0.15	2.94	0.42	1.03	0.55	1.00	0.70	0.46	0.03	0.57	0.21	0.36	0.00	1.90	0.73	0.31	2.72	1.90	0.73	0.31	2.72
Chlorophyll (µg/L)	6.10	5.15	0.17	0.01	0.38	0.09	0.03	0.03	1.39	0.11	0.04	0.00	0.25	0.04	0.02	0.07	7.72	4.79	0.87	0.03	7.72	4.79	0.87	0.03
Phaeophytin (µg/L)	4.02	1.83	0.57	0.02	1.01	0.19	0.08	0.04	0.51	0.25	0.15	0.01	0.45	0.16	0.10	0.03	1.10	2.00	1.26	0.05	1.10	2.00	1.26	0.05
DOC (mg/L)	1.57	1.60	1.31	1.29	1.80	1.19	1.43	2.12	1.24	0.92	0.93	1.16	0.79	1.07	0.89	0.70	0.90	0.82	0.76	0.86	0.90	0.82	0.76	0.86
TDN (µM)	41.6	42.8	ND	49.6	33.4	38.5	35.4	31.6	ND	34.8	ND	47.5	41.2	36.4	38.4	41.8	22.5	36.6	37.8	50.3	22.5	36.6	37.8	50.3
TDP (µM)	3.1	3.4	ND	3.3	2.6	2.7	2.8	2.3	ND	2.4	ND	3.2	2.5	2.5	2.6	2.8	1.9	2.9	2.8	3.6	1.9	2.9	2.8	3.6
BP (µg/L/hr)	0.51	0.26	0.15	0.03	0.04	0.01	ND	0.01	0.07	ND	ND	ND	0.04	0.01	0.02	ND	0.11	0.13	0.07	ND	0.11	0.13	0.07	ND
POC (µg/L)	300.5	237.5	74.3	37.7	249.3	110.3	48.5	29.5	ND	ND	ND	ND	80.6	60.9	55.4	30.3	469.2	228.5	105.2	118.1	469.2	228.5	105.2	118.1
PON (µg/L)	57.5	43.1	8.3	2.5	26.4	11.2	3.9	2.8	ND	ND	ND	ND	10.1	7.2	6.5	2.3	76.6	36.1	13.2	7.9	76.6	36.1	13.2	7.9
$PO^{13}C$	-20.2	-19.9	-23.2	-28.7	-23.9	-24.6	-25.1	-25.2	ND	ND	ND	ND	-23.4	-23.1	-23.8	-25.3	-18.7	-18.2	-21.3	-26.1	-18.7	-18.2	-21.3	-26.1
$PO^{15}N$	7.4	9.0	11.0	9.0	1.5	-13.0	8.1	7.2	ND	ND	ND	ND	9.6	3.5	1.9	-5.2	5.7	6.8	7.9	6.3	5.7	6.8	7.9	6.3
Average Wind Speed (km/hr)	13.0	ND	15.2	18.1	ND	ND	ND	ND	9.3	10.3	18.2	16.5	17.8	19.2	20.2	ND	3.3	9.5	10.3	12.4	3.3	9.5	10.3	12.4
Average Wind Direction	183.4	ND	163.6	173.7	118.4	150.4	201.1	255.5	260.9	68.1	16.0	33.2	356.5	27.0	177.8	ND	191.5	161.2	160.4	171.5	191.5	161.2	160.4	171.5
Coastal Upwelling Index 45°N	-32	ND	-32	-32	11	11	11	11	16	16	16	16	105	105	105	82	-8	-8	-8	-8	-8	-8	-8	-8
Coastal Upwelling Index 48°N	-10	ND	-10	-10	-4	-4	-4	-4	-6	-6	-6	-6	22	22	22	38	-3	-3	-3	-3	-3	-3	-3	-3

Table 3.3 Metadata for CR samples collected from August 2007 to September 2008

	Sample Site																
	Aug-07			Nov-07				Apr-08			Jul-08				Sep-08	
Environmental Parameter	CR-7	CR-15	CR-40	CR-7	CR-15	CR-30	CR-40	CR-7	CR-15	CR-40	CR-7	CR-15	CR-30	CR-40	CR-15	CR-30
Depth (m)	55	100	860	46	80	139	1000	54	107	850	56	114	318	885	109	317
Salinity (psu)	31.5	33.9	34.3	33.7	33.9	34.0	34.4	33.6	33.9	34.3	34.0	34.0	34.1	34.3	33.9	34.0
Temperature (°C)	15.4	7.2	4.0	8.5	8.1	7.6	3.7	8.1	7.3	4.1	6.7	6.6	6.1	4.2	7.5	7.0
Dissolved Oxygen (mg/L)	0.9	0.9	0.4	3.5	3.0	2.5	0.4	3.4	2.1	0.3	1.0	1.3	1.5	0.3	1.6	2.4
PO^4 (µM)	0.3	2.9	3.3	2.4	2.5	2.5	3.3	1.8	2.0	3.0	2.6	2.2	2.2	2.7	2.7	2.0
Nitrate + Nitrite (µM)	2.78	35.25	43.49	30.37	31.39	31.71	42.72	21.03	25.38	39.60	31.42	26.51	28.79	35.41	31.65	23.40
Silicate (µM)	18.19	62.89	115.48	45.23	44.51	46.65	120.64	33.25	42.28	105.48	59.09	49.27	52.70	91.70	51.38	35.21
NO^2 (µM)	0.06	0.07	0.04	0.21	0.10	0.12	0.09	0.10	0.09	0.04	0.18	0.17	0.10	0.02	0.33	0.04
NH_4 (µM)	0.48	0.30	0.23	1.68	0.38	0.73	2.95	1.03	1.56	0.47	0.38	0.97	1.23	0.38	1.24	0.04
Chlorophyll (µg/L)	14.72	0.08	0.00	0.40	0.16	0.15	0.07	0.25	0.07	0.00	0.07	0.05	1.28	0.02	0.24	0.16
Phaeophytin (µg/L)	0.32	0.29	0.00	1.32	0.84	0.18	0.03	0.50	0.24	0.02	0.23	0.20	0.52	0.02	0.67	0.63
DOC (mg/L)	1.16	ND	1.05	1.04	1.22	1.19	1.47	0.86	2.50	3.88	0.97	0.75	0.76	0.60	1.03	0.84
TDN (µM)	12.5	42.2	51.5	32.4	35.4	34.6	46.6	ND	ND	ND	41	38.7	38.15	43.9	40.65	37.9
TDP (µM)	0.7	2.2	3.2	2.4	2.5	2.6	3.3	ND	ND	ND	2.79	2.86	2.82	3.02	3.02	2.56
BP (µg/L/hr)	0.97	0.04	ND	0.03	0.02	0.02	ND	0.03	ND	ND	0.05	0.06	0.01	0.01	0.08	0.02
POC (µg/L)	415.2	62.1	26.8	201.5	81.6	59.3	26	144.5	83.3	24.9	117.1	83.5	74.9	28.5	95.2	41.6
PON (µg/L)	66	7.1	2.2	25.2	10.7	8.9	2.3	14.6	9.5	2.7	15.1	9.1	7.4	2.9	12.9	4.9
$PO^{13}C$	-18.35	-24.17	-28.45	-22.8	-22.8	-22.9	-25.4	-25.2	-24.8	-26.3	-23	-23.3	-24.3	-25.2	-22.4	-24.2
$PO^{15}N$	7.8	12.4	9.7	6.5	3.6	7.7	5.3	9.5	8.3	4.3	5.9	4.0	8.0	6.1	10.1	8.4
Average Wind Speed (km/hr)	15.6	16.0	5.5	ND	ND	ND	21.9	12.4	11.9	9.9	ND	ND	ND	ND	5.3	3.2
Average Wind Direction	184.0	177.2	213.7	18.1	27.4	65.3	24.3	298.7	273.4	243.6	ND	ND	ND	ND	58.3	105.7
Coastal Upwelling Index 45°N	-8	-8	-8	48	48	48	48	7	7	7	67	67	67	67	3	3
Coastal Upwelling Index 48°N	-3	-3	-3	23	23	23	23	28	28	28	57	57	57	57	0	0

Table 3.4 Metadata for LP samples collected from August 2007 to July 2008

	Sample Site									
	Aug-07				Nov-07				Jul-08	
Environmental Parameter	LP-6	LP-17	LP-32	LP-52	LP-6	LP-17	LP-32	LP-52	LP-32	LP-52
Depth (m)	50	100	775	1500	50	130	600	1000	791	1000
Salinity (psu)	33.5	33.9	34.3	34.5	31.9	33.7	34.2	34.4	34.3	34.6
Temperature (°C)	7.3	7.1	4.2	2.6	10.9	7.7	4.9	3.5	4.5	2.4
Dissolved Oxygen (mg/L)	2.0	1.4	0.4	1.0	5.7	2.0	0.5	0.3	0.3	1.2
PO^4 (µM)	2.99	2.41	3.36	3.20	0.88	2.38	3.02	2.29	2.66	3.27
Nitrate + Nitrite (µM)	31.16	28.58	42.98	43.28	6.01	28.16	39.46	28.94	34.08	43.76
Silicate (µM)	55.44	47.00	108.63	155.01	16.38	43.13	88.47	87.07	90.79	163.17
NO^2 (µM)	0.33	0.05	0.05	0.03	0.35	0.03	0.01	0.00	0.07	0.03
NH_4 (µM)	0.64	0.13	2.74	0.43	1.57	0.80	1.73	0.96	0.17	0.37
Chlorophyll (µg/L)	0.54	0.10	0.00	0.00	0.61	0.05	0.00	0.01	0.01	0.01
Phaeophytin (µg/L)	2.76	0.46	0.02	0.00	0.87	0.21	0.03	0.02	0.06	0.03
DOC (mg/L)	1.26	1.67	1.69	2.33	0.97	0.89	1.00	0.73	1.34	1.17
TDN (µM)	41.8	42.6	52.3	56.1	ND	ND	ND	ND	16.0	14.6
TDP (µM)	3.1	2.9	3.3	3.1	ND	ND	ND	ND	1.0	0.5
BP (µg/L/hr)	0.07	0.03	0.03	0.01	0.03	0.01	ND	ND	0.01	0.01
POC (µg/L)	146	108.6	54.6	32.3	193.9	87.7	34.4	45.9	ND	ND
PON (µg/L)	19.5	13.8	5.7	2.4	26.1	10.5	3.4	3.1	ND	ND
$PO^{13}C$	-22.5	-25.4	-26.3	-27.5	-22.4	-23.4	-25.5	-26.8	ND	ND
$PO^{15}N$	8.5	9.8	8.6	9.1	6.0	6.6	10.6	4.4	ND	ND
Average Wind Speed (km/hr)	12.1	12.6	13.3	19.5	16.8	27.1	21.4	19.0	13.3	ND
Average Wind Direction	169.6	143.3	145.9	187.3	271.6	295.3	153.2	156.1	309.7	ND
Coastal Upwelling Index 45°N	-16	-16	-16	-16	-9	-9	-9	-9	55	55
Coastal Upwelling Index 48°N	-13	-13	-13	-13	7	7	7	7	45	45

Table 3.5 Mean copy number/mL of ARCTIC96BD-19 16S rDNA for samples collected on the Newport Hydroline, Columbia River Line and LaPush Line from August 2007 through September 2009. (-) denotes no sample was available.

	Mean Copy #/ml					
Sample Site	August-07	November-07	April-08	July-08	September-08	September-09
NH-5	1.2E+04	-	4.5E+06	1.0E+07	1.9E+05	2.5E+05
NH-10	3.4E+05	1.5E+06	1.2E+05	2.1E+07	1.3E+06	3.3E+07
NH-20	1.3E+06	3.0E+06	1.0E+06	3.0E+07	1.8E+06	3.1E+06
NH-55	3.9E+04	1.0E+05	2.3E+04	2.4E+06	3.1E+05	4.1E+05
CR-7	7.3E+05	3.0E+06	5.7E+05	3.4E+07	-	-
CR-15	6.9E+05	1.7E+06	6.2E+04	3.7E+07	1.8E+07	-
CR-30	-	4.2E+08	-	2.4E+07	-	-
CR-40	7.2E+05	3.2E+08	2.2E+05	2.8E+07	2.0E+07	-
LP6	1.5E+03	1.9E+05	-	-	-	-
LP17	3.0E+05	7.3E+05	-	-	-	-
LP32	4.7E+05	6.4E+05	-	3.0E+06	-	-
LP52	1.0E+04	9.1E+03	-	2.4E+06	-	-

Table 3.6 Spearman correlation analysis results for environmental parameters collected during this study vs. SUP05 and ARCTIC96BD-19 16S rDNA copy number/mL.

		vs. Sampling Depth	vs. Salinity	vs. Temperature	vs. Oxygen	vs. Turbidity	vs. PO_4	vs. N+N	vs. Silicate	vs. NO_2
SUP05	r	0.208	0.2895	-0.3676	-0.3518	-0.5497	0.1268	0.2006	0.22	-0.1325
	95% confidence interval	-0.08633 to 0.4689	0.0004042 to 0.5339	-0.5936 to -0.08777	-0.5818 to -0.06983	-0.7936 to -0.1536	-0.1684 to 0.4012	-0.09393 to 0.4629	-0.07379 to 0.4787	-0.4117 to 0.1693
	P value	0.1516	0.0436	0.0094	0.0132	0.008	0.3851	0.1669	0.1288	0.3745
	Number of Pairs	49	49	49	49	22	49	49	49	47
ARCTIC96BD-19	r	0.1115	0.1648	-0.04985	-0.03383	-0.07303	-0.06246	-0.06246	0.003164	-0.03715
	95% confidence interval	-0.1835 to 0.3881	-0.1306 to 0.4332	-0.3342 to 0.2428	-0.3198 to 0.2678	-0.4901 to 0.3713	-0.3453 to 0.2308	-0.3453 to 0.2308	-0.2862 to 0.2920	-0.3228 to 0.2547
	P value	0.4455	0.2579	0.7337	0.8175	0.7467	0.6699	0.6699	0.9828	0.8
	Number of Pairs	49	49	49	49	22	49	49	49	49

		vs. NH_4	vs. Chlorophyll	vs. Phaeophytin	vs. DOC	vs. TDN	vs. TDP	vs. BP	vs. POC	vs. PON
SUP05	r	-0.2429	-0.3335	-0.3539	0.06536	0.2433	0.05631	-0.3444	-0.4295	-0.4024
	95% confidence interval	-0.5021 to 0.05642	-0.5701 to -0.04584	-0.5924 to -0.05837	-0.2312 to 0.3508	-0.08792 to 0.5260	-0.2729 to 0.3737	-0.6216 to 0.009412	-0.6518 to -0.1393	-0.6325 to -0.1076
	P value	0.1	0.0205	0.0171	0.659	0.1356	0.7335	0.0497	0.004	0.0075
	Number of Pairs	47	48	45	48	39	39	33	43	43
ARCTIC96BD-19	r	-0.2019	-0.0373	-0.029	-0.201	-0.2004	-0.2769	-0.195	-0.3021	-0.2572
	95% confidence interval	-0.4692 to 0.09923	-0.3259 to 0.2576	-0.3279 to 0.2752	-0.4658 to 0.09680	-0.4926 to 0.1325	-0.5516 to 0.05206	-0.5124 to 0.1693	0.5587 to 0.007277	-0.5243 to 0.05603
	P value	0.1735	0.8013	0.85	0.1707	0.2213	0.0879	0.2768	0.0489	0.096
	Number of Pairs	47	48	45	48	39	39	33	43	43

		vs. PO^3N	vs. Wind Speed	vs. Wind Direction	vs. Coastal Upwelling Index 45°N	vs. Coastal Upwelling Index 48°N
SUP05	r	0.267	0.1499	0.03614	0.09386	-0.04458
	95% confidence interval	-0.04548 to 0.5319	-0.1705 to 0.4416	-0.2794 to 0.3447	-0.2039 to 0.3757	-0.3324 to 0.2508
	P value	0.0835	0.3435	0.8202	0.5257	0.7635
	Number of Pairs	43	42	42	48	48
ARCTIC96BD-19	r	-0.1572	-0.16	-0.1867	0.5681	0.3936
	95% confidence interval	-0.4443 to 0.1593	-0.4499 to 0.1604	-0.4716 to 0.1335	0.3309 to 0.7378	0.1147 to 0.6150
	P value	0.3141	0.3114	0.2364	< 0.0001	0.0056
	Number of Pairs	43	42	42	48	48

3.7 **Figures**

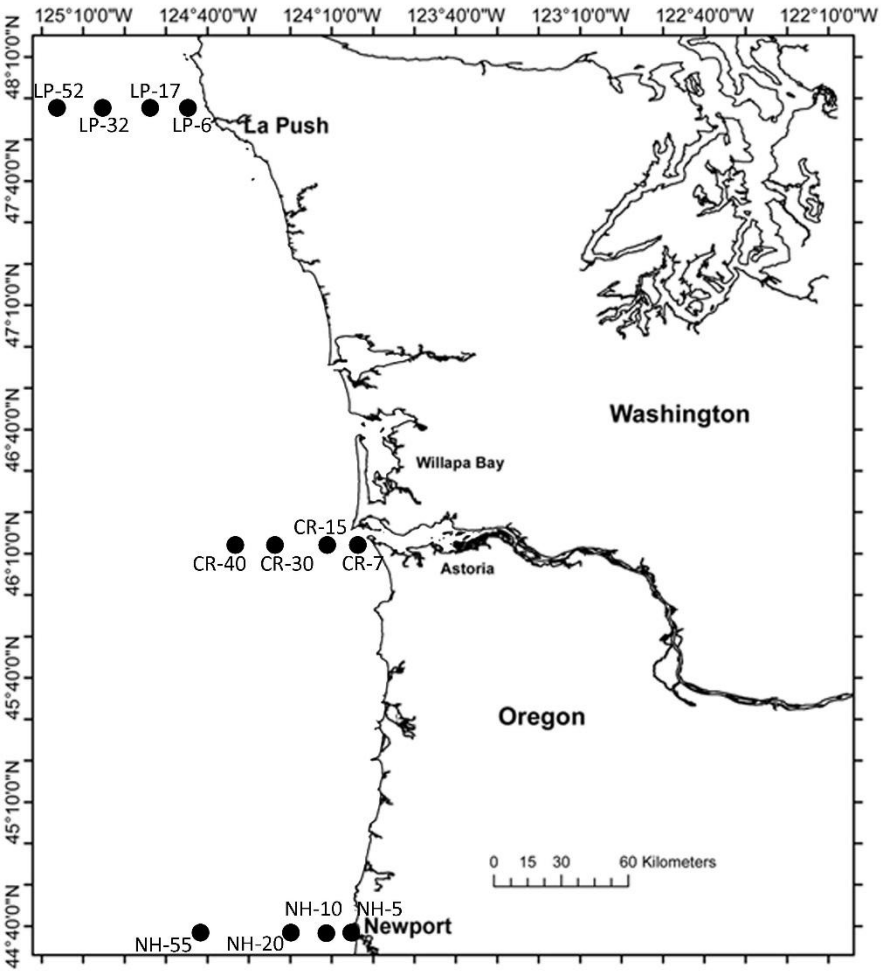

Figure 3.1 Map of sampling lines and individual sample stations, from North to South: LaPush Line, Columbia River Line, and the Newport Hydroline

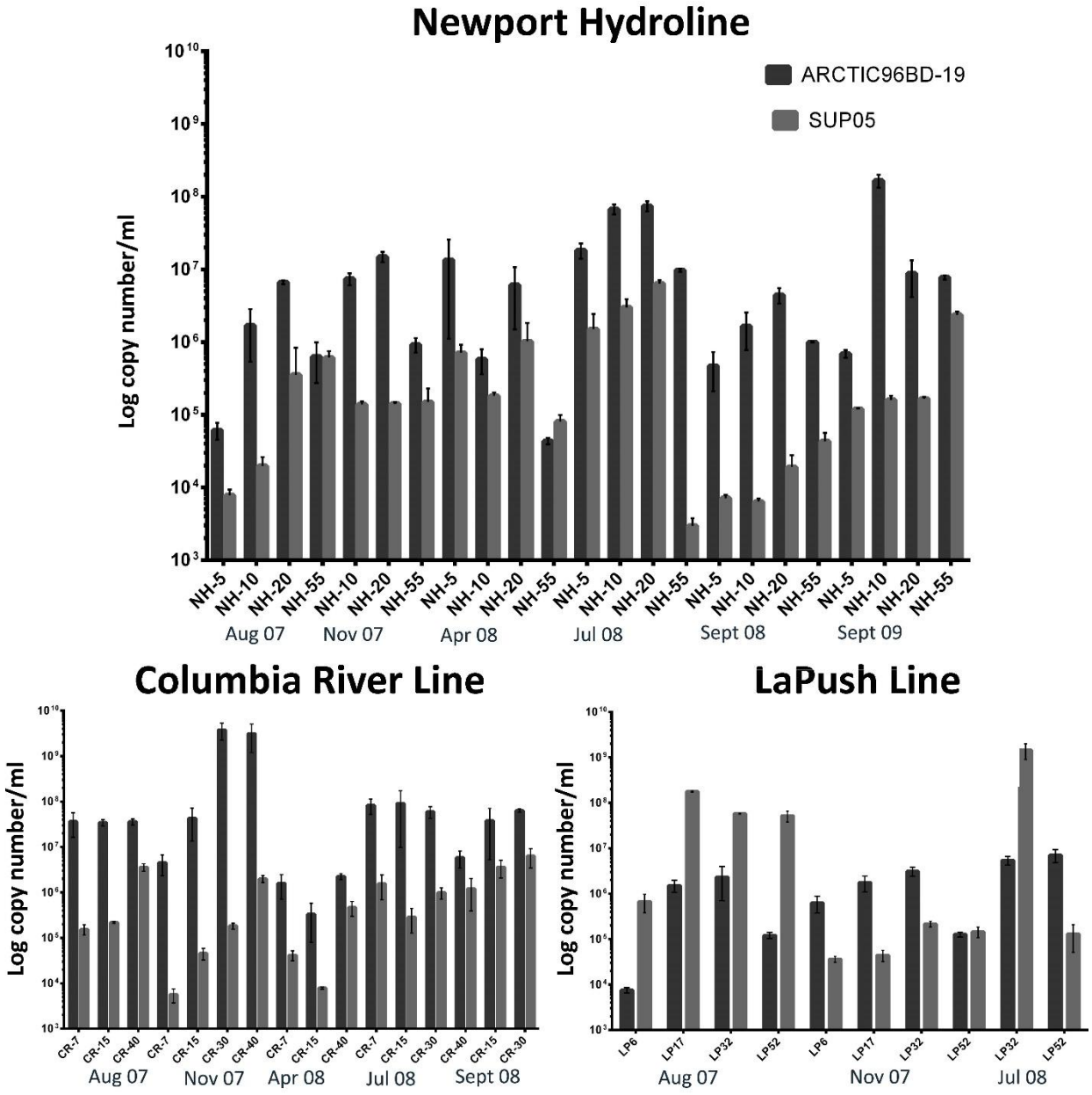

Figure 3.2 ARCTIC96BD-19 and SUP05 log 16S rDNA copy number/ml data for a. Newport Hydroline (NH), b. Columbia River (CR) Line, and c. LaPush (LP) Line for all sampling locations and seasons.

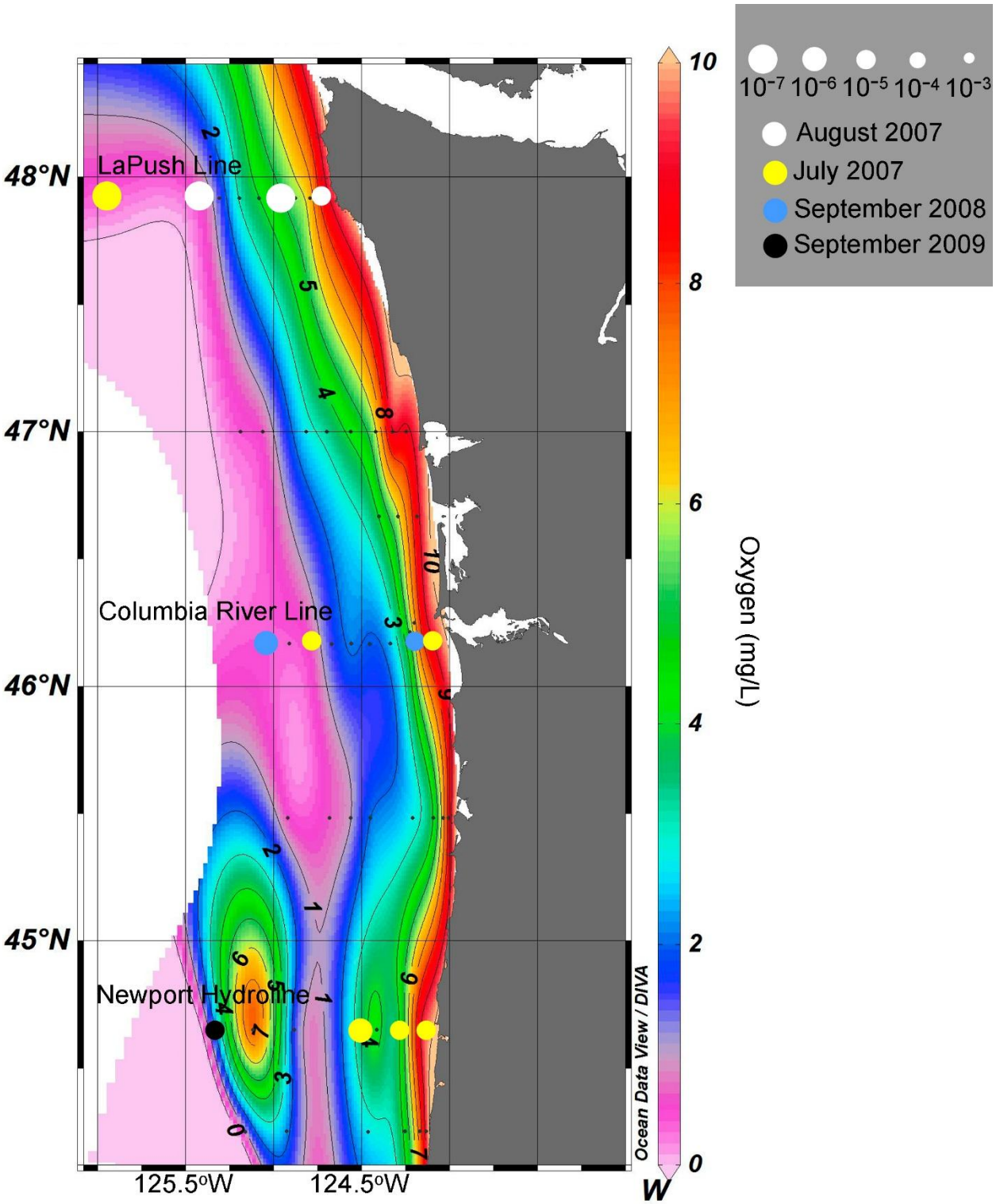

Figure 3.3 The highest SUP05 mean 16S rDNA copy number/ml observed during this study are plotted by location and average dissovled oxygen at bottom depth. SUP05 maximums range from 10^3 to 10^7 and were mostly observed in summer samples.

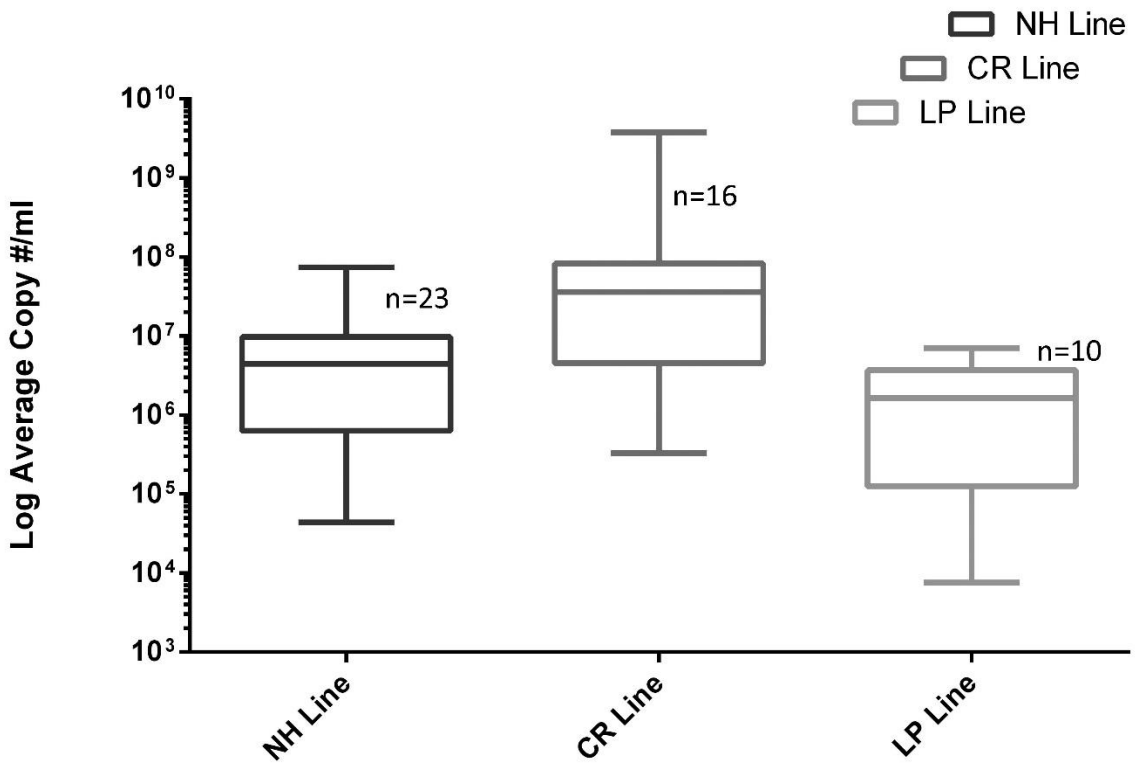

Figure 3.4 Box plot of log average 16S rDNA copy number/mL of ARCTIC96BD-19 for all sampling sites on the NH Line, CR Line, and LP Line. The mean is denoted by the solid line.

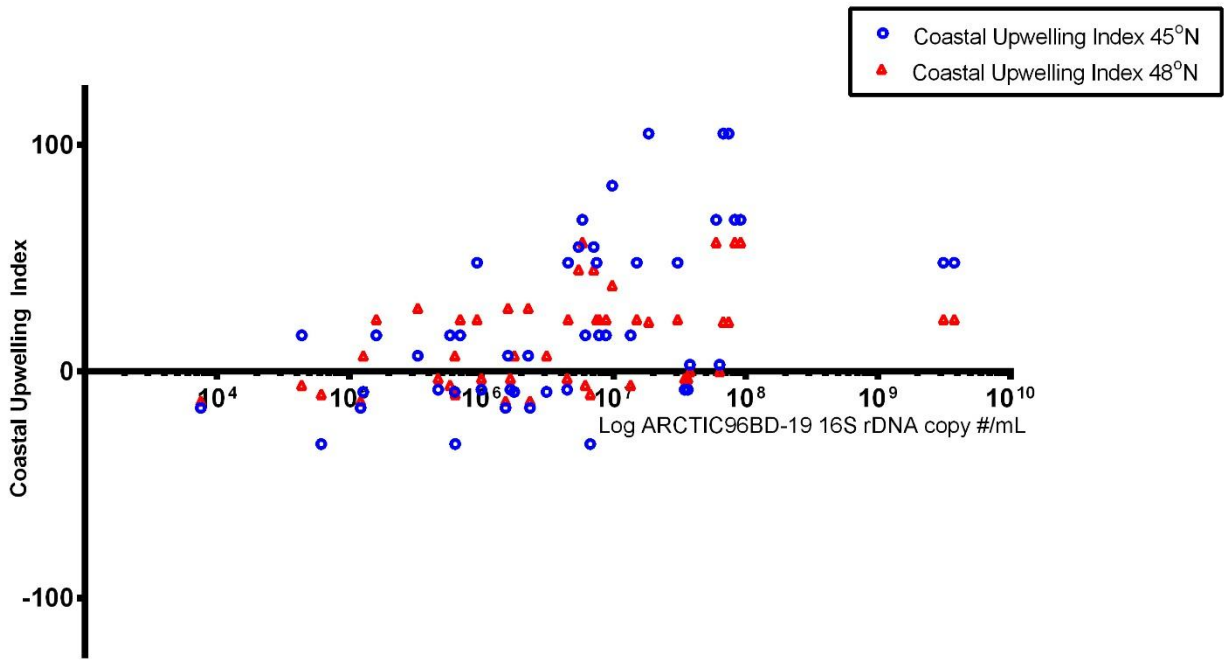

Figure 3.5 Log ARCTIC96BD-19 16S rDNA gene copy number/mL vs. coastal upwelling index at 45°N and 48°N, the Oregon and Washington coasts respectively.

Chapter 4: Bacterial and archaeal amoA gene expression in the coastal northeast Pacific Ocean

4.1 Abstract

Ammonia oxidation was studied in the coastal northeast Pacific Ocean by RT-qPCR amplification of *amoA* gene transcripts to assess the abundance and distribution of Betaproteobacterial ammonia oxidizing bacteria (AOB), ammonia oxidizing archaea Group A (AOA-A) and ammonia oxidizing archaea Group B (AOA-B) *amoA* gene expression in water collected at two different depths in May and July 2010 from two sampling lines subject to varying influence from the Columbia River plume. Variation in *amoA* gene expression was observed with location, depth, sampling period, and shifts in nutrient concentration and dissolved oxygen levels. At sites with minor riverine influence from an aging Columbia River plume AOA-A activity consistently dominated total *amoA* activity. At sites characterized by major freshwater inputs more variability was detected, with the AOA-A *amoA* gene being equal or less abundant than those of AOB in the pre-freshet period of May but dominant during the low flow period of July. AOA-A, and AOA-B *amoA* ratios to marine group 1 *Crenarchaea* 16S rRNA transcripts suggested that ammonia oxidation activity in archaea was more active in bottom waters. Furthermore, nearest the river mouth AOA-A *amoA* ratios to marine group 1 *Crenarchaea* 16S rRNA changed from 2.9 in May to 0.2 in July as AOA-B influence increased concurrently with low flow and moderate upwelling conditions. AOA-B was highly correlated to changes with nitrate + nitrite, particulate organic carbon, salinity, temperature, depth, and dissolved oxygen indicative of deep ocean water. Overall, our results suggest that both coastal

upwelling events and freshwater discharge of the Columbia River have a profound influence on microbial-mediated ammonia oxidation in Northeast Pacific coastal zone.

4.2 **Introduction**

Ammonia oxidation, the microbial-mediated process in which ammonium is oxidized to nitrite and then to nitrate, is carried out by a few lineages in the Beta- and Gammaproteobacteria and by members of the ammonia oxidizing archaea Group A (AOA-A) and B (AOA-B) of the Thaumarchaeota clade within the phylum *Crenarchaeota*. The contribution of ammonia-oxidizing bacteria (AOB) to nitrification has been well documented over the past two decades (Ward, 2008; Ward et al., 1984), however ammonia-oxidizing archaea (AOA) were only recently discovered. The identification of the ammonia-monooxygenase subunit A (*amoA*) gene on archaeal metagenomic scaffolds (Venter et al., 2004) led to the subsequent identification of archaeal *amoA* in a variety of environments such as coastal and marine waters (Beman et al., 2008; Coolen et al., 2007; Francis et al., 2005; Herfort et al., 2007; Lam et al., 2007; Mincer et al., 2007; Wuchter et al., 2006), coastal and estuarine sediments (Beman and Francis, 2006; Caffrey et al., 2007; Francis et al., 2005; Mosier and Francis, 2008; Park et al., 2008), lakes, rivers, and freshwater sediment (Auguet et al., 2011; Francis et al., 2005; Herfort et al., 2007; Herrmann et al., 2008; Vissers et al., 2013), revealing the expansive breadth of nitrification in the microbial world.

Archaeal *amoA* has been observed in marine environments at 10–1,000 times greater abundance than *amoA* from AOB, suggesting that the AOA play a key role in the marine nitrogen cycle (Francis et al., 2005; Mincer et al., 2007; Prosser and Nicol, 2008; Santoro et al., 2010; Wuchter et al., 2006). Pelagic marine *Thaumarchaeota* are typically most abundant below ~100m depth in the water column (Church et al., 2010; DeLong, 1992; Mincer et al., 2007; Santoro et al.,

2010), in surface waters at higher latitudes and polar oceans (Alonso-Sáez et al., 2008; Kalanetra et al., 2009; Massana et al., 1997; Murray et al., 1999a, 1999b), and in hypoxic regions and oxygen minimum zones ($[O_2] \leq 0.5$mg/L) such as the Black Sea, Baltic Sea, Gulf of California, Arabian Sea, and the eastern tropical Pacific Ocean (Beman et al., 2010; Coolen et al., 2007; Labrenz et al., 2010; Lam et al., 2007, 2009; Molina et al., 2010). *Thaumarchaea* are now recognized as the dominant ammonia oxidizers in marine environments, including oxygen minimum zones, while AOBs tend to occupy different aquatic niches, such as freshwater systems (Bouskill et al., 2012; Mincer et al., 2007). This niche differentiation may be due to the high ammonium affinity (Km = 133 nM NH_4^+) and low thresholds ($\leq$10 nM) of AOA for its substrate (Martens-Habbena et al., 2009). However, in San Francisco Bay sediments, AOB *amoA* outnumbered archaeal *amoA* at higher-salinity locations (Mosier and Francis, 2008), which raises the question of whether AOB *amoA* genes may also be more abundant than archaeal *amoA* genes in environments where ammonia-oxidizing archaea are expected to prevail. Furthermore, niche differentiation, specifically with depth, has been observed within the *Thaumarchaea*, as AOA-A *amoA* have been described as a "shallow" gene, with transcripts primarily found in the shallow, euphotic zone of the ocean (<200 m), while AOA-B are considered to be a "deep clade" sequence more characteristic of deep water (>200 m depth) (Beman et al., 2008; Mincer et al., 2007; Mosier and Francis, 2011; Santoro et al., 2010). Additional studies are, therefore, needed to discern the physical and biogeochemical conditions under which AOA-A, AOA-B, or AOB predominate, especially in dynamic coastal environments affected by multiple forcings.

Pacific Northwest coastal waters constitute a complex environment strongly influenced by wind-forced upwelling dynamics (Huyer, 1983; Landry and Hickey, 1989). During upwelling events, south winds draw nutrient-rich, low oxygen, subsurface water into the photic zone, where

microbial growth is stimulated. Low dissolved oxygen levels have been detected in deep water off the continental shelf year-round, but more recently low O_2 levels were also observed in shallower shelf waters during upwelling periods (Chan et al., 2008; Hales et al., 2006; Roegner et al., 2002; Wheeler et al., 2003). The coast is further impacted by the Columbia River, the second-largest freshwater discharge in the United States (Simenstad et al., 1990). Late spring mountain snow-melt results in high river discharge from May through June known as the freshet. The river reaches its lowest discharge volume from September through October as snowpack is depleted.

The Columbia River has a significant influence on biogeochemical processes in the coastal ocean as the estuarine waters mix with the coastal waters and form a lower-salinity plume that carries dissolved metals, nutrients and sediments from the estuary into coastal Pacific waters (Aguilar-Islas and Bruland, 2006; Bruland et al., 2008b; Hickey and Banas, 2003; Hill and Wheeler, 2002; Lohan and Bruland, 2006). In the plume, stratification, nutrient pathways, light and circulation are altered by wind and ambient currents (Hickey et al., 2005). In general the Columbia River plume is oriented southwest offshore of the Oregon shelf in summer and north or northwest along the Washington shelf in winter, but is frequently bi-directional, branching both north and south off the river mouth. In the summer, during a downwelling event, the southwest plume moves onshore over the Oregon shelf as a new plume forms north of the river mouth over the Washington shelf, trapped within~20–30 km of the coast (Hickey et al., 2005). When winds become upwelling-favorable, inner shelf currents reverse and flow to the south, while the shallow plume is advected offshore where it moves farther south, influenced by the seasonal mean ambient flow (Hickey et al., 2005).

To determine the physical and biogeochemical conditions that may influence the levels of AOA vs. AOB activity in such a complex coastal environment we examined the abundance and distribution of AOA and AOB *amoA* gene transcripts in the surface and bottom waters of four coastal Pacific Ocean sites subjected to seasonal upwelling and associated hypoxia but differentially influenced by the Columbia River plume (Fig. 4.1). The analysis of *amoA* gene transcripts was chosen over *amoA* DNA, because it reveals *amoA* expression which is suggestive of active ammonia oxidation. DNA analysis, in contrast, only reveals the potential for activity. Analyses were performed on samples collected in 2010 in pre- (May) and post- (July) freshet when river discharge ranged from 6244 - 7037 m^3/s to 3828 - 4070 m^3/s respectively. In order to normalize data relative to archaeal transcriptional activity we also quantified Marine Group 1 (MG1) *Crenarchaeota* gene transcripts to estimate the fraction of marine bacteria and *Crenarchaeota* that are ammonia oxidizers. To gain a better understanding of the physical and biogeochemical forcing of coastal AOA or AOB communities, we related these transcript abundances and ratios to a suite of environmental parameters.

4.3 **Methods**

4.3.1 **Environmental Setting**

The Columbia River runs 1,200 miles through Canada and the US draining a basin of 259,000 square miles of freshwater into the Northeast Pacific Ocean at Astoria, Oregon and Ilwaco, Washington. The Columbia River has a strong influence on biogeochemical processes in the coastal ocean through the delivery of nutrients in a plume that, during times of high discharge, can extend hundreds of kilometers from the river mouth (Barnes et al., 1970; Bruland et al., 2008b; Hickey and Banas, 2003).

In this study, sampling sites covered two distinct regions: the outer reaches of the near-field Columbia River plume (stations CR-7 and CR-15) and 184 km south off the coast of Newport, Oregon (stations NH-10 and NH-20) (Fig. 4.1). Samples were collected in May 2010 during high flow pre-freshet conditions (Columbia River flow at Bonneville Dam May 22-31, 2010 ranged from 6244 – 7037 m^3/s) and in July 2010, a post freshet period characterized by low river discharge (Columbia River flow at Bonneville Dam measured between July 22-26, 2010 ranged from 3828 – 4070 m^3/s).

The Center for Coastal Margin Observation and Prediction Virtual Columbia River model DB14 was used to assess plume salinity at the sampling time points (http://www.stccmop.org/datamart/virtualcolumbiariver/simulationdatabases). The Virtual Columbia River includes self-redundant long-term simulation databases of 4D (space-time) circulation that are designed to characterize variability and change using realistic bathymetry and external forcings characteristic of the Columbia River coastal margin.

4.3.2 **Sample Collection**

Samples were collected on 22-26 May 2010 and 30-31 July 2010 during two CMOP (Center for Coastal Margin Observation and Prediction) cruises aboard the R/V Wecoma in the Pacific Northwest coastal margin off Newport in Oregon (USA) at sites NH-10 (80 m water column depth) and NH-20 (135 m water column depth) and off the Columbia River estuary at sites CR-7 (56 m water column depth) and CR-15 (113 m water column depth) (Fig. 4.1). Water samples were collected 2m below the surface and 5m above the seafloor with 10 L Niskin sampling bottles attached to a SeaBird CTD (conductivity-depth-temperature) rosette equipped with a suite of sensors including an O_2 sensor. Water was immediately filtered through a 0.2-µm pore-size

Sterivex filter (PES, ESTAR, Millipore) using a peristaltic pump, fixed with 2 mL RNAlater (Ambion) and frozen at −80°C until extraction.

4.3.3 **Environmental Parameters**

Water was also collected and analyzed for environmental parameters including nutrients, pigments, and rate of bacterial production as described below. For collection of chlorophyll *a* 0.5–2 L of water was filtered through 25 mm GF/F (Whatman) filters and frozen in liquid nitrogen. Concentrations were determined using high performance liquid chromatography (HPLC) (Wright et al., 1991). Filtrate from combusted (4.5 h at 500 °C) 25 mm GF/F (Whatman) filters was collected for analysis of dissolved organic carbon (DOC), total dissolved nitrogen (TDN), and nutrients. 20 mL of filtrate were stored at −20°C and analyzed for DOC by Horn Point Laboratory (HPL) analytical services with a Shimadzu TOC-5000 total organic carbon analyzer (Sugimura and Suzuki, 1988). 20 mL of filtrate for TDN was collected in 30 mL, stored at −20°C, and analyzed by HPL analytical services (Valderrama, 1981). For dissolved nitrate + nitrite (N+N) measurements, 25 mL of filtrate was collected in acid-washed polyethylene vials, stored at −20°C, and analyzed using a standard continuous segmented-flow autoanalyzer for N + N at Oregon Health & Science University (Gordon et al., 1993).

Bacterial carbon production (BP) was calculated as previously described by Fortunato and Crump, 2011.

Particulate organic carbon (POC) and particulate nitrogen (PN) were collected by filtering 250–1000 mL of water through a 25 mm diameter combusted (4.5 h at 500 °C) GF/F filter (Whatman). Filters were stored at −20 °C. POC and PN content of the suspended particulate matter on acid-fumed filters (Hedges and Stern, 1984) were determined at the University of

California, Davis using a Carlo Erba NA-1500 Elemental Analyzer system (CE Elantech, Lakewood, NJ, USA) (Verardo et al., 1990).

Upwelling index at latitude 45°N (m^3 s^{-1} per 100 m) was reported by the US National Oceanographic and Atmospheric Administration (NOAA, http://las.pfeg.noaa.gov/) (Schwing and Mendelssohn, 1997).

4.3.4 **Nucleic acid extraction**

Water for DNA samples (2-6 L per sample) was filtered through a 0.2-µm pore-size Sterivex filter (PES, ESTAR, Millipore) using a peristaltic pump and frozen at −80°C. Total nucleic acid was extracted using a phenol chloroform method previously described by Herfort et al., 2011. Extracts were treated with DNase I (Invitrogen) according to the manufacturer's protocol to remove DNA prior to reverse transcription and cDNA synthesis. Purified RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

4.3.5 **Reverse transcription and cDNA synthesis**

cDNA was synthesized using the OmniScript RT Kit (Qiagen) according to the manufacturer's protocol. Each priming reaction contained 2 µl of RNA extract (RNA concentration), 1 µM of corresponding reverse primer (Table 2), 0.5 µM of each dNTP, 1X RT buffer, 10U RNaseOUT (Invitrogen), and 4U OmniScript RT. Blank reverse transcription controls contained the aforementioned reaction mixture substituting DEPC-treated water for the RT enzyme. The reverse transcription reaction products and blank reverse transcription controls were stored at -20^{o}C until use in qPCR.

4.3.6 **Quantitative PCR**

All qPCR assays were carried out in 25 µl reactions using Dynamo Flash SYBR Green MasterMix (Thermo Scientific) on a StepOne Plus real-time PCR machine (Applied Biosystems).

Plasmids containing cloned inserts of the target gene (pCR 2.1, Invitrogen) were used as standards. All reactions were run in triplicate with a standard curve spanning 10^4-10^9 templates. Fresh standards were made from frozen stocks each day of analysis.

Gene-specific primers are listed in Table 2. The primer concentrations were 0.16 µM in each 25-µl reaction, and the following cycling times were used: 95°C for 10 min, followed by 45 individual cycles of 95°C for 15 s, 55°C (AOA-A *amoA*), 56°C (AOA-B *amoA*), 58°C (AOB *amoA*), 58°C (MG1 16S) and 55°C (Bacterial 16S), for 15 s, and 72°C for 15 s. Standard curve coefficients of variation and efficiencies were as follows: Betaproteobacterial *amoA* (r^2 = 0.985 efficiency = 88.7%), archaeal *amoA* group A (r^2 = 0.964, efficiency = 92.9%), archaeal *amoA* group B (r^2 = 0.999, efficiency = 88.5%), *Crenarchaea* marine group 1 16S rRNA (r^2 = 0.996, efficiency = 95.6%), and bacterial 16S rRNA (r^2 = 0.994, efficiency = 112.0%). Melting curve and sequence analyses of qPCR products were used to test for nonspecific amplification. Transcripts abundance reported as zero for AOA-B were below the detection limit which is 2.0 x 10^0 copies/mL as determined by the mean of triplicates.

4.3.7 **Statistical Analysis**

Spearman Correlation analysis was conducted using GraphPad Prism Version 6 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

4.4 **Results and Discussion**

AOA-A numerically dominated the total *amoA* gene transcripts in surface and bottom waters (Fig. 4.2) of the Newport Hydroline sites NH-10 and NH-20, which are coastal environments characterized by minor riverine influence form the Columbia River (Huyer et al., 2007) (Fig. 4.1). Surface samples in May 2010 were 3.1 x 10^4 copies/mL at NH-10 and 1.8 x 10^3 copies/mL at NH-20, and 3.1 x 10^4 copies/mL at NH-10 and 2.8 x 10^4 copies/mL at NH-20 in July. AOA-A at NH-10 was consistently higher compared to NH-20 at bottom depth, with 9.5 x 10^4 versus 4.8 x 10^4 copies/mL in May and 2.4 x 10^4 versus 2.2 x 10^4 copies/mL in July respectively (Fig. 4.2 and Table 4.3). The prominence of AOA-A over AOB in the marine environment is consistent with previous studies that found AOA dominate in marine waters (Coolen et al., 2007; Lam et al., 2007; Mincer et al., 2007).

In contrast, *amoA* transcript abundance at sites where the Columbia River has major impacts on coastal waters showed more variability (Fig. 4.1). On the Columbia River line (CR-7 and CR-15) AOB *amoA* gene transcripts were, on average, equal to or more abundant than those of AOA in both bottom and surface waters in May 2010 (Fig. 4.2). The exception to these patterns was the high copy numbers of AOA-A *amoA* gene transcripts measured in surface water at CR-7 (1.7 x 10^3 copies/mL) and CR-15 (6.9 x 10^3) in July 2010 that were above those of AOB (Fig. 4.2). This might be related to the elevated secondary microbial production measured at that site which was 0.79 µg C L^{-1} h^{-1} at CR-7 and 0.69 µg C L^{-1} h^{-1} at CR-15 (Table 4.1); the highest values recorded during this study. AOB *amoA* has been found to be negatively correlated with bacterial production in Arctic seawater (Christman, 2009), and in good agreement in the present study, AOB *amoA* transcript levels normalized to bacterial 16S rRNA gene transcript copy numbers

(the ratio of AOB *amoA*:16S rRNA) was negatively correlated to bacterial production (p= 0.0014; *rho*= -0.7441; n= 16).

The link between AOB dominance and freshwater input was not only apparent spatially but also temporally. In surface waters, the transcript level of AOB was one order of magnitude higher at the Columbia River line sites during increased freshwater discharge in May 2010 (6244 – 7037 m^3/s), when salinity in May 2010 at CR-7 was 28.4 psu and 30.4 psu at CR-15. This is compared to periods of low discharge (3828 – 4070 m^3/s) in July 2010, when salinity was 30.4 at CR-7, and 28.9 psu at CR-15 (Fig. 4.2). This suggests that the level of AOB *amoA* activity in surface coastal waters is influenced by the Columbia River outflow. It is difficult to ascertain, however, if the increased activity of AOB *amoA* is due to nutrients exported during the high discharge periods or if AOB are flushed into the plume with freshwater from the river, either as free-living organisms or attached to particles. Given the instability of RNA, however, it is likely that the AOB *amoA* activity observed during this study is the result of nutrient discharge. The Columbia River is noted for particle discharge to the plume particularly during periods of high river flow (Hill and Wheeler, 2002; Lohan and Bruland, 2006; Spahn et al., 2009) and is considered to be a significant source of nutrients to the California Current (Aguilar-Islas and Bruland, 2006). AOB *amoA* have been found to be associated more often with freshwater niches (Beman et al., 2008; Caffrey et al., 2007) and within the Columbia River estuary, AOB *amoA* genes amplified by qPCR were highest only in surface water and decreased with increasing salinity (Moeller, unpublished). The prominence of AOB over AOA in estuarine samples influenced by freshwater is in contrast to previous studies where AOA were more abundant in the freshwater regions of the San Francisco Bay Estuary (Mosier and Francis, 2008), the Fitzroy River Estuary (Abell et al., 2010) and Huntington Beach subterranean estuary (Santoro et al., 2008). Yet, our data aligns

with recent research from the Chesapeake Bay estuary where AOA only dominated in more saline, mesotrophic levels of the water column towards the mouth of the Bay compared to AOB (Bouskill et al., 2012).

Normalized AOA *amoA* gene transcription at CR-7 and CR-15 exhibited variability compared to Newport samples that seem to reflect a seasonal (high flow or upwelling) dependency. Surprisingly, values derived from normalized transcript levels did not necessarily align with patterns of transcript copy numbers. The most dramatic response was observed at CR-7. Between May 2010 and July 2010, at bottom depth CR-7 underwent a decrease in dissolved oxygen from 7.0 mg/L to 2.0 mg/L (Fig. 4.3d), which was concomitant with a large increase in TDN (5.0 µM to 39.3 µM), and nitrate + nitrite (3.8 µM to 34.3 µM), (Table 1). Likewise the ratio of Group A AOA to MG1 16S shifted from 2.9 to 0.17 during the same period while the ratio of Group B AOA to MG1 16S increased from below the limit of detection to 0.09. However, bacterial production measured at the site only increased from 0.03 to 0.05 µg/C/L/hr suggesting that these shifts in *amoA* contribution may be due more to changes in nutrients than microbial dynamics. These shifts in environmental parameters and contributions of AOA-A and AOA-B *amoA* transcription may be indicative of reduced river discharge and upwelled coastal waters common in the Pacific Northwest Coastal Margin that bring cold, nutrient rich, oxygen poor water from the oxygen minimum zone onto the shelf. Indeed, AOA-B are associated with sequences derived from deep water (>200 m depth) ecotypes (Hatzenpichler, 2012). In our study AOA-B *amoA*:bacterial 16S was found to be positively correlated with NO_3^-+NO_2^- (p= 0.0009; *rho*= 0.7645; n= 16), TDN (p=0.0018; *rho*= 0.7322; n= 16), salinity (p= 0.0025; *rho*= 0.7159; n= 16) and depth (p=0.0158; *rho*= 0.6013; n= 16), and negatively correlated with dissolved oxygen (p= 0.0017; *rho*= -0.7289; n= 16), temperature (p= 0.0011; *rho*= -0.7431; n= 16), and POC (p=

0.032; *rho*= -0.5345; n=16) suggesting that the bulk of these organisms are likely to have moved with upwelled water masses that typically intrude into the Columbia River estuary during the low-flow summer months (Roegner et al., 2011). While very low numbers (approaching the limit of detection) of AOA-B *amoA* gene transcripts were found in surface waters, this is not surprising as AOA-B sequences have been identified throughout the water column in areas prone to upwelling (Santoro et al., 2010).

Shifts in AOA *amoA* contribution to nitrification at CR-7 are likely to be the result of the influence of river discharge and associated plume characteristics. During the periods of high discharge the plume is large, diffuse and can extend to the seafloor at shallower depths (Spahn et al., 2009), as evidenced by lower salinity and decreased transmissivity at bottom depth at CR-7 in May 2010 (Table 1 and Fig. 4.3b and 4.3c). The late summer plume however is thin, detached from the seafloor, and largely relegated to surface waters, with particles being sheared off from bottom sediments with the ebb tide from the estuary (Spahn et al., 2009). The dramatic increase in normalized *amoA* transcript levels at CR-7 may be the result of upwelling of cold, nutrient rich deep ocean water, observed partially by a decrease in temperature at bottom depth in July (Table 1 and Fig 4.3a), in combination with reduced Columbia River discharge, which allows for greater stratification within the water column.

In the Columbia River estuary, as in other estuaries in this region (Hickey and Banas, 2003) river flows dominate nitrogen inputs into the estuary in winter and spring, while upwelling has a greater influence in summer and fall. Previous gene expression studies in the Columbia River coastal margin have suggested that the microbial response to these nutrient inputs may also exhibit seasonal differences (Smith et al., 2010). Research regarding the influence of river plumes on AOA *amoA* gene transcription is lacking, however our data aligns with recent

research in the Gulf of Mexico that found elevated AOA *amoA* gene copies of DNA relative to AOB in the bottom waters of the Mississippi River Plume, which suffers seasonal hypoxia (Tolar et al., 2013). The hypoxia observed at bottom depths in the Columbia River plume, unlike the Mississippi, is not due to eutrophic conditions, but rather seasonal upwelling of OMZ waters. Therefore this research suggests that the interaction of OMZs with river to ocean plume environments may influence biogeochemical cycles and provides evidence that plume environments may harbor unique microbial communities relative to the surrounding coastal environments. As OMZs expand (Stramma et al., 2008b) and global climate change alters patterns of river discharge (van Vliet et al., 2013) these microbial processes may be significantly impacted. While we cannot conclude with confidence that the elevated AOA-A and AOA-B *amoA* gene transcription relative to total MG1 community is due directly to the influence of seasonal riverine-derived and/or upwelling-derived nutrient inputs in the Columbia River coastal margin, we present evidence that the Columbia River near-field plume can be an area of elevated Thaumarchaeal contribution to the marine nitrogen cycle and thus warrants greater investigation.

4.5 **Conclusion**

The Columbia River coastal margin is a dynamic environment and our analyses of bacterial and archaeal *amoA* transcripts highlight the complexity of its coastal zone. RT-qPCR amplification of *amoA* gene transcripts provided insight into the distribution and abundance of Betaproteobacteria and Thaumarchaeal *amoA* gene transcription at two distinct sampling lines in the coastal northeast Pacific Ocean. Overall, our results suggest differential contributions of the AOB and AOA (AOA-A and AOA-B) to nitrogen cycling in the Columbia River coastal margin, particularly at sites located in the Columbia River plume. In most cases, samples collected near the benthic boundary layer, 5m from the ocean floor, and particularly at Newport Hydroline

sample sites NH-10 and NH-20, had higher transcript copy numbers of AOB and AOA *amoA* by one to two orders of magnitude compared to surface samples. Columbia River Line samples collected during the freshet in May however showed the inverse pattern, suggesting that the freshwater discharge of the Columbia River may influence ammonia oxidation in the plume region (Fig. 4.2).

4.6 **Acknowledgments**

This work was carried out within the context of the Science and Technology Center for Coastal Margin Observation and Prediction (CMOP). We would like to thank Captain Rick Verlini and the crew of the R/V Wecoma. We would also like to thank the CMOP Cyberinfrastructure team especially Paul Turner for providing access to the Columbia River plume models.

4.7 **Tables**

Table 4.1 Environmental parameters measured at the sampling locations given on Fig. 4.1.

Date	Site	Depth (m)	Temperature (°C)	Salinity (psu)	DO (mg/L)	BP (µg/C/L/hr)	DOC (mg/L)	POC (µg/L)	PON (µg/L)	TDN (µM)	$NO_3^- + NO_2^-$ (µM)
May-10	CR-7	2	11.37	28.4	9.2	0.23	1.14	292	103	0.0	0.5
		46	10.47	31.7	7.0	0.03	0.94	121	43	5.0	3.8
	CR-15	2	11.24	30.6	8.8	0.39	0.82	229	61	3.8	1.3
		104	7.38	33.8	2.3	0.06	0.92	188	45	33.4	21.6
	NH-10	2	11.55	30.9	8.3	0.28	0.91	542	122	4.8	2.5
		75	7.85	33.8	3.0	0.01	0.95	199	57	28.7	26.2
	NH-20	2	11.57	30.7	8.7	0.07	0.89	292	68	0.0	4.9
		127	7.64	33.8	3.0	0.02	0.72	109	35	24.5	15.3
Jul-10	CR-7	2	11.50	30.4	10.0	0.74	1.10	962	164	12.2	6.1
		50	6.80	33.9	1.9	0.05	1.05	104	49	39.3	34.3
	CR-15	2	12.87	28.9	9.5	0.69	0.99	954	223	10.6	3.2
		100	6.56	34.0	1.2	0.03	1.11	94	25	39.3	35.6
	NH-10	2	9.38	32.3	9.3	0.34	1.16	1184	297	13.0	7.8
		76	6.83	33.9	2.2	0.01	1.09	57	40	41.9	29.5
	NH-20	2	11.43	32.3	7.4	0.17	1.33	863	231	8.9	1.1
		138	6.89	33.9	2.3	0.02	0.98	41	11	42.0	31.9

Table 4.2 Sequences, melting temperature, and references of primers used for RT-qPCR in this study.

Gene	Group	Primer Name	Sequence (5'-3')	Annealing Temp.	Reference
16S rRNA	Total *Bacteria*	533F 684R	GTGYCAGCMGCCGCGGTAA TCTACSSATTTYACYSCTAC	55°C	Weisburg et al., 1991 Bandi et al., 1994
16S rRNA	*Crenarchaea* marine group 1	GI_751F GI_956R	GTCTACCAGAACAYGTTC HGGCGTTGACTCCAATTG	58°C	Mincer et al., 2007
AmoA	*Betaproteobacteria*	AmoA-1F AmoA-2R	GGGGHTTYTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	58°C	Rotthauwe et al., 1997
AmoA	Thaumarchaea group A	Arch-amoAFA Arch-amoAR	ACACCAGTTTGGYTACCWTCDGC GCGGCCATCCATCTGTATGT	55°C	Beman et al., 2008 Francis et al., 2005
AmoA	Thaumarchaea group B	Arch-amoAFB Arch-amoAR	CATCCRATGTGGATTCCATCDTG GCGGCCATCCATCTGTATGT	56°C	Beman et al., 2008 Francis et al., 2005

Table 4.3 Mean copy number/mL of AOB, AOA-A, and AOA-B *amoA* gene transcripts

			Mean Copy Number/mL		
Date	Site	Depth	β amoA	Group A amoA	Group B amoA
May-10	CR-7	2	2.2E+05	7.0E+03	1.5E+01
		46	1.5E+02	2.7E+00	0.0E+00
	CR-15	2	3.0E+04	5.5E+03	1.5E+01
		104	8.5E+03	5.1E+01	4.0E+01
	NH-10	2	1.5E+04	3.1E+04	3.7E+01
		75	2.5E+04	9.5E+04	3.9E+00
	NH-20	2	3.8E+03	1.8E+03	0.0E+00
		127	4.7E+04	4.8E+04	3.1E+00
Jul-10	CR-7	2	1.3E+03	1.7E+03	9.3E+00
		50	5.8E+04	3.9E+02	1.5E+02
	CR-15	2	5.6E+03	6.9E+03	0.0E+00
		100	3.7E+03	8.3E+02	8.6E+01
	NH-10	2	1.4E+04	3.1E+04	3.3E+01
		76	6.8E+03	2.4E+04	1.0E+01
	NH-20	2	2.5E+03	2.8E+04	0.0E+00
		138	5.4E+03	2.2E+04	8.7E+00

4.8 Figures

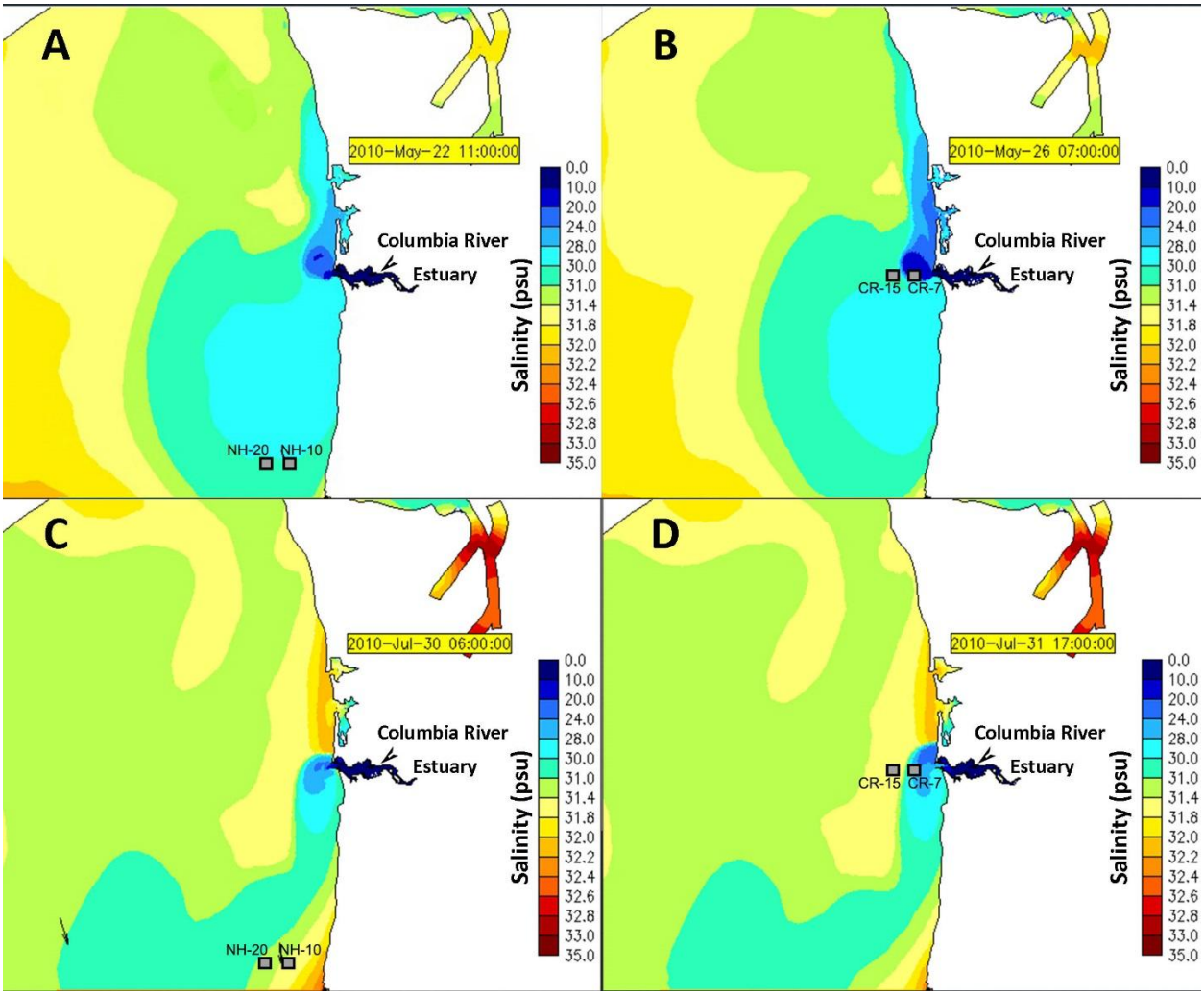

Figure 4.1 Models of surface salinity influenced by the Columbia River plume in the Pacific Northwest Coastal Margin for A. sampling at NH-10 and NH-20 on 22 May 2010, B. sampling at CR-7 and CR-15 on 26 May 2010, C. sampling at NH-10 on NH-20 on 30 July 2010, and D. sampling at CR-7 and CR-15 on 31 July 2010. Models were generated for approximately 1 hour within sample collection time.

From the CMOP Virtual Columbia River model DB 14:
http://www.stccmop.org/datamart/virtualcolumbiariver/simulationdatabases

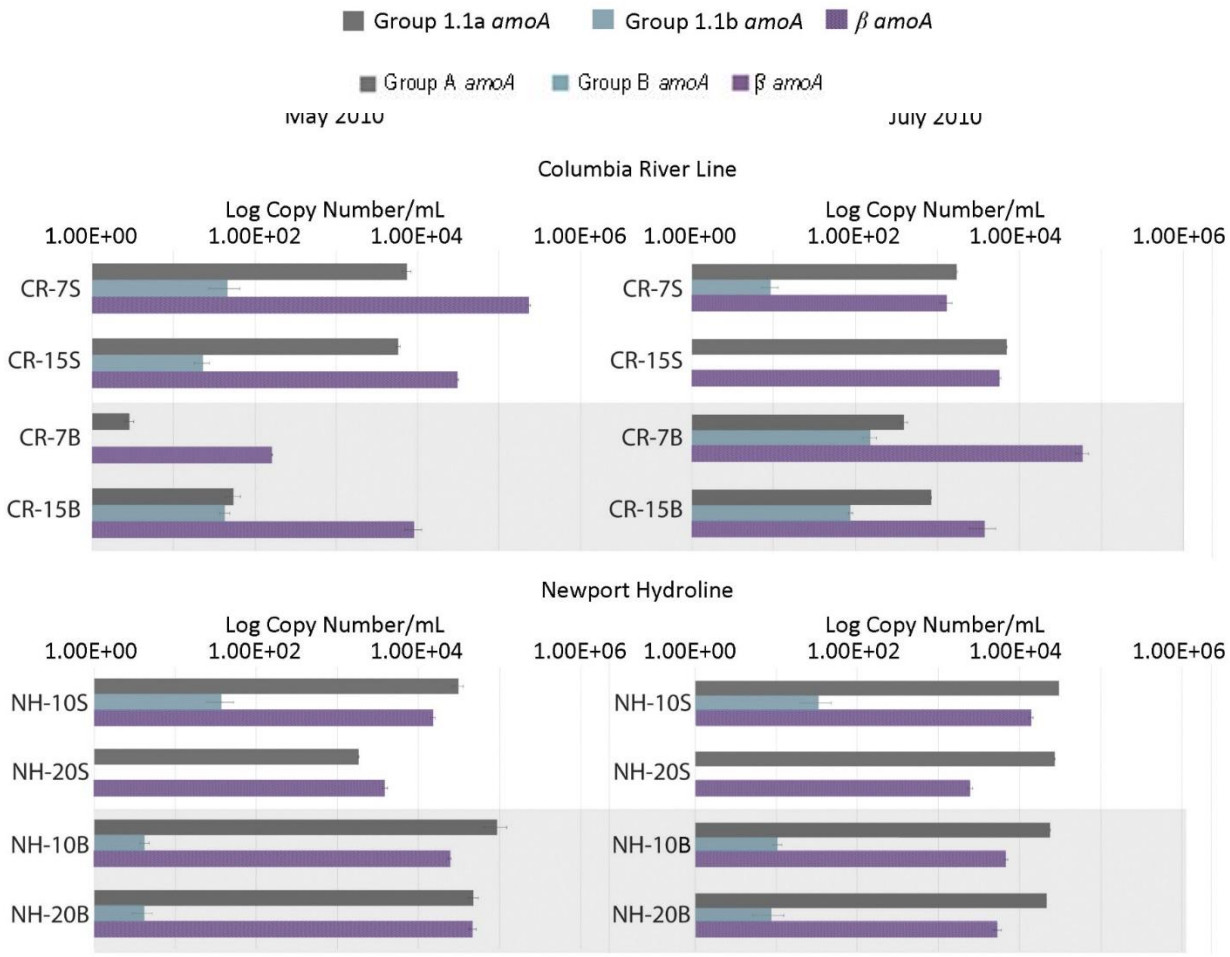

Figure 4.2 Log[10] copy number per mL of AOB, AOA-A, and AOA-B *amoA* gene transcripts determined by RT-qPCR for surface and bottom samples collected at CR-7 and CR-15 (top) and NH-10 and NH-10 (bottom) in May 2010 and July 2010. Grey shading denotes bottom samples.

.

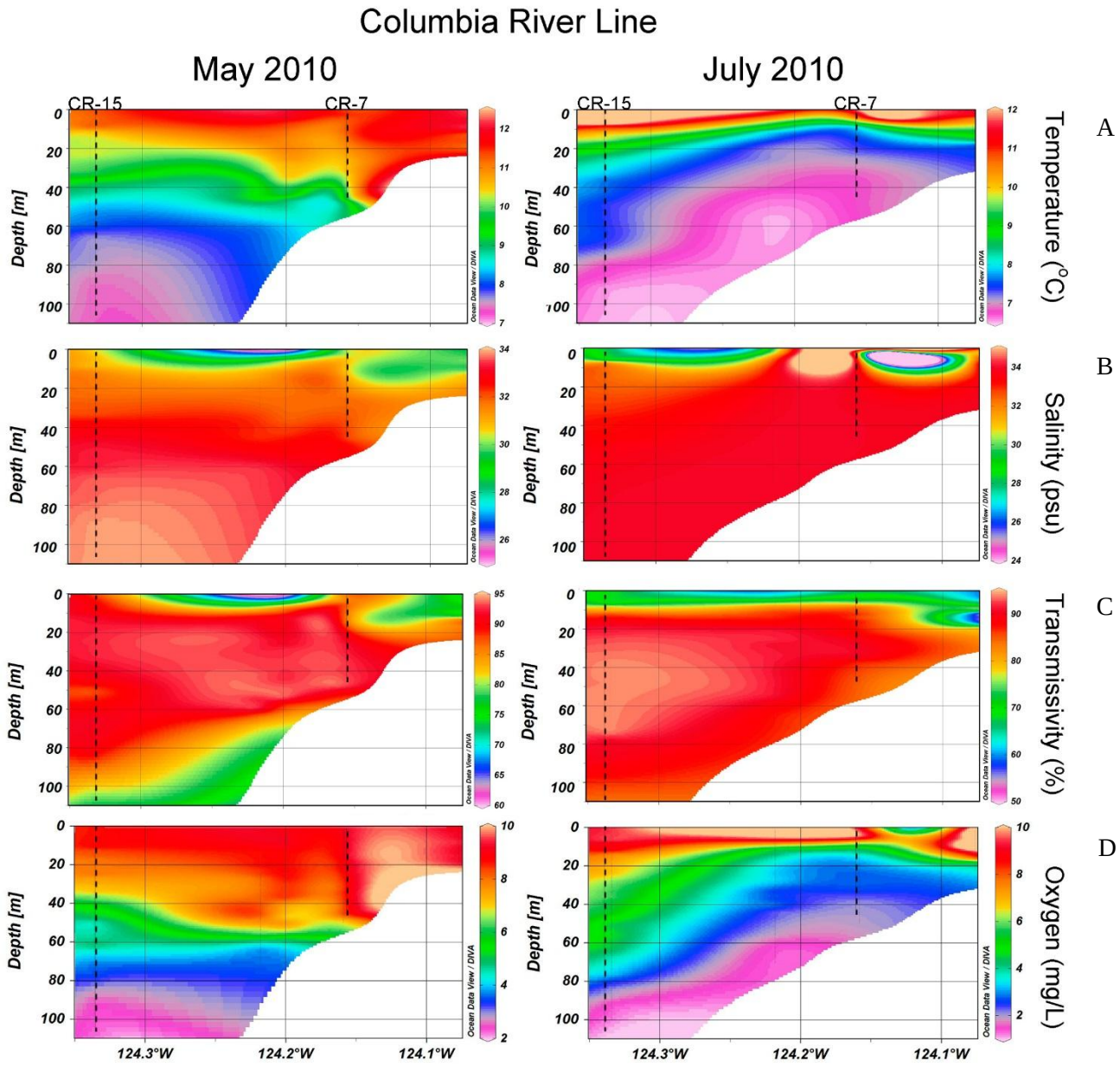

Figure 4.3 Comparison of Columbia River Line water column profiles of temperature (^{o}C), salinity (psu), transmissivity (%), and oxygen (mg/L) from May and July samples.

Chapter 5: Concluding Remarks

The research conducted for this dissertation presents an evaluation of DIC-assimilating microbial communities and their role in microbial-mediated biogeochemical cycles within the Pacific Northwest Coastal Margin, focusing on inner shelf environments that, over the past decade, have experienced increased occurrences of seasonal hypoxia. Stable isotope probing (SIP) was initially used to test the hypothesis that on the Oregon and Washington coasts, transient hypoxic and low oxygen events induced from upwelling and ocean circulation would select for autotrophic or facultative autotrophic organisms, the result of which formed the foundation for the subsequent studies. Three specific questions were addressed:

1 What organisms are actively assimilating DIC in the Pacific Northwest Coastal Margin?

Analyses of bacterial 16S rRNA genes acquired from active fractions of SIP analysis with ^{13}C-$NaHCO_3$ in Chapter 2 revealed that the organisms actively assimilating DIC on the Pacific Northwest Coastal Margin were not limited to autotrophic communities. Bacterial DIC-assimilation operates in Oregon coastal waters across a wide variety of microorganisms: Gammaproteobacteria, Alphaproteobacteria, and Bacteroidetes dominated the DIC-assimilating community regardless of sample location or time. This result did not necessarily support our initial hypothesis that the hypoxic waters of the Pacific Northwest coastal margin would favor autotrophic organisms capable of oxygen tolerant DIC assimilation. The diversity of organisms identified actively assimilating DIC, however, resembled data obtained from SIP incubations conducted in the sulfidic zone of a Black Sea redoxcline. In that study clades of Gammaproteobacteria such as *Alteromonadales* and *Oceanospirillales*, primarily considered to be dominated by heterotrophs, were also implicated in DIC-assimilation (Glaubitz et al., 2010). Considering the complex nature of Pacific Northwest coastal waters - which are subject to

periods of elevated primary productivity, OMZ influence, hypoxia, as well as riverine and physical processes that can result in alterations in the composition or delivery of labile DOC - Chapter 2 suggests that the ability to assimilate DIC may be important for marine bacteria to thrive in a dynamic environment. Data from active SIP fractions also revealed the activity of organisms potentially involved in transformations of inorganic sulfur and nitrogen compounds.

2 *Are any of the organisms identified through SIP associated with low-oxygen or hypoxic environments?*

The results in Chapter 2 led to qPCR analyses of 16S rDNA in Chapter 3 which in turn provided insights into the distribution of co-occurring SUP05 and ARCTIC96BD-19 clade bacteria in coastal waters, both considered to be associated with hypoxic and anoxic environments (Fuchs et al., 2005; Lavik et al., 2009b; Stevens and Ulloa, 2008; Walsh et al., 2009a). Our results indicate that both SUP05 and ARCTIC96BD-19 have a consistent presence in the inner continental shelf of the Northeast Pacific Ocean. As anticipated, the presence of SUP05 correlated negatively with dissolved oxygen, but ARCTIC96BD-19 was much more prominent, particularly in inner-shelf samples, and was strongly correlated with the coastal upwelling index. The coastal upwelling index is a calculation based upon Ekman's theory of mass transport due to wind stress. Ekman mass transport, defined as the wind stress divided by the Coriolis parameter (a function of the earth's rotation and latitude), is then resolved into components parallel and normal to the local coastline orientation. The magnitude of the offshore component is considered to be an index of the amount of water upwelled from the base of the Ekman layer. Positive values are the result of upwelling while negative values suggest downwelling (NOAA, http://las.pfeg.noaa.gov/). This suggests that wind-driven upwelling events, which pull nutrient rich OMZ waters often

associated with hypoxia on the continental shelf, influence the presence of ARCTIC96BD-19 in the coastal margin.

3 What is the influence of coastal upwelling of OMZ water and the Columbia River on nitrogen processes in the coastal environment?

Building upon observations of the activity of potential organisms involved in nitrogen cycling identified within the Pacific Northwest coastal zone in Chapter 2, RT-qPCR amplification of *amoA* gene transcripts provided insight into the distribution and abundance of Betaproteobacteria and Thaumarchaeal *amoA* northeast Pacific Ocean. Data suggested differential contributions of the AOB and AOA (AOA-A and AOA-B) to nitrogen cycling in the Columbia River coastal margin, particularly at sites located in the Columbia River plume. Samples collected at bottom depth at sites that were only mildly influenced by the Columbia River plume had higher transcript copy numbers of AOA *amoA* in the majority of the samples. Samples collected at sites strongly influenced by the Columbia River plume during high flow conditions in May, however, show higher AOB *amoA* transcript copy numbers suggesting that the freshwater discharge of the Columbia River may influence ammonia oxidation in the plume region. Furthermore, AOA-A and AOA-B comprised a significantly higher proportion of the total Marine Group 1 archaeal population at bottom depths in locations strongly influenced by the plume, but subjected to stronger seasonal hypoxia associated with upwelling conditions. Areas subject to intense seasonal fluxes of freshwater input, nutrients, and dissolved oxygen, such as Columbia River Line sites, may also be areas of significant biogeochemical cycling relative to the surrounding coastal zone as the changing environment may select for organisms able to tolerate nutrient limitation, enrichment, and diminished oxygen levels, in particular chemoautotrophic organisms. This is further supported by the results of Appendix A where shifts in microbial communities

actively assimilating DIC were observed (Figs. A.1 – A.4) when high flow conditions characterized by particulate discharge from the Columbia River changed to low flow conditions with great upwelling influence.

5.1 **Recommendations for Future Research**

This work highlights the complexity of Pacific Northwest coastal zone and the dramatic influence the physical and biochemical parameters can have on local biogeochemical cycles. The coastal zone is characterized by upwelling and high oceanic productivity, which supports a robust ecosystem that is particularly vulnerable to environmental perturbations such as hypoxia, ocean acidification, and alterations in biogeochemical cycles. The continued progress of global climate change may have a profound influence on coastal ecosystems. Microbes can be sentinels of change. An understanding of microbial response to changing environments may provide the means to predict the impact environmental perturbations will have on higher trophic levels, possibly even on a global scale.

The diverse community assimilating DIC, as detailed in Chapter 2, suggests many organisms typically associated with heterotrophic metabolism have the ability to assimilate DIC, further suggesting that there is much still to be learned about marine microbial metabolism and that microorganisms may be more adaptive than previously believed. It also begs the question of how this DIC uptake may affect the balance of ocean carbon cycles. For example, if a greater number of organisms are taking up DIC than previously assumed, DIC-assimilation may help buffer the effects of ocean acidification (the direct result of increased CO_2 in the atmosphere and marine environment). Furthermore, it suggests that changes in the availability of quality labile carbon may force many microorganisms to utilize alternative energy pathways, or select for organisms capable of traditional autotrophic growth.

Chapter 2, showed the widespread occurrence of GSO bacteria within the inner shelf of the Northeast Pacific coastal zone. The assertion that the GSO clade could serve as sentinels for hypoxia remains unresolved, however the ubiquity of SUP05 in the coastal zone suggests that these organisms may not be optimal sentinels for hypoxia or OMZ expansion in near-shore environments. While our work lays a foundation for explorations of the spatial patterns of GSO bacteria on the coast, a more robust analysis of the occurrence and activity of these organisms should be conducted and assessed. A strong focus on sulfur and nitrogen cycling of these organisms would also be of interest to coastal management, as the potential for a versatile energy metabolism suggests multiple pathways for sulfur oxidation and denitrification. The continued propagation of organisms such as ARCTIC96BD-19 bacteria in low oxygen shelf waters may strongly influence biogeochemical properties in the area.

As OMZ expansion and variations in river flow may directly influence the marine nitrogen cycle, a more detailed assessment of AOA-A and AOA-B in the coastal margin should be conducted. While we presented compelling evidence that the Columbia River near field plume may be an area of elevated archaeal *amoA* gene transcription relative to total MG1 community in Chapter 3, a larger dataset spanning multiple time points is suggested. Furthermore, while ammonia oxidation within the Columbia River estuary has been studied (see Moeller, unpublished), the influence of seasonal riverine-derived and/or upwelling-derived nutrient inputs in the Columbia River coastal margin on ammonia oxidation warrants greater investigation. Furthermore, the limited data provided in Appendix A suggests that microbial community housed along the benthic boundary layer beneath the Columbia River plume may be influenced by particulate discharge. Future work should focus on the analysis of *amoA* gene transcript levels in relation to *amoA* gene copy number to determine if *amoA* genes are induced in response to environmental

conditions influenced by the Columbia River plume or if changes observed were due to the result of upwelling and hypoxia.

Marine microbes, are responsible for approximately half of the Earth's primary production and play a critical role in global nutrient cycling. Understanding and establishing the specific roles these organisms play in carbon and nutrients is particularly important in the face of global climate change. This dissertation demonstrated that the biogeochemical potential of DIC-assimilating microbial communities within the Pacific Northwest coastal margin. Over the next century the coastal environment will likely undergo dramatic changes, along with changes in ocean circulation and physical forcings. The distribution and activity of microorganisms will have a significant impact on ocean and atmospheric chemistry as nutrient cycles shift under the influence of the responding microbial community. Our data, and emerging data on microbial biogeochemical processes in the coastal environment, will be invaluable when assessing the potential impact of changes associated with global climate change such as decreases in DO and alterations of marine nutrient cycles.

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Appendix A: Variations in DIC-assimilating communities at CR-20

Building upon data observed in Chapter 2 at CR-20 in May 2010, SIP with ^{13}C-$NaHCO_3$ was also performed at the site in July 2010. However, nutrient samples for this location were lost in transit and while CR-15 nutrient data (Table 3.2) could be used as a proxy, it is not an accurate reflection of CR-20. As a result, data comparing CR-20 in May 2010 vs. July 2010 is highlighted briefly and presented as an appendix.

Samples were collected at the oxygen minimum of CR-20, 128m, in both May, during high river flow conditions (from 6244 – 7037 m^3/s), and July 2010, during low flow river flow conditions (3828 – 4070 m^3/s), see section 4.3.1 for more detail. Upwelling Index at the time of sampling was 9 in May 2010, but shifting out of a strong downwelling event that occurred the previous four days that averaged -104. In July 2010 the upwelling index was 83 at the time of sampling suggesting this sample was significantly more influenced by an upwelling event. Samples were collected and extracted as described in Chapter 2.9. SIP experiments and subsequent molecular analyses were conducted as described in Chapter 2.10.

As stated in Chapter 4, the Columbia River has a strong influence on biogeochemical processes in the coastal ocean through the delivery of nutrients in a plume that, during times of high discharge, can extend hundreds of kilometers from the river mouth (Barnes et al., 1970; Bruland et al., 2008; Hickey and Banas, 2003). The Columbia River is noted for its particle discharge, averaging 8 million tons of sediment each year (Spahn et al., 2009). As sediment particles are expelled from the estuary into coastal waters, the balance of the incorporation of particles into the coastal ecosystem versus burial on the shelf or in the deep ocean depends to a large extent on the rate at which they are removed from the water column. It also depends on if, or when, the particles are re-suspended from the bottom at which time they contribute benthic particle-

associated nutrients to coastal environment (Spahn et al., 2009). The factors controlling sediment resuspension and deposition are complex but can include upwelling and downwelling process among other. The flux of resuspended particles during high flow periods is out of the plume (Spahn et al., 2009).

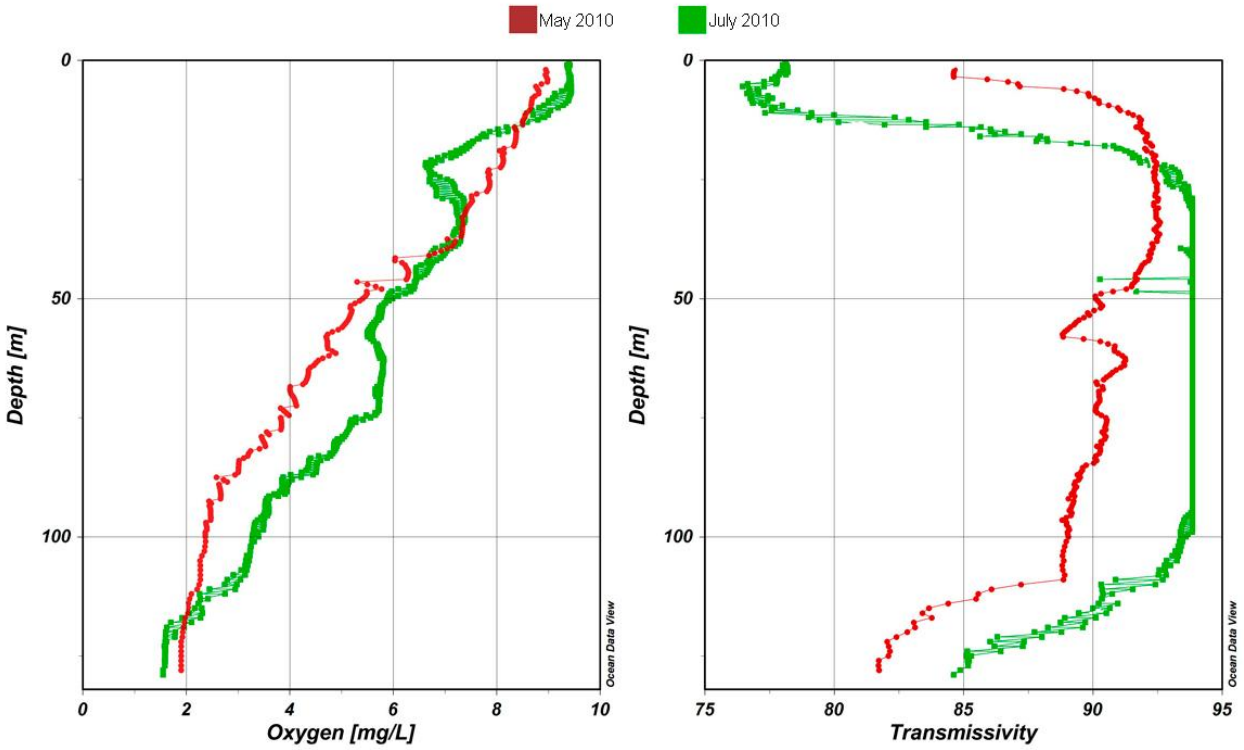

Figure A-6 CTD profiles of dissolved oxygen (mg/L) and transmissivity collected at CR-20 in May 2010 (red) and July 2010 (green).

The bottom nepheloid layer (BNL), the turbulent region of the water column above the benthos in the ocean, is characterized by elevated particulate organic matter suspended by turbulence relative to the water column (Bertagnolli et al., 2011). These areas are also known for enhanced microbial activity close to the seafloor is mainly supported by the resuspension of small phytodetrital particles, nutrients, and by the DOC flux (Boetius et al., 2000).

The Columbia River benthic nepheloid layer was identified at CR-20 in May 2010 and July 2010 through the observation of decreased *in situ* light transmissivity (Fig. A-1). Transmissivity data from both sampling campaigns show a 10 to 20% decrease in light transmissivity along the shelf indicating elevated levels of particulate matter comprising the benthic boundary layer (Fig. A-1). Transmissivity was reduced throughout the majority of the water column at CR-20 in May 2010, suggesting the development of a benthic nepheloid layer, a turbid layer which extended 15 or greater above the bottom (Fig. A-1).

The goal of this portion of the dissertation is to examine the microbial carbon fixing activity in the benthic boundary layer of the Columbia River line through stable isotope probing (SIP) with ^{13}C-$NaHCO_3$ to determine the potential effect of dissolved inorganic carbon (DIC) assimilation on the biogeochemistry of the plume's particle-laden bottom water which may be an area of enhanced microbial activity supported by the resuspension of particles (Boetius et al., 2000) and coastal upwelling events. CR-20 was singled out for SIP analysis due to the hypoxic environment observed during May 2010 and July 2010 sampling efforts as well as the exceptional region of particulate matter observed suspended throughout the water column in the May 2010.

A.1 Results and Discussion

Bacterial class distributions among the active DIC-assimilating fractions in May and July at CR-20 shift from the high-flow spring environment to the low flow summer environment (Fig. A-2), most notably the Gammaproteobacteria are reduced from 67% of the active DIC-assimilating community to only 26% in July and Betaproteobacteria decreased from 4% of the active DIC-assimilating community to 1%. Deltaproteobacteria increased between seasons from 1% to 9% of the DIC-assimilating community. Actinobacteria and Planctomycetes also increased from

May to July. Bacteroidetes and Alphaproteobacteria were consistently represented at 14% to 15% of the DIC-assimilating community. Key changes in DIC-assimilating species are highlighted below.

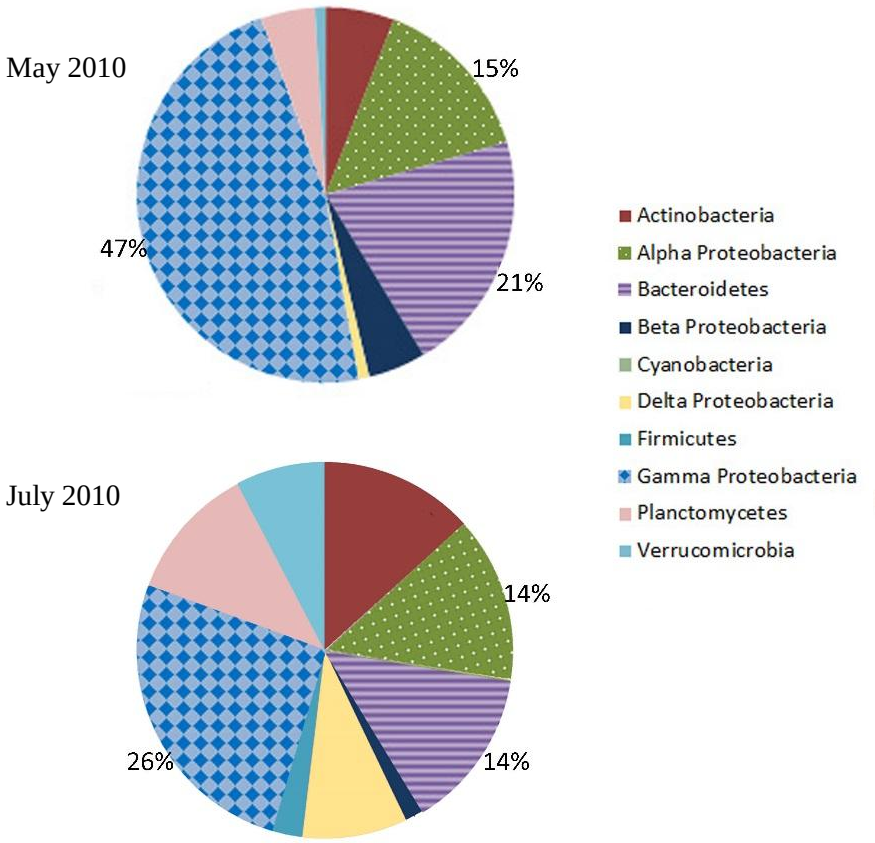

Figure A-7 Class distributions of 16S rRNA gene clone libraries of ^{13}C fractions from all ^{13}C-$NaHCO_3$ incubations at CR-20 in May and July 2010

A.1.1 Betaproteobacteria

Clones identified at CR-20 in May 2010 were related to *Dechloromonas sp.*, *Diaphorobacter nitroreducens*, *Acidivorax temperans*, and *Aquabacterium sp.* These organisms are absent in the SIP clone libraries in July as the community is predominantly related to *Methylophilus sp.* (Fig. A-3).

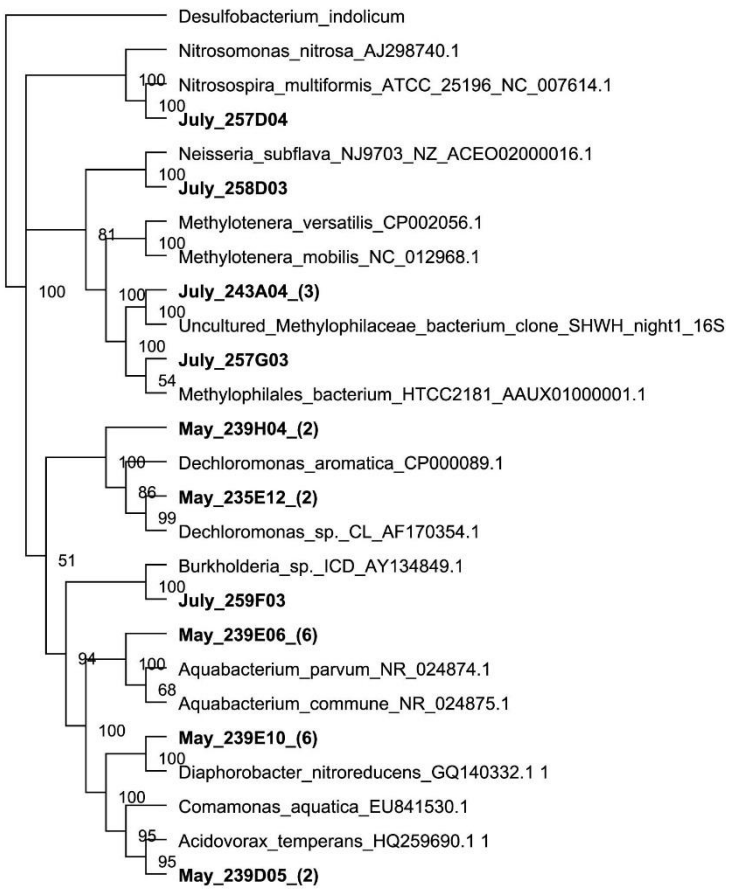

Figure A-8 Rooted neighbor-joining phylogenetic tree of Deltaproteobacteria involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of ^{13}C from SIP experiments conducted at CR-20 in May 2010 and July 2010

This shift in community composition is important as May 2010 organisms are known to participate in the nitrogen cycle from nitrification through denitrification, but the predominance of *Methylophilus sp*. combined with the decrease in Betaproteobacteria in July suggested a significantly reduced role for Betaproteobacteria involved in nitrogen cycling. *Methylophilus sp.* can use reduced one-carbon compounds, such as methanol or methane, as the carbon source for their growth and some *Methylophilus* sp. are also capable of denitrification (Jenkins et al., 1987). A shift from Betaproteobacteria involved in nitrogen cycling processes to those involved in methylotrophy suggests changes in available carbon sources between May and July.

A.1.2 Firmicutes

In July 2010 active SIP fractions Firmicutes, which were not found in May 2010, were actively assimilating DIC (Fig. A-3). Nearly all the sequences recovered were related to *Dialister microaerophilus* (data not shown). *D. microaerophilus* was first isolated from human clinical samples (Jumas-Bilak et al., 2005) but isolates have been found in marine environments (Dang et al., 2013). These organisms have been described as being involved in sediment microbial nitrogen gas fixation (Dang et al., 2013). Considering these communities were only found actively assimilating DIC in the July sample, they may be an indication that these organisms were sheared off the bottom sediment and suspended in the benthic boundary layer or particle attached.

A.1.3 Deltaproteobacteria

Deltaproteobacteria increased between seasons from 1% to 9% of the DIC-assimilating community (Fig A-2). In May 2010 16S rRNA gene a small number of sequences in the active fractions were affiliated with the SAR324 cluster and also *Nitrospina sp.* While the SAR324 and *Nitrospina sp.* continued to be active in DIC-assimilation in July a greater abundance was observed. Organisms related to *Desulfobacteraceae* also emerged in July (Fig. A-4). *Desulfobacteraceae* are sulfate reducers and have been identified in river-dominated coastal margin of the Pacific Northwest as well as within the estuary (Smith et al., 2013). Considering the DO at the time of collection was 1.2 mg/L it may be possible that these organisms where particle attached and functioning with an anoxic microzone (Smith et al., 2013).

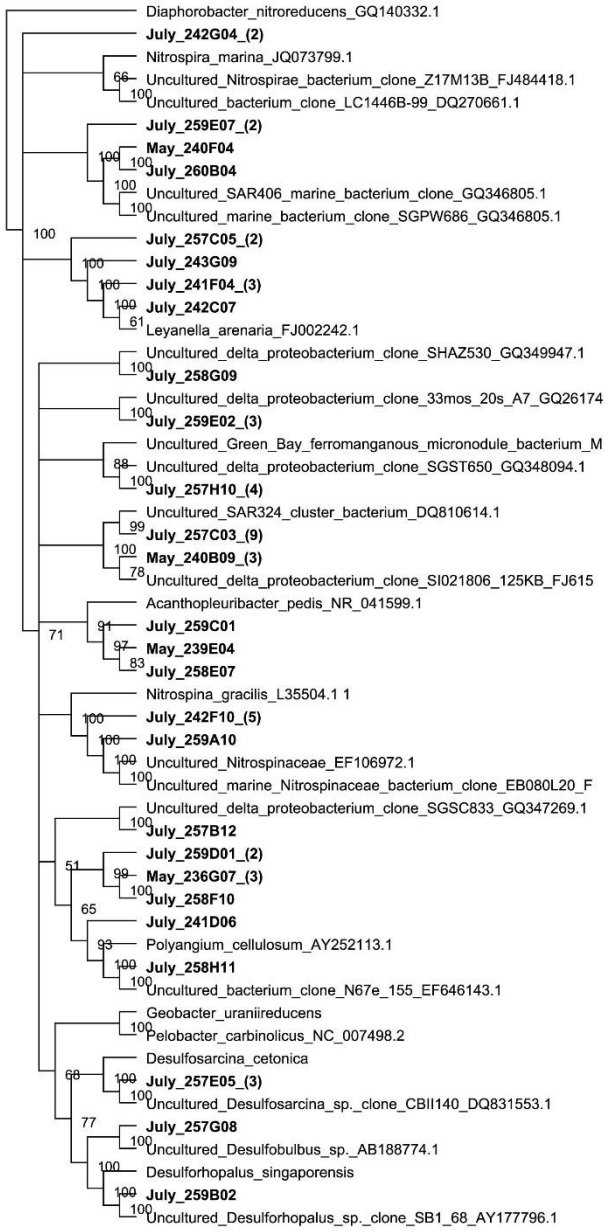

Figure A-9 Rooted neighbor-joining phylogenetic tree of Deltaproteobacteria involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of ^{13}C from SIP experiments conducted at CR-20 in May 2010 and July 2010

A.1.4 Gammaproteobacteria

In May 2010 *Alteromonadales* and *Oceanospirillales,* were most prevalent among the heterotrophic DIC-assimilating community; these organisms remained significantly abundant in July 2010. Gammaproteobacteria, however, decreased in overall abundance with regards to the

total DIC-assimilating community from 47% in May to 26% in July (Fig. A-2). This decrease was is observed with a reduction in unclassified *Oceanospirillales* from May to July. Gammaproteobacteria sulfur oxidizing symbionts, SUP05 and ARCTIC96BD-10, continued to be abundant among active DIC-assimilating organisms regardless of season, consistent with observations in Chapter 2 and Chapter 3.

Organisms closely related to *Pseudomonas aeruginosa*, present in May 2010, were noticeably absent from July 2010 clone libraries (data not shown). *P. aeruginosa* has been isolated from the open ocean, but is more commonly associated with soils and freshwater (Khan et al., 2007). Given that *P. aeruginosa* what found to be assimilating DIC in May during high flow conditions, but not in July during low flow conditions, we hypothesize that the organisms may have been flushed out with Columbia River plume particulate discharge.

Unique to July, however, a small number of organisms associated to Methylococcaceae and Crenotrichaceae bacteria were observed assimilating DIC (Fig. A-5). Members of these families are generally methane oxidizing bacteria, and utilize methane as a substrate in conjunction with the reduction of sulfate and nitrate. The potential appearance of methylotrophy and methanotrophy in July 2010 suggests that that conditions in the benthic environment may have changed in favor of organisms able to utilize alternative carbon sources, possibly as a result of a decrease in quality of labile carbon, or these organisms where sheared off the bottom sediment during upwelling events. Methylotrophic and methanotrophic bacteria use C-1 carbon, although for very different purposes. Methanotrophs obtain energy and carbon from C-1 carbon whereas methylotrophs use C-1 carbon compounds, such as methanol or methane, as the carbon source but not for energy.

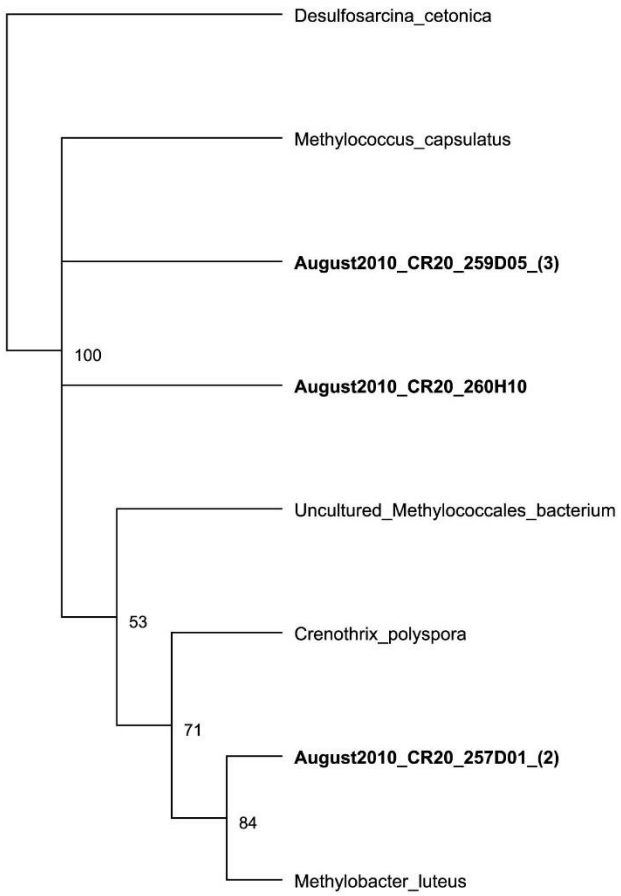

Figure A-10 Rooted neighbor-joining phylogenetic tree of organisms related to *Methylococcaceae* and *Crenotrichaceae* Gammaproteobacteria involved in DIC-assimilation obtained from 16S rRNA gene clone libraries of ^{13}C from SIP experiments conducted at CR-20 in July 2010

A.2 Conclusion

It is difficult to ascertain if the changes in microbial communities are due to settling particulate discharge, the delivery of nutrients from the OMZ through upwelling, or shearing from the benthic boundary as the result of physical processes. Considering that the Pacific Northwest coastal ocean is a dynamic environment subject to multiple processes, it is highly likely that the results presented in this Appendix are due to a combination of each process to a varying degree. The observations made within this appendix suggest that the DIC-assimilating populations subject to influence by Columbia River discharge may shift to populations that are able to utilize

alternative carbon and energy sources, or inorganic electron donors, once the system is subject to low flow conditions and nutrient replenishment is largely relegated to upwelling processes. Overall, this data shows biogeochemical potential in the Columbia River benthic nepheloid layer. However, the lack of nutrient data for the CR-20 July 2010 sample makes comparison with the May 2010 sample difficult. If data CR-15 reviewed in Chapter 4 (Table 4.1) is used as a proxy it's clear that CR-20 may have the potential to undergo dramatic shifts in nutrient concentration that can alter microbial communities and their biogeochemical responses. The Columbia River benthic nepheloid layer may be an area of biogeochemical interest that responds dramatically to changes in Columbia River flow and particle deposition warranting further investigation.