AN ABSTRACT OF THE THESIS OF

Katie S. Watkins-Brandt for the degree of Master of Science in Oceanography presented on August 2, 2010.

Title: Phosphorus Control of Nitrogen Fixation

Abstract approved:

Angelicque E. White Ricardo M. Letelier Yvette H. Spitz

Under conditions of fixed N-limitation, as with most oligotrophic systems, the process of biological N_2 fixation (diazotrophy) is favored, provided the necessary trace elements and vitamins are sufficient. Despite the well understood contributions of N_2 fixation in oligotrophic regions, the nutritional and ecological controls of marine diazotrophs have not been well characterized. The aim of this thesis is to explore the role of phosphorus (P) in regulating productivity and nitrogen fixation rates of diazotrophs and to examine the method of phosphorus fractionation as a proxy for P-status. Phosphorus is an essential element required by all marine organisms for many components of the cell. Herein, we demonstrate that P plays an important role in regulating productivity and nitrogen fixation rates of diazotrophs. Chapter two characterizes the spatial variation of nitrogen and carbon fixation rates and presents a series of experiments designed to determine the role of inorganic and organic phosphorus limitation in diazotrophic productivity. We present a spatially extensive record of dinitrogen (N_2) fixation rates and distributions of N_2 fixing microorganisms along with the results of exogenous P addition experiments conducted during a series of cruises in the North Pacific Subtropical Gyre (NPSG). These experiments produced three major findings: (1) methylphosphonate (MPn) and dissolved inorganic phosphorus (DIP) were utilized with equal metabolic efficiency over a single photoperiod, (2) the bulk of the enhanced N_2 fixation rates were within the range

reported for the Hawaii Ocean Time-series (HOT) suggesting that P levels in this region can be saturating but were not at the time of sampling and (3) MPn and DIP additions stimulated C fixation rates beyond estimated contributions by diazotrophs, and hence both DIP and bioavailable dissolved organic phosphorus (DOP) additions could lead to enhancement of net primary productivity on short time-scales. Chapter three presents an initial investigation into compartmentalization of phosphorus in natural assemblages of *Trichodesmium* in relation to the bulk plankton community and explores the use of a phosphorus fractionation method as a potential proxy for P-status. We evaluated intracellular P compartmentalization by both the bulk natural assemblage of plankton and isolated colonies of *Trichodesmium* from five cruises within the North and South Pacific subtropical Gyres (NPSG and SPSG, respectively). Using a trichloroacetic acid extraction method, samples were analyzed for total and acid soluble fractions of particulate P. Field data suggest that natural populations of *Trichodesmium* demonstrate a unique internal composition of primarily inorganic phosphorus while the bulk plankton assemblage are composed of primarily organic P pools.

Phosphorus Control of Nitrogen Fixation

by

Katie S. Watkins-Brandt

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented August 2, 2010
Commencement June 2011

Master of Science thesis of Katie S. Watkins-Brandt presented on August 2, 2010

APPROVED:

_____

Co-Major Professor, representing Oceanography

_____

Co-Major Professor, representing Oceanography

_____

Co-Major Professor, representing Oceanography

_____

Dean of the College of Oceanic and Atmospheric Sciences

_____

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

_____

Katie S. Watkins-Brandt, Author

ACKNOWLEDGMENTS

I thank:

Dr. Angelicque White, Dr. Ricardo Letelier and Dr. Yvette Spitz for all of their support and guidance, their time and their criticism and most of all for never giving up on me. The National Science Foundation (OCE-0623596) grant supporting my research and providing the means necessary for an excellent experience.

Dr. Evelyn Sherr for serving as a committee member and an outside view and Dr. Julie Pett-Ridge for serving as my graduate council representative.

Jasmine Nahorniak for her endless patience in teaching me to work with remote sensing and Matlab. Dave Karl, Matt Church and Daniela Böttjer, of the University of Hawaii, Mike Brzezinski chief scientist of the Pacific Open Ocean Bloom cruise and Zbigniew Kolber chief scientist of the Ocean PERturbation Experiment for the cruise opportunities and valuable collaborations. All of the folks in the Ocean Microbial Ecology Laboratory who have been like family to me and have always been engaging and encouraging. Dr. Tim Cowles for bringing me to Oregon State as a student intern, the experience that started it all. To all my colleagues for the stimulating conversations that have been had.

A special thanks to the COVEN: those strong and wonderful women whose friendships and support throughout the years has kept me going. My family, especially my mother Julie, my father Tom and my grandfather John whom have always believed in me. Kat Stevens for being a great friend and always being there when I need her. Most of all to my husband Craig for putting up with me, always knowing how to make me laugh, understanding me and picking me up when I fall down.

CONTRIBUTION OF AUTHORS

Yvette Spitz and Ricardo Letelier contributed intellectually and to the editing process for material presented in Chapter 2 and 3. Angelicque White also contributed to the design and provided intellectual contributions for Chapter 2 and 3 along with Figure 1-1, and data used in Chapter 2. Matt Church and Daniela Böttjer provided *nifH* gene abundance data and contributed to the writing effort for the manuscript presented in Chapter 2. Joe Jennings ran a subset of the nutrient samples and Zanna Chase tested stocks for trace metal contamination. Each of the aforementioned is acknowledged and/or co-authors for the developing or submitted or manuscripts of these respective chapters.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure Page

LIST OF TABLES

1 Introduction

"Without phosphorus there would be no thoughts."

- Fredrich Karl Christian Ludwig Buchner

1.1 Phosphorus: sources and analysis

1.1.1 Phosphorus sources

Phosphorus (P) is an essential macronutrient required by all marine organisms for structural and functional components of the cell. From a physiological and biochemical perspective, P is fundamental to growth and uptake kinetics and microbial production. Cytological adaptations such as the storage of P in inclusions and vacuoles for later use are crucial to marine organisms in low P environments. The ecological role of P in the marine environment in terms of both seasonal distributions and bioavailability of P compounds is of great interest in understanding the adaptive strategies of P utilization and the coping mechanisms of organisms during P depletion.

The role of P in phytoplankton productivity in the marine environment has long been perceived as limiting over geological time scales (Codispoti, 1989), however P also limits the productivity of specific functional groups, dinitrogen fixing organisms in particular, on shorter time-scales as these organisms have the capacity to acquire nitrogen from N_2 which is always available in excess in surface waters (Tyrell, 1999). In this respect, it is important to characterize P transformations from cellular to ecosystem scales in order to better understand the role of P in regulation of marine productivity. The primary oceanic source of P is riverine input via continental weathering of the Earths' crust, with smaller contributions from atmospheric deposition and volcanic eruptions (Benitez-Nelson, 2000). The recycling of P in the marine environment is a rapid process and regeneration of P can account for a substantial portion of utilizable P within a system (Griffith et al. 1973). Figure 1-1 depicts a broad representation of the marine phosphorus cycle (provided by A.E.

White). Once P enters the marine environment it is cycled through intracellular, organic and inorganic reservoirs as it is transformed by microorganisms.

1.1.2 Phosphorus: definitions and analysis

Phosphorus in the marine environment is present in many molecular forms and is comprised of a dissolved and particulate pool, each of which includes an organic and inorganic fraction. As with other methods of chemical analysis, phosphorus pools are operationally defined. The total dissolved phosphorus (TDP) pool is defined as both dissolved inorganic phosphorus (DIP) and dissolved organic phosphorus (DOP). Inorganic P residues consist of orthophosphate (PO_4) and pyrophosphates with PO_4 widely accepted as the most readily bioavailable pool for photoautotrophs. The high molecular weight fraction of the organic P pool is comprised of roughly 25% phosphonates and 75% esters (Kolowith et al. 2001). Methodological limitations restrict our ability to fully characterize the low molecular weight P pools. The functional classes of DOP compounds are listed in Table 1-1 and include phosphate monoesters and diesters (C-O-P bond class) and phosphonates (C-P bond). Also included within this table is a summary of the marine diazotrophs capable of utilizing the specific DOP compounds listed (adapted from White et al. 2010). Despite the cryptic nature of the DOP pool, a growing body of literature has documented that both inorganic and organic P are used to fuel primary productivity in the upper ocean (Orchard et al. 2010; White et al. 2010; See Chapter 2). One study of note in this canon is that of Bjorkman et al. (2003); these authors have demonstrated simultaneous utilization of DOP and DIP occurring at equivalent specific rates per unit biomass in the upper euphotic zone of the oligotrophic North Pacific.

As previously mentioned, the analytical methods available for the chemical analysis of different conceptual phosphorus pools are operationally defined. The DIP portion of the dissolved pool, further denoted as soluble reactive phosphorus (SRP), is the portion of the TDP pool that is reactive to the molybdenum blue forming complex (Murphy and Riley, 1962). During the analysis of SRP, it has been observed that a

fraction of inorganic condensed phosphates (polyphosphate and pyrophosphate) and select organic P compounds may be hydrolyzed (Rigler, 1968; Thomson-Bulldis and Karl, 1998). With this in mind, the DOP pool, also known as the soluble non-reactive pool (SNP) is calculated as the TDP less the SRP. While the SRP pool contains some organic phosphorus components, the SNP pool is not limited to DOP and may include some inorganic condensed phosphates and particulate contaminants. Due to the solubilization of compounds within each pool, SRP is considered roughly equivalent to DIP while SNP is roughly equivalent to DOP (for further reviews refer to Baldwin (1998) and Karl (2007)). While there are various oxidation procedures for TDP analysis, herein, we employ the basic persulfate oxidation method of Valderramma (1981) due to its efficiency in hydrolyzing a suite of commercially available phosphonate compounds.

The particulate phosphorus (PP) pool, also termed the total particulate phosphorus (TPP) pool, is divided into an inorganic (PIP) and organic (POP) fraction. PP pools reflect the P investments in the cellular machinery necessary for basal metabolism, growth and reproduction and include P bound in nucleic acids, mono- and di-esters, polyphosphate, phospholipids and other molecular forms. In a broader context, the PIP fraction includes dissolved phosphorus that has been adsorbed to abiotic and biotic particles along with the acid-labile fraction of intracellular storage components such as orthophosphate, polyphosphate and pyrophosphate. TPP also includes living material and refractory components within detrital particles.

Several analytical procedures are available to quantify the TPP pool and specifically the PIP and POP fractions within the total pool (Aspila et al. 1976; Miyata and Hattori, 1986; Solorzano and Sharp, 1980; Yoshimura et al. 2007). Multiple sequential extraction procedures are a very useful tool in determining the PIP and POP fractionation in samples but require large volumes of water and extensive chemical analysis. In our work, we have adopted a simpler acid-extraction method modified from the method of Miyata and Hattori (1986). The analysis of these analytically defined phosphorus pools through chemical fractionation of TPP to PIP and POP allows us to better characterize the intracellular P composition of phytoplankton.

Given that PIP and POP pools are operationally defined by acid lability, we have used commercially available inorganic and organic P compounds as external standards.

1.2 Phosphorus as a potential control of diazotrophy

Under conditions of fixed N-limitation, as with most oligotrophic systems, the growth of organisms capable of utilizing the near limitless pools of N_2 (diazotrophs) is favored, provided the necessary trace elements and vitamins are sufficient. The process of nitrogen fixation entails the biological reduction of atmospheric N_2 to ammonium (NH_4^+), ammonia (NH_3) and dissolved organic nitrogen (DON). The annual input of N via N_2 fixation ranges from 90 to 130 Tg N yr^{-1} (Galloway et al. 2004). This range includes estimates based on direct measurements of nitrogen fixation and geochemical approximations (Galloway et al. 2004; Bates and Hansell, 2004). The rate of nitrogen fixation throughout the marine environment varies significantly on both temporal and spatial scales (Galloway et al. 2004). Recent discoveries of previously unknown diazotrophs and expansion of the known spatial boundaries of pelagic N_2 fixation have suggested that previous estimates of N_2 fixation are low relative to actual global rates (Capone, 2001; Moisander et al. 2010; Montoya et al. 2004).

Biological N_2-fixation is an inefficient process with gross N_2 fixation in excess of N demand and concomitant excretion or NH_4 and DON, which may lead to short-lived mitigation of community scale N-limitation (Karl, 2002; Karl et al. 1997). However, under N-limiting conditions the growth of diazotrophs is ultimately limited by other elements such as phosphorus (P), iron (Fe) and possibly inorganic carbon (Karl et al. 1997; Sañudo-Wilhelmy et al. 2001; Hutchins et al. 2007). Numerous studies have investigated potential controls of diazotrophic production in oligotrophic systems, and have concluded that the intensity of water column stratification (White et al. 2007), changes in surface N:P ratios via mixing events (Karl and Letelier, 2008) and the role of spring iron deposition in relation to a shoaling of the mixed layer (Dore et al. 2008) are all potential controls of diazotrophy. The previous work of Sañudo-

Wilhelmy et al (2001) has also suggested that phosphorus and iron may co-limit nitrogen fixation. Chapter two examines P restriction on carbon and nitrogen fixation rates in the North Pacific subtropical gyre.

1.3 Trichodesmium: unique adaptations in P-restricted environments

The marine cyanobacterium *Trichodesmium* is a globally significant bloom forming diazotroph residing primarily in the euphotic zone of subtropical and oligotrophic waters (Capone, 2001; Karl et al. 2002). Recognition of *Trichodesmium* as an ecologically important organism is a relatively recent phenomenon. While observations date back to the days of Captain Cook (Ship logs in the 1700's) and Darwin (Observations in the Red Sea during the Voyage of the Beagle), only since the 1960's has *Trichodesmium* truly become a focus of research. This diazotroph possess several unique physiological adaptations to cope in oligotrophic environments such as the ability to fix dinitrogen gas (N_2) under aerobic conditions (Berman-Frank et al. 2001), hydrolyze a variety of dissolved organic phosphorus (DOP) compounds (White et al. 2010), exhibit a broad C:P and N:P stoichiometry (White et al. 2006) and regulate buoyancy through carbohydrate ballasting (Romans et al. 1994; Villareal and Carpenter, 2003). The combination of these adaptations has undoubtedly contributed to their often dominant presence in late summer within the North Pacific Subtropical gyre (NPSG) (Carpenter, 1983; Carpenter et al. 1992).

In the context of the work presented in this thesis, *Trichodesmium* has evolved mechanisms to deal with the chronically low levels of DIP (< 100 nmol L^{-1}) within the NPSG including their ability to hydrolyze organic phosphorus compounds, their ability to maintain maximal growth rates with low cellular P quotas and the ability to store P in times of excess (White et al. 2010; White et al. 2006; Letelier and Karl, 1998). DOP concentrations in surface waters of the NPSG are two to three times greater than that of DIP and therefore represent a large potential bioavailable P pool (Björkman et al. 2000). The ability to hydrolyze DOP compounds may be crucial to supplementing or fulfilling P requirements necessary for growth, particularly for an

organism such as *Trichodesmium,* which are characterized by a low affinity for DIP (Sohm and Capone, 2006; Dyhrman et al. 2007).

Another unique adaptation of *Trichodesmium* is its ability to exhibit flexible elemental stoichiometry. *Trichodesmium* in nature are characterized by a low internal P cell quota relative to carbon or nitrogen (White et al. 2006; Sohm et al. 2006). The ecological significance of a low cell quota implies that *Trichodesmium* is well-adapted for survival in oligotrophic systems characterized by low P concentrations. The aim of chapter three is to explore the intracellular phosphorus content of bulk seawater samples in comparison to picked colonies of *Trichodesmium* and to investigate the relationship between relative intracellular P content and the production of organic matter by *Trichodesmium*.

1.4 Thesis objectives

The aim of this thesis is to contribute to the existing literature regarding phosphorus regulation of carbon and nitrogen fixation in natural systems. Chapter two provides insight into the spatial variation of nitrogen and carbon fixation rates in the North Pacific and presents a series of experiments designed to examine the potential role of inorganic and organic phosphorus limitation in diazotrophic productivity. Chapter three presents an initial investigation into compartmentalization of phosphorus in natural assemblages of *Trichodesmium* in relation to the bulk phytoplankton community.

1.5 Literature cited

Aspila KI, Agemian H, Chau ASY (1976) A semi-automated method for determination of inorganic, organic and total phosphate in sediments. Analyst 101:187-197

Baldwin D (1998) Reactive “organic” phosphorus revisited. Water Research 32:2265-2270

Bates NR, Hansell DA (2004) Temporal variability of excess nitrate in the subtropical mode water of the North Atlantic Ocean. Marine Chemistry 84:225-241

Benitez-Nelson C (2000) The biogeochemical cycling of phosphorus in marine systems. Earth Science Reviews 51:109-135

Berman-Frank I, Lundgren P, Chen Y-B, Kuepper H, Kolber Z, Bergman B, Falkowski P (2001) Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. Science 294:1534-1537

Björkman KM, Karl DM (2003) Bioavailability of dissolved organic phosphorus in the euphotic zone at Station ALOHA, North Pacific Subtropical Gyre. Limnology and Oceanography 48:1049-1057

Björkman KM, Thomson-Bulldis AL, Karl DM (2000) Phosphorus dynamics in the North Pacific subtropical gyre. Aquatic Microbial Ecology 22:185-198

Capone DG (2001) Marine nitrogen fixation: What's the fuss? Current Opinions in Microbiology 4:341-348

Carpenter EJ (1983) Physiology and ecology of marine *Oscillatoria* (*Trichodesmium*). Marine Biology Letters 4:69-85

Carpenter EJ, Capone DG, Rueter JG (1992) Marine pelagic cyanobacteria: *Trichodesmium* and other diazotrophs. NATO Ser CV 362 Kluwer Acdemic Publishing, Dordrecht The Netherlands

Codispoti L (1989) Phosphorus vs. nitrogen limitation of new and export production. John Wiley & Sons, p 377

Dore J, Brum J, Tupas L, Karl DM (2002) Seasonal and interannual variability in sources of nitrogen supporting export in the oligotrophic subtropical North Pacific Ocean. Limnology and Oceanography 47:1595-1607

Dore J, Letelier R, Church M, Lukas R, Karl D (2008) Summer phytoplankton blooms in the oligotrophic North Pacific Subtropical Gyre: historical perspective and recent observations. Progress in Oceanography 76:2-38

Dyhrman S, Ammerman J, Van Mooy B (2007) Microbes and the marine phosphorus cycle. Oceanography 20:110-116.

Dyhrman ST, Chappell PD, Haley ST, Moffett JW, Orchard ED, Waterbury JB, Webb EA (2006) Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. Nature 439:68-70

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA, Karl DM, Michaels AF, Porter JH, Townsend AR, Vöosmarty CJ (2004) Nitrogen Cycles: Past, Present, and Future. Biogeochemistry 70:153-226

Griffith E, Beeton A, Spencer J, Mitchell D Environmental Phosphorus Handbook, Vol. John Wiley & Sons.

Hutchins DA, Fu F, Zhang Y, Warner ME, Feng Y, Portune K, Bernhardt PW, Mulholland MR (2007) CO_2 control of *Trichodesmium* N_2 fixation, photosynthesis, growth rates, and elemental ratios: Implications for past, present, and future ocean biogeochemistry. Limnology and Oceanography 52:1293-1304.

Karl D, Letelier R (2008) Nitrogen fixation-enhanced carbon sequestration in low nitrate, low chlorophyll seascapes. Marine Ecology Progress Series 364:257-268

Karl D, Letelier R, Tupas L, Dore J, Christian J, Hebel D (1997) The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. Nature 388:533-538

Karl DM (2007) The marine phosphorus cycle. In: Hurst CJ, Crawford RL, Garland JL, Lipson DA, Mills AL, Stetzenbach LD (eds) Manual of environmental microbiology. American Society of Microbiology, p 523-539.

Karl DM, Michaels A, Bergman B, Capone DG, Carpenter EJ, Letelier RM, Lipschultz F, Paerl H, Sigman D, Stal L (2002) Dinitrogen fixation in the world's oceans. Biogeochemistry 57/58:47-98

Karl DM (2002) Nutrient dynamics in the deep blue sea. Trends in Microbiology 10:410-418

Kolowith LC, Ingall ED, Benner R (2001) Composition and cycling of marine organic phosphorus. Limnology and Oceanography 46:309-320

Letelier RM, Karl DM (1998) *Trichodesmium* spp. physiology and nutrient fluxes in the North Pacific subtropical gyre. Aquatic Microbial Ecology 15:265-276

Miyata K, Hattori A (1986) A simple fractionation method for determination of phosphorus components in phytoplankton: Application to natural populations of phytoplankton in summer surface waters of Tokyo Bay. Journal of Oceanography 42:255-265

Moisander PH, Beinart RA, Hewson I, White AE, Johnson KS, Carlson CA, Montoya JP (2010) Unicellular cyanobacterial distributions broaden the ocean N_2 fixation domain. Science 327:1512-1514

Montoya JP, Holl CM, Zehr JP, Hansen A, Villareal TA, Capone DG (2004) High rates of N_2 fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. Nature. 430:1027-1032

Mulholland MR, Floge S, Carpenter EJ, Capone DG (2002) Phosphorus dynamics in cultures and natural populations of *Trichodesmium* spp. Marine Ecology Progress Series 239:45-55

Murphy J, Riley JP (1962) A modified single solution method for determination of phosphate in natural waters. Analytical Chimica Acta 24:31-36

Orchard ED (2010) Phosphorus physiology of the marine cyanobacterium *Trichodesimum*. Woods Hole Oceanographic Institution

Rigler F (1968) Further observations inconsistent with the hypothesis that the molybdenum blue method measures orthophosphate in lake water. Limnology and Oceanography 13:7-13

Romans KM, Carpenter EJ, Bergman B (1994) Buoyancy regulation in the colonial diazotrophic cyanobacterium *Trichodesmium tenue*: Ultrastructure and storage of carbohydrates, polyphosphate and nitrogen. Journal of Phycology 30:935-942

Sañudo-Wilhelmy SA, Kustka AB, Gobler CJ, Hutchins DA, Yang M, Lwiza K, Burns J, Capone DG, Raven JA, Carpenter EJ (2001) Phosphorus limitation of

nitrogen fixation by *Trichodesmium* in the central Atlantic Ocean. Nature 411:66-69

Sohm JA, Capone DG (2006) Phosphorus dynamics of the tropical and subtropical north Atlantic: *Trichdesmium spp.* versus bulk phytoplankton. Marine Ecology Progress Series 317:21-28

Solorzano L, Sharp JH (1980) Determination of total dissolved phosphorus and particulate phosphorus in natural waters. Limnology and Oceanography 25:754-758

Thomson-Bulldis A, Karl D (1998) Application of a novel method for phosphorus determinations in the oligotrophic North Pacific Ocean. Limnology and Oceanography 43:1565-1577

Tyrrell T (1999) The relative influences of nitrogen and phosphorus on oceanic primary production. Nature 400:525-531

Valderrama JC (1981) The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Marine Chemistry 10:109-122

Villareal TA, Carpenter, EJ (2003) Buoyancy regulation and the potential for vertical migration in the oceanic cyanobacterium *Trichodesmium*. Microbial Ecology 45:1-10.

White A, Karl D, Björkman K, Beversdorf L, Letelier R (2010) Production of organic matter by *Trichodesmium* IMS101 as a function of phosphorus source. Limnology and Oceanography in press

White AE, Spitz YH, Karl DM, Letelier RM (2006) Flexible elemental stoichiometry in *Trichodesmium* spp. and its ecological implications. Limnology and Oceanography 51:1777-1790

White AE, Spitz YH, Letelier RM (2007) What factors are driving summer phytoplankton blooms in the North Pacific Subtropical Gyre. Journal of Geophysical Research 112:C1200

Yoshimura T, Nishioka J, Saito H, Takeda S, Tsuda A, Wells M (2007) Distributions of particulate and dissolved organic and inorganic phosphorus in North Pacific surface waters. Marine Chemistry 103:112-121

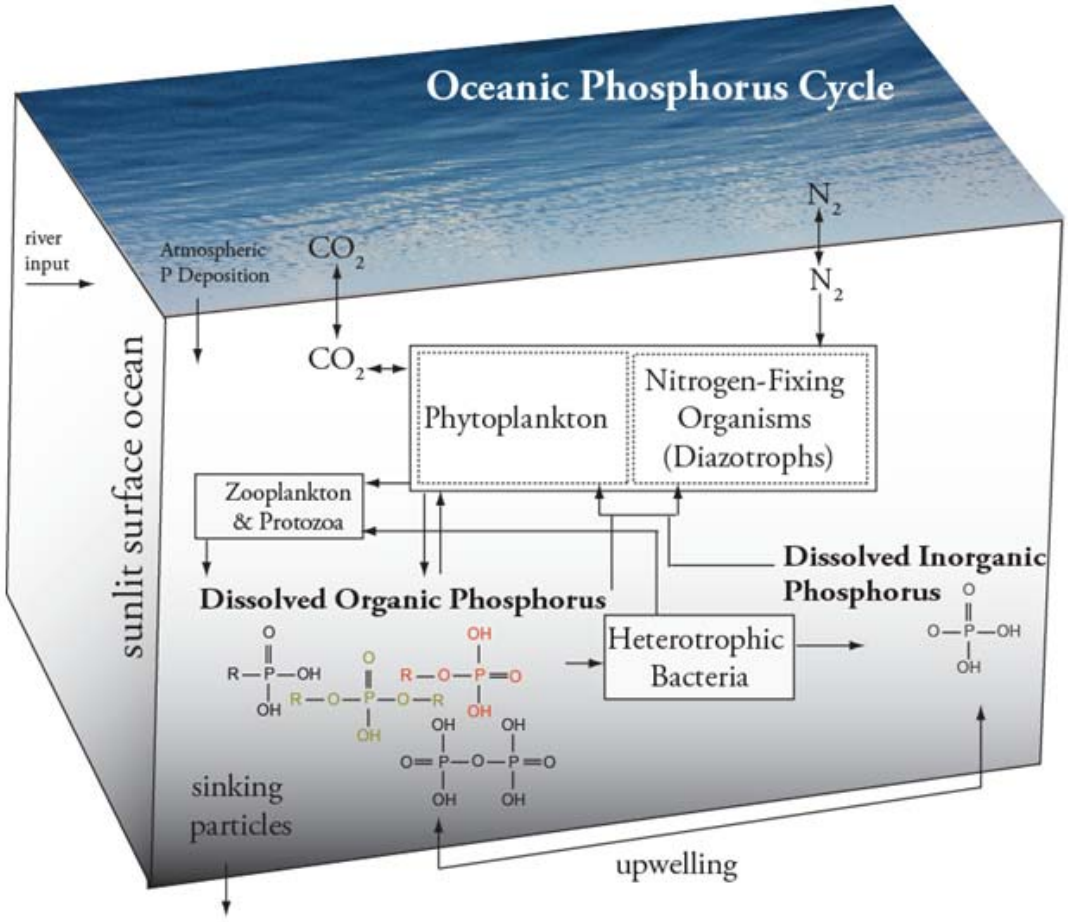

Figure 1-1. A broad view of the oceanic phosphorus cycle.

Table 1-1. A list of organic phosphorus compound classes including the diazotrophs known to be capable of metabolizing specific compounds (adapted from White et al. 2010)

P-Compound Class	Empirical Formula	Metabolizing Diazotroph
PHOSPHATE ESTERS [C-O-P BOND CLASS]		
Glucose 6-phosphate (G6P)	$C_6H_{13}O_9P$	*Trichodesmium* (4)
Adenosine 5' monophosphate (AMP)	$C_{10}H_{14}N_5O_7P$	*Crocosphaera* (1); *Trichodesmium* (4)
Glycerophosphate (GP)	$C_3H_7MgO_6P\ xH_20$ (various salts)	*Crocosphaera* (1); *Trichodesmium* (2)
myo -Inositol hexakisphosphate (InsP6)	$C_6H_{16}CaO_{24}P_6$	*Crocosphaera* (1)
PHOSPHONATE [C-P BOND CLASS]		
Methylphosphonic Acid (MPN)	CH_5O_2P	*Trichodesmium* (4,3)
2-Aminoethyl phosphonic acid (2AEP)	$C_2H_8NO_2P$	*Trichodesmium* (4)
Ethylphosphonate (EPN)	$C_2H_7O_2P$	*Trichodesmium* (4)
2-Amino-3 phosphonopropionic acid (2A3P)	$C_3H_8NO_5P$	no diazotrophs to date (4)

† 1. Dyhrman & Haley (2006) 2. Mulholland et al. (2002) 3. Karl et al. (2008) 4. White et al. (in prep)

Enhancement of nitrogen fixation in the oligotrophic North Pacific via inorganic and organic phosphorus additions

Katie S. Watkins-Brandt, Ricardo M. Letelier, Yvette H. Spitz, Matthew J. Church, Daniela Böttjer and Angelicque E. White

In preparation for submission to Marine Ecology Progress Series
International Ecology Institute
Nordbunte 23 (+3,5,28,30) 21385 Oldendorf/Luhe Germany

2 Enhancement of nitrogen and carbon fixation in the oligotrophic North Pacific via inorganic and organic phosphorus additions

2.1 Abstract

We present a spatially extensive record of dinitrogen (N_2) fixation rates and distributions of N_2 fixing microorganisms along with the results of exogenous phosphorus (P) addition experiments conducted during a series of cruises in the North Pacific Subtropical Gyre (NPSG). We measured the N_2 and carbon (C) fixation rates of natural plankton assemblages in response to the addition of methylphosphonate (MPn), a dissolved organic phosphorus (DOP) compound, and dissolved inorganic phosphorus (DIP). Results are compared to parallel unamended controls. These experiments produced three major findings: (1) MPn and DIP were utilized with equal metabolic efficiency over a single photoperiod, (2) the bulk of the enhanced N_2 fixation rates were within the range reported for the Hawaii Ocean Time-series (HOT) suggesting that P levels in this region can be saturating but were not at the time of sampling and (3) MPn and DIP additions stimulated C fixation rates beyond estimated contributions by diazotrophs, and hence both DIP and bioavailable DOP additions could lead to enhancement of net primary productivity on short time-scales. Our results suggest that the rate of N_2 fixation in our study region may have been restricted by the availability and composition of the total P pool (inorganic and organic P) during our field season.

KEY WORDS: Phosphorus · Nitrogen fixation · Dissolved organic matter

2.2 Introduction

The surface mixed layer of subtropical oceanic gyres is typically characterized by a deficit of dissolved inorganic nitrogen (DIN) relative to inorganic phosphorus (P), where DIN concentrations are nearly always at or below the detection limit of standard autoanalyzer technology (~30 nmol L^{-1}). For this reason the rate of primary productivity in the oligotrophic subtropical gyres of the Pacific and Atlantic basins has long been held to be restricted by the supply of reduced nitrogen (N) (Perry and Eppley 1981; Wu et al. 2000; Karl 2002). Under such conditions biological dinitrogen (N_2) fixation, the process by which select genera of marine microorganisms convert N_2 into cellular N, can act as a source of new N and support a significant fraction of net primary productivity in DIN-limited systems (Capone 2001; Karl et al. 2002). Despite the well understood contributions of N_2 fixation to productivity, elemental cycles and particle export in oligotrophic regions, the nutritional and ecological controls of marine diazotrophs have not been well characterized (Vitousek and Howarth 1991; Zehr and Ward 2002).

While N_2 fixation may lead to short-lived mitigation of community scale N-limitation, the growth of diazotrophs is ultimately limited by other elements such as phosphorus (P), iron (Fe) and inorganic carbon (Karl et al. 1997; Sañudo-Wilhelmy et al. 2001; Hutchins et al. 2007). In the North Pacific subtropical gyre (NPSG), Karl et al. (2001b) have described climate-driven shifts in the ecology of the NPSG early in this century that led to enhanced N_2 fixation and a concomitant drawdown of P. The authors further hypothesized that this 'domain shift' would result in a progressive shift from N-limitation to P-limitation of primary productivity (Karl et al. 2001b) and eventually to P-limitation of diazotrophs. To address the potential P-limitation of any functional group in pelagic ecosystems, one must also consider that while inorganic P (phosphate) is often preferred for growth, a subset of P bound in organic complexes are bioavailable (Cembella et al. 1982; Dyhrman et al. 2007) and potentially present in greater concentrations than inorganic pools. In the NPSG, dissolved inorganic P (DIP) concentrations in surface waters are low (<100 nmol L^{-1}) relative to biological

demand (Karl et al. 2001a; Karl and Björkman 2002) and relative to dissolved organic P (DOP) pools which range from 200 to 300 nmol L^{-1} and represent a large potentially bioavailable P pool (Björkman et al. 2000). Partial characterization of the high molecular weight fraction of marine DOP by nuclear magnetic resonance (NMR) spectroscopy reveals a resource comprised of esters and phosphonates in a ratio of 3:1, respectively (Kolowith et al. 2001).While both of these compound classes are bioavailable to microbes, including diazotrophs, considerable uncertainty remains regarding the extent to which bioavailable P (inorganic + labile organic) regulates diazotrophy, net productivity, and plankton community structure in the oligotrophic ocean.

In the NPSG, Björkman et al. (2000) have shown that the addition of certain DOP compounds to seawater samples resulted in hydrolysis rates 50 times or more in excess of ambient P uptake rates. Later, using an isotope dilution technique, Björkman and Karl (2003) demonstrated that the microbial community at Station ALOHA (22.45°N, 158°W), the sampling station for the Hawaii Ocean Time-series (HOT) program, simultaneously utilized DOP and DIP for P nutrition, with rates of DOP uptake equivalent to DIP uptake. This work and others (Karl et al. 2008; Duhamel et al. 2010) elucidated the importance of DOP as a source of P supporting plankton nutrition in the NPSG. Yet, to date studies examining how natural assemblages of diazotrophs respond to additions of DOP relative to DIP are lacking.

In this study, we conducted a series of experiments aimed at (1) characterizing the spatial variability of N_2 fixation and diazotroph community structure in the NPSG and (2) assessing the short-term (24-hr) N_2 fixation and C productivity responses of natural plankton populations to saturating additions of DIP and a model DOP compound, the phosphonate methylphosphonate (MPn). MPn was selected as a target compound based on previous laboratory and *in situ* studies confirming active MPn hydrolysis by the diazotroph *Trichodesmium* (Dyhrman et al. 2006; Karl et al. 2008; White et al. 2010). The overarching objective of this work was to measure the response of microbial communities to the addition of a potentially limiting nutrient (P) as a direct test of contemporaneous resource limitation.

2.3 Materials and Methods

In July through August of 2008, aboard the R/V *Kilo Moana*, a series of experiments were conducted near (1) the frontal boundary separating the North Pacific subtropical and subarctic gyres [Pacific Open Ocean Bloom (POOB) cruise (28°N to 32°N, July 2- July 16)] and (2) near the Hawaiian Islands [Ocean PERturbation EXperiment (OPEREX) cruise (22°N to 26°N, July 30- August 14)]. The regional mean satellite-derived sea surface temperature (SST) and chlorophyll *a* (Chl) fields for these cruise periods were obtained from the 4-km, level-3 MODIS AQUA data provided by NASA/Goddard Space Flight Center and accessed via http://oceancolor.gsfc.nasa.gov. Each cruise track is shown relative to the mean July-August SST and Chl fields averaged over the specific cruise duration (Fig. 2-1). The sea surface height anomaly field relative to the POOB and OPEREX cruise efforts (Fig. 2-2) was obtained from the ⅓º by ⅓º resolution, merged TOPEX/Poseidon and ERS satellite altimetry data for July-August, 2008. These altimeter products were produced by Ssalto/Duacs and distributed by AVISO, with support from Cnes.

2.3.1 Experimental design

Experimental manipulations were designed to examine the change in C and N_2 fixation rates over a 24-hr period in response to additions of either KH_2PO_4 (Fisher Scientific 7778, 99%, further denoted as DIP) or MPn (Sigma-Aldrich 64259, $\geq$ 98%). The rates of N_2 and C fixation in these treatments were compared to rates measured in parallel incubations with no added P. At all stations, water was collected using a Sea-Bird CTD (conductivity, temperature and depth) rosette equipped with 24 PVC 12-L sampling bottles. Polycarbonate bottles (4.4 L) were filled to overflowing with whole seawater (no pre-screening) collected from within the surface mixed layer. Care was taken to ensure that no bubbles were present before carefully sealing each bottle with a septum cap. All samples were collected from the upper 50 m of the water where irradiance varied between approximately 20-60% of the surface flux.

Incubation bottles were transferred to on-deck incubators plumbed with surface seawater and shaded to approximate light levels from which the samples originated. Two incubators were employed; one screened to ~30% surface irradiance and the other screened to ~60% of the surface irradiance. Whenever possible, controls (no P additions) as well as experimental treatments (MPn and/or DIP additions) were incubated in duplicate. All incubations began as close to dawn as practically possible, and lasted a full 24-hr period.

2.3.2 Nitrogen and carbon fixation rate determinations

All N_2 fixation rate measurements employed the $^{15}N_2$ isotopic tracer method described by Montoya et al. (1996) in parallel with ^{13}C-productivity measurements with rate calculations as per Legendre and Gosselin (1997). Using a gas tight syringe, 2.0 ml of $^{15}N_2$ gas (99 atom %, Cambridge Scientific) was injected into each bottle and the bottles were gently inverted several times. Immediately following $^{15}N_2$ additions, 0.5 ml of 47 mM bicarbonate ($NaH^{13}CO_3$) stock was injected into each bottle with a plunger-type syringe and the bottles were again gently mixed by inversion. For experimental treatments only, following the injection of both $^{15}N_2$ and $NaH^{13}CO_3$, each 4.4 L polycarbonate bottle was spiked using a Leur-lock syringe with either DIP or MPn to reach a final concentration of 5 μM above ambient P levels. The reported half-saturation coefficients (K_s) for specific-uptake of DIP in natural populations of *Trichodesmium* range from 0.4-9.0 μmol L^{-1} (McCarthy and Carpenter 1979; Sohm & Capone 2006); values of K_s for labile DOP uptake are presently unknown for *in situ* populations of oceanic diazotrophs, as are DIP uptake kinetics for uncultured diazotrophic phylotypes (including Group A and *Crocosphaera* unicellular diazotrophs and *Richelia*/diatom symbioses). Given the low affinity for DIP reported for *Trichodesmium* and the uncertainty of MPn uptake kinetics, we selected a level of P addition (5 μM) intended to ensure that the full microbial assemblage was exposed to saturating, but not inhibitory, concentrations of DIP or DOP. Controls were treated

and incubated under identical conditions as manipulations but without an external addition of P to approximate the ambient rate of C and N_2 fixation in surface waters.

At the termination of each incubation, bottles were filtered onto a 25 mm pre-combusted glass fiber filter (GFF, Whatman) and stored in a -20°C freezer until later analysis. Once ashore, samples were acid-fumed, dried overnight at 60°C and then encapsulated in tin and silver capsules. Particulate C, N and the isotopic composition of particulate material ($\delta^{15}N_{PN}$ and $\delta^{13}C_{PC}$) were determined using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer in the University of California Davis stable isotope facility.

To contextualize our results, we also present rates of C and N_2 fixation rates from unamended seawater samples measured at or around Station ALOHA during the 2004-2007 field seasons (Fong et al. 2008; Grabowski et al. 2008; Church et al. 2009). These rate measurements were conducted using methodology similar to what has been outlined above although a subset of samples were incubated using *in situ* arrays rather than on deck incubations (see Church et al. 2009). Lastly, during the OPEREX cruise, as we crossed through mesoscale features that coincided with visible *Trichodesmium* biomass, we collected duplicate samples from 25 m and 45 m for subsequent size-fractionated C and N_2 fixation rate determinations. For these measurements, two treatments were prepared: (1) whole water incubations filtered onto a combusted GFF filter (total) and (2) incubations pre-filtered through a 10 μm polycarbonate with the filtrate subsequently retained onto a GFF filter (<10 μm). N_2 fixation rates in the large (>10 μm) size fraction were calculated as the difference between rate measurements in these two samples (>10 μm = Total – <10 μm). Determination of size-fractionated rates was intended to provide insight into the distribution of N_2 fixation between small unicellular diazotrophs and large filamentous diazotrophs along this section of our study region.

2.3.3 Diazotroph distributions and *nifH* gene quantification during POOB 2008

Distributions of several groups of diazotrophic cyanobacteria common to the upper ocean waters of the NPSG were identified based on quantitative polymerase chain (QPCR) amplification of *nifH* genes. Seawater samples for subsequent *nifH* gene QPCR amplification were collected from the POOB 2008 cruise at 4 depths representing the ~60%, ~20%, ~10%, and ~1% light levels (depths varied from 9-20 m, 33-47 m, 46-65 m, and 102-143 m, respectively). Water was subsampled from the CTD rosette bottles into acid-washed 10 liter polyethylene carboys and filtered onto 0.22 μm pore size Sterivex-GS (Millipore) filter capsules. Filters were capped, flash frozen in liquid nitrogen and stored frozen at -80^{o}C until extraction.

In the shore-based laboratory, 1 ml of a cell lysis buffer (20 mM Tris-HCL, pH 8.0; 2 mM EDTA, pH 8.0; 1.2% Triton X and 20 mg ml^{-1} lysozyme) was injected into each filter capsule using a 5 ml syringe. Filters (with syringes still attached) were placed into a hybridization oven at 37^{o}C, vortexing at ~15 minute intervals for 1.5 hours. Filters were removed from the oven and the buffer was removed from the filter capsule by syringe and dispensed into 2 ml microcentrifuge tubes; 42 μl of proteinase K and 334 μl Buffer AL (Qiagen) was added to each sample and samples were vortexed and placed in a hybridization oven at 70^{o}C for 30 minutes. Following this incubation, 700 μl of 100% ethanol was added to each sample, the microcentrifuge tubes were vortexed and samples were transferred to the DNeasy spin columns (Qiagen) where DNA was purified following the manufacturer's recommended protocols.

Abundances of *nifH* genes were determined using the QPCR assay described in Church et al. (2005). For this study, we examined the spatial variability of 6 phylotypes frequently retrieved from PCR amplified *nifH* gene clone libraries at Station ALOHA (Dominic et al. 1998; Zehr et al. 2001; Church et al. 2008). QPCR primer and probe sets targeting the following *nifH* phylotypes were used: *Trichodesmium* spp.; three groups of heterocystous cyanobacteria (termed Het1, Het2,

and Het3, respectively); and two groups of *nifH* containing unicellular cyanobacteria including the uncultivated phylotype termed Group A and phylotypes whose *nifH* sequences are 97-99% identical to *Crocosphaera watsonii*. Descriptions of the primers and probes used for these reactions can be found in Church et al. (2005, 2008). All QPCR reactions were run in duplicate; the detection limit for these reactions corresponded to ~30 gene copies L^{-1} of seawater.

2.3.4 Ancillary Measurements

Whole seawater samples were collected for nutrient analyses from each depth sampled (all within the upper 50 m) in acid-washed polycarbonate bottles and stored at -20°C until later analyses. In the laboratory, after thawing, subsamples of these collections were transferred to acid-cleaned Teflon® digestion bombs and oxidized via the alkaline persulfate digestion described by Valderrama (1981). After oxidation, total dissolved P (TDP) was measured as described by Strickland and Parsons (1972). Low level soluble reactive P (assumed to be equivalent to DIP) was measured via the MAGIC (MAGnesium-Induced Coprecipitation) method of Karl and Tien (1992). Dissolved organic phosphorus (DOP) concentrations were calculated as the difference between TDP and DIP concentrations. Chlorophyll *a* concentrations were determined fluorometrically using a Turner Designs 10-AU fluorometer. Samples were extracted in 90% acetone for 24 hours at -20°C in the dark (Strickland and Parsons 1972).

2.3.5 Stock Purity

All liquid stocks used for manipulations including DIP (K_2HPO_4), MPn and $NaH^{13}CO_3$ were tested post-cruise for potential contamination of iron (Fe) and reactive N concentrations. Total Fe concentrations in these stocks were determined by isotope dilution (^{57}Fe) ICP-MS after pre-concentration and matrix removal using NTA (Lohan et al. 2005). Nitrate + nitrite (N+N) and ammonium (NH_4) concentrations in all stocks were measured by standard colorimetric methods adapted to an autoanalyzer

(Strickland & Parsons 1972). These analyses indicated that the addition of liquid stocks (DIP + ^{13}C and MPn + ^{13}C) to 4.4 L incubation bottles led to Fe enhancements of 2.7 pmol L^{-1} and 3.3 pmol L^{-1} above ambient Fe concentrations, respectively. This level of Fe contamination is minimal given that total Fe in the surface waters of the NPSG region are generally between 0.5 – 1.0 nmol L^{-1} (Boyle et al. 2005). Total N contamination (N+N+NH_4) in each 4.4 L incubation bottle (DIP + ^{13}C and MPn + ^{13}C) was determined to be 0.30 nmol L^{-1} and 0.38 nmol L^{-1}, respectively. For comparison, the mean N+N concentrations (not including NH_4) in the upper 45 m measured by the Hawaiian Ocean Time-Series (HOT) program in July- August of 2008 were 2-3 nmol L^{-1} (data from http://hahana.soest.hawaii.edu/hot/hot-dogs), again suggesting minimal addition of N+N by the addition of stocks. The MPn stock was also tested for the presence of DIP via the MAGIC method of Karl and Tien (1992); concentrations of DIP were found to be below the detection limit (< 1 nmol L^{-1}) of this technique. Thus, despite the relatively high levels of P additions made in these experiments (5 μmol L^{-1} final concentration above ambient levels), the relative purity of stocks as compared to *in situ* Fe and N+N concentrations provides confidence that enhanced C or N_2 fixation in experimental treatments derived from the amendment of P.

2.4 Results

2.4.1 Phytoplankton standing stock and P concentrations

Remote sensing chlorophyll retrievals indicate mesoscale surface enhancements during the July-August 2008 period within each of our cruise regions (Fig. 2-1 B and C). Shipboard determinations, summarized for both cruises in Table 2-2, indicated *in situ* mixed layer chlorophyll concentrations were greatest at depths of 25 m and 45 m during August 2008 within a mesoscale patch proximate to the Hawaiian Islands (0.10 to 0.18 μg L^{-1}) Concentrations of satellite-derived chlorophyll did not strictly match measured *in situ* chlorophyll, presumably stemming from offsets in timing of sampling and the limitation of remote sensing chlorophyll retrievals to the first optical depth (see White et al. 2007). In July 2008, *in situ* chlorophyll

concentrations in the upper mixed layer (~45 m) near the subtropical front (28-32^{o}N, 140-150^{o}W) to the north-east of the Hawaiian Islands were generally lower than those measured in August, with concentrations ranging from 0.06 to 0.10 μg L^{-1}. During this period, chlorophyll concentrations were greatest (~0.10 μg L^{-1}) at stations 7, 15, 18, and 19 (Table 2-2). DIP concentrations within the mixed layer of this region ranged from 15 to 61 nmol L^{-1}, averaging 30 ± 11 nmol L^{-1}. In contrast, DOP concentrations were significantly greater (181 to 339 nmol L^{-1}, averaging 249 ± 37 nmol L^{-1}). No significant differences were found in ambient P concentrations within the upper mixed layer during the OPEREX and POOB stations (t-test $p > 0.05$, n = 34) despite these studies occurring in hydrographically distinct regions of the NPSG (Fig. 2-1A).

2.4.2 Diazotrophic Community Structure

Diazotrophic community structure either was assessed via QPCR amplification of *nifH* genes (POOB) or inferred from direct visual observations and size-fractionated N_2 rate measurements (OPEREX, discussed in subsequent sections). For the July 2008 expedition (POOB), the spatial distributions of six *nifH* phylotypes were determined. In the well-lit regions of the euphotic zone (~60% surface irradiance, 9-20 m) of this region, *nifH* gene abundances ranged approximately an order of magnitude between stations (1.1 x 10^2 to 1.6 x 10^3 gene L^{-1}), with roughly equal contributions from unicellular and filamentous *nifH*-containing cyanobacterial phylotypes (Fig. 2-3A). Among the unicellular N_2 fixing cyanobacteria the uncultivated Group A phylotype (UCYN-A) dominated the *nifH* gene abundances (ranging 2.4 x 10^2 to 1.6 x 10^3 *nifH* genes L^{-1}), while the Het2 phylotype (identified as a symbiont of open ocean diatoms belonging to the genera *Hemiaulus* spp.) was generally the most abundant filamentous cyanobacterial phylotype (ranging from 4.0 x 10^2 to 7.5 x 10^2 of the *nifH* genes L^{-1}). *nifH* gene abundances of the unicellular Group A phylotype increased more than an order of magnitude (ranging 2.6 x 10^3 to 7.6 x 10^4 *nifH* genes L^{-1}) in the deeper mid-euphotic zone waters (33-65 m) where irradiance declined to ~5-10% of the surface flux (Fig. 2-3B). In contrast, gene abundances of the filamentous cyanobacterial

phylotypes decreased below the well-lit, near-surface waters. Relative to the balanced contributions of unicellular and filamentous cyanobacterial phylotypes observed in the well-lit upper ocean, the increase in UCYN-A and concomitant decrease in filamentous phylotypes (specifically Het2) resulted in the numerical dominance of unicellular N_2 fixers in the mid to deep euphotic zone waters.

2.4.3 Carbon and Nitrogen Fixation Rates: Controls

Significant variability in the magnitude of C and N_2 fixation rates of the unamended controls was observed within and between cruises in our study region (Table 2-1, Fig. 2-4A). The highest rates of N_2 and C fixation (8-21 nmol N L^{-1} d^{-1} and 602-934 nmol C L^{-1} d^{-1} respectively) were recorded in the upper 25 m of waters near the Hawaiian Islands, in a transition zone between a cyclonic and anticyclonic eddy (Fig. 2-2B, Table 2-1). At select stations during this cruise (stations 41-45) size-fractionated rate determinations (Fig. 2-5) indicated that N_2 fixation in the >10 μM size fraction ranged from 4.92 to 15.34 nmol N L^{-1} d^{-1}, with lower rates measured in the <10 μM size fraction (2.24 to 5.52 nmol N L^{-1} d^{-1}). The resulting rates of N_2 fixation in > 10 μm size fraction accounted for ~63-80% of the total N_2 fixation at these stations. This finding is consistent with the observation of visible *Trichodesmium* biomass in this region. Total C fixation rates were also elevated along this transect (744 ± 163 nmol C L^{-1} d^{-1}) with the <10 μm size fraction accounting for 657 ± 105 nmol C L^{-1} d^{-1} or 88% of the total C fixation (Fig. 2-5).

In contrast to the relatively high rates of N_2 fixation observed in the waters near Hawaii, rates of N_2 and C fixation were substantially lower (0.02 to 2.37 nmol N L^{-1} d^{-1} and 167- 647 nmol C L^{-1} d^{-1}, see Fig. 2-4A and Fig. 2-5) in the northern NPSG waters, along the edge of the subtropical front. Rates of N_2 fixation in this region were greatest near the edge of an anticyclonic eddy (Stations 7, 10; Fig. 2-2A and Table 2-1). Figure 2-4A shows the full range of N_2 fixation rates measured in the upper 45 m for both cruises relative to $^{15}N_2$ fixation data obtained from the prior literature for the NPSG region.

2.4.4 Carbon and Nitrogen Fixation Rates: Treatments

Enhancement of C and N_2 fixation rates in experimental treatments were calculated by subtracting the rate measured in the manipulation (+DIP or +MPn) from that measured in the control (no P-addition) (data are shown in Fig. 2-4B-C; Fig. 2-5A-B and Table 2-1). Both DIP and MPn amendments displayed significantly enhanced C and N_2 fixation relative to controls. Comparison of the responses to MPn and DIP treatments within each cruise revealed no significant difference in N_2 fixation stimulated by these exogenous P additions (POOB t-test $p = 0.91$, n = 34; OPEREX t-test, $p = 0.99$, n = 12). Similarly, enhancement in rates of C fixation were statistically similar when treatments were compared for each cruise (POOB t-test $p = 0.80$, n = 34; OPEREX t-test, $p = 0.55$, n = 12). These data suggest that within a single photoperiod, MPn and DIP contributed equally to the observed stimulation of N_2 and C fixation. Within the POOB dataset, only station 1 did not demonstrate enhanced C fixation rates in response to either MPn or DIP, albeit only DIP treatments and controls were performed at stations 17 through 21. Notably, despite the high levels of P added, the magnitude of enhanced N_2 fixation rates was within the range (+/- 2.75σ) of historical rate measurements for the NPSG measured at Station ALOHA (Fig. 2-5A).

By examining the C:N fixation ratios, our data lend insight into the relative contribution of diazotrophs to total C production. Molar C: N fixation ratios (Fig. 2-6 A-C) were calculated as the absolute difference of C fixation rates (treatment less control) divided by the absolute difference of N_2 fixation rates (treatment less control). In the paired MPn and DIP treatments (n=6 pairs) conducted during the OPEREX cruise, the ratio of C:N fixation ranged from 14-47 mol C : mol N for MPn treatments and 13-59 mol C : mol N for DIP treatments. One station (the 45 m sample at station 36) exhibited an order of magnitude increase in C: N fixation ratios (346 and 293 mol C:mol N for MPn and DIP treatments, respectively) driven by low rates of N_2 fixation at this depth (Fig. 2-6C and Table 2-1). Within the POOB dataset for the frontal region of the NPSG, the magnitude and variability of C fixation in response to MPn and DIP

additions drove the ratio of C:N fixation (Fig. 2-6). The lowest C:N enhancement ratios observed within this cruise region were at Stations 5-12 and 19-21 and corresponded to the most significant enhancements of N_2 fixation (Fig. 2-5). At these stations (5-12 and 19-21) the ratio of enhanced C:N fixation ranged from 2-70 mol C:mol N with a mean of 30±19 mol C : mol N for pooled DIP and MPn treatments. For the remainder of the POOB dataset, where N_2 fixation rates were relatively low, the ratio of enhanced C : N fixation exceeded 50 mol C : mol N (Fig. 2-6).

2.5 Discussion

Variability in volumetric N_2 fixation rates in nature is fundamentally driven by changes in either cell specific growth rates, diazotrophic biomass, or both. The diverse assemblage of microbes having the capacity to fix N_2 in the upper ocean span a wide range of cell specific rates of N_2 fixation (Goebel et al. 2008) so that the absolute rate of N_2 fixation measured at any one time and location is partially a reflection of diazotrophic community structure. In the open ocean, three major classes of diazotrophs have been identified to date: (1) species of the non-heterocystous, filamentous cyanobacterium *Trichodesmium* (2) *Richelia intracellularis*, a heterocystous endosymbiont of select diatom species and (3) groups of small unicellular diazotrophs (Zehr and Ward 2002; Church et al. 2008). In this study, we found the surface waters (< 20 m) of the northern edge of the NPSG (SST ~ 22ºC) to be dominated by both the unicellular Group A phylotype and heterocystous *Richelia* with Group A becoming more abundant in the mid-euphotic zone (33-65 m, Fig. 2-3). Visual observations of surface slicks and size-fractionated rate measurements indicate that transects along the more central NPSG region near the Hawaiian Islands (SST ~ 26ºC) sampled in August 2008 were conversely dominated by large colony-forming *Trichodesmium* spp. The salient differences in the size and physiology of each of these diazotrophic phylotypes suggests that they may differentially impact primary productivity and bulk N_2 fixation in the surface ocean. While we do not have any reliable means to examine the total number of N_2 fixing cells and thus normalize our

rate measurements, our results indicate that at the time of our sampling, the warmer central NSPG supported a higher mean rate of N_2 and C fixation than the cooler boundary waters to the north during our sampling period (Fig. 2-4 and 2-5). Moreover, enhanced N_2 fixation was observed in association with mesoscale features (Fig. 2-2) a finding that is consistent with previous studies (Davis and McGillicuddy 2006; Fong et al. 2008; Church et al. 2009) and likely reflects physical aggregation of diazotrophic cells.

Beyond observation of the variability of diazotrophic diversity and activity in the NPSG, a central objective of this research was to determine if the known heterogeneity of ambient N_2 and C fixation in this region was a consequence of differential P resource limitation. To examine whether P concentrations limited natural populations of diazotrophs, we conducted a series of P amendment experiments over a spatially extensive area of the NPSG. In contrast to previous experiments in oligotrophic surface waters that focused on DIP (and/or Fe) additions (e.g. Mills et al. 2004; Rees et al. 2006; Grabowski et al. 2008) we also sought to investigate the response of N_2 and C fixation rates to the addition of a presumably bioavailable DOP compound, MPn, relative to the response to DIP additions. MPn was selected as a test compound since it is known to be readily utilized by the diazotroph *Trichodesmium* (Karl et al. 2008; White et al. 2010). Relatively high doses of P (5 μmol L^{-1}) were added to ensure that saturating but not inhibitory levels of P were achieved. These experiments yielded three major findings: (1) when added in excess, MPn and DIP stimulated approximately equal rates of C and N_2 fixation over a single photoperiod such that both compounds, when added in excess, can support primary productivity and diazotrophy with equal efficiency; (2) resultant rates of N_2 fixation from the experimental amendments were within the range reported for Station ALOHA (Fig. 2-5), suggesting that despite observing stimulation of diazotrophy by additions of P in the current study, P concentrations in this region may often be sufficient to support the nutritional demands of diazotrophs, and (3) MPn and DIP additions stimulated C fixation rates at levels exceeding the estimated contribution of diazotrophs and hence

the availability of both DIP and DOP can regulate primary production and N_2 fixation on daily time-scales in this ecosystem.

Several other investigators have examined the role of P in regulating N_2 fixation in the subtropical gyres. Grabowski et al. (2008) conducted a series of manipulation experiments (+DIP, +Fe, +DIP and Fe) in May 2004 to March 2005 at Station ALOHA in the NPSG, the time-series station of the HOT program. In their study, the addition of 160-320 nmol L^{-1} P and/or 20 nmol L^{-1} Fe and following a 24 hr incubation period, resulted in significant variability in the relative response of N_2 fixation rates to nutrient amendments, ranging from insignificant changes in N_2 fixation to a near doubling of N_2 fixation rates in response to Fe, DIP, and dual amendments. Heterogeneity of the microbial response to nutrient amendments was also observed in the North Atlantic where Mills et al. (2004) observed a doubling of N_2 fixation rates at one site in response to both P and Fe additions while two other sites sampled did not differ from that measured in unamended controls. These previous studies, in conjunction with our results indicate that P availability can restrict rates of N_2 fixation in the NPSG. Yet, our experimental manipulations also revealed that MPn and DIP additions led to stimulation of C fixation rates (Fig. 2-6) in excess of those that could be attributed to diazotrophic activity (C:N fixation ratios >>100). Such uncoupling of C and N_2 fixation may reflect bulk community P limitation or diazotroph-mediated production of bioavailable nitrogen (DIN or DON) which stimulated plankton community growth, or both. We simply do not have sufficient data to resolve this question. However, in complementary work Van Mooy and Devol (2008) have shown that NH_4 additions rather than DIP additions led to enhanced DIP uptake in the > 2 μm size fraction of plankton populations at Station ALOHA. This observation coupled with similar results for *Prochlorococcus* specific RNA synthesis rates (N rather than P stimulated a positive response) led the authors to conclude that at the time of sampling (July 2003, 2004), the NPSG was in a state of proximate N-limitation. If N_2 fixation rates in the NPSG are limited by P as our results suggest for our sampling period, then it difficult to conceptualize a system where diazotrophy has pushed the system to a state of prolonged P limitation. Rather P limitation of

diazotrophs, would imply that new N inputs via N_2 fixation are restricted at times and that the NPSG may indeed be in a state of N limitation as indicated by earlier results of Van Mooy and Devol (2008). These inferences are of course subject to the spatial and temporal limitations of our sampling protocols, however they do provide insight into the complexities inherent in the ascription of the proximate growth limiting resource in the contemporary oligotrophic ocean.

Addition experiments such as those described above provide a snapshot of the nutritional status of resident microbial populations. To further contextualize these vignettes, a historical record of the temporal and spatial variability of diazotrophy is necessary and in our case available via the Hawaii Ocean Time-series record. The majority of N_2 fixation rates achieved by either MPn or DIP additions fell within the range of rates measured in the NPSG (Fig. 2-5, median enhancements for each cruise are within 95% confidence intervals for HOT data). These results indicate that P limitation may be a transitory occurrence in this region; i.e. P may be only one of several factors regulating N_2 fixation in the NPSG. Other resources such as Fe as well as food web dynamics (*e.g.* predation, lysis) and physical aggregation of diazotrophic biomass by mesoscale forcing also likely constrain diazotroph biomass and rates of N_2 fixation in this region. It is entirely likely that there is no single limiting factor driving the order of magnitude variability observed for both N_2 fixation rates and the abundance of N_2 fixing organisms (Dore et al. 2008; Church et al. 2009) in this region. In summary, results of these P amendment experiments demonstrated that at the time of sampling, the productivity of microbial communities in the NPSG was restricted by the availability of bioavailable P. While we do not yet have a mechanistic model for constraining the observed variability of oceanic N_2 fixation, the data presented here indicate that the physical and biological processes that regulate DOP composition may in turn play an important role in the regulation of C and N_2 fixation in the NPSG.

Acknowledgements: Support for this project was provided by the National Science Foundation (OCE-0623596 and OCE-0850827). We would like to thank chief scientist

Mark Brzezinski, of the Pacific Open Ocean Bloom cruise; chief scientist Zbigniew Kolber, of the Ocean PERturbation EXperiment cruise; the Captain and crew of the Kilo Moana and numerous colleagues from the University of Hawai'i, the Center for Microbial Oceanography: Research and Education (C-MORE) and Oregon State University Ocean Microbial Ecology Laboratory (OMEL). We also thank Zanna Chase and Joe Jennings for trace element and nutrient analyses, respectively.

2.6 Literature cited

Björkman KM, Karl DM (2003) Bioavailability of dissolved organic phosphorus in the euphotic zone at Station ALOHA, North Pacific Subtropical Gyre. Limnology and Oceanography 48:1049-1057

Björkman KM, Thomson-Bulldis AL, Karl DM (2000) Phosphorus dynamics in the North Pacific subtropical gyre. Aquatic Microbial Ecology 22:185-198

Boyle E, Bergquist BA, Kayser RA, Mahowald N (2005) Iron, Manganese, and Lead at Hawaii Ocean Timeseries Station ALOHA: Temporal Variability and an Intermediate Water Hydrothermal Plume. Geochimica et Cosmochimica Acta 69:933-952

Capone DG (2001) Marine nitrogen fixation: What's the fuss? Current Opinions in Microbiology 4:341-348

Cembella AD, Antia NJ, Harrison PJ (1982) The utilization of inorganic and organic phosphorous compounds as nutrients by eukaryotic microalgae: a multidisciplinary perspective: Part I. Critical Reviews in Microbiology 10:317-391

Church M, Björkman K, Karl D, Saito M, Zehr J (2008) Regional distributions of nitrogen-fixing bacteria in the Pacific Ocean. Limnology and Oceanography 53:63

Church M, Mahaffey C, Letelier R, Lukas R, Zehr J, Karl D (2009) Physical forcing of nitrogen fixation and diazotroph community structure in the North Pacific subtropical gyre. Global Biogeochemical Cycles 23:doi:10.1029/2008GB003418

Church MJ, Jenkins BD, Karl DM, Zehr JP (2005) Vertical distributions of nitrogen-fixing phylotypes at Stn ALOHA in the oligotrophic North Pacific Ocean. Aquatic Microbial Ecology 38:3-14

Davis CS, McGillicuddy DJ (2006) Transatlantic abundance of the N_2-fixing colonial cyanobacterium *Trichodesmium*. Science 312:1517-1520

Dominic B, Chen Y, Zehr J (1998) Cloning and transcriptional analysis of the nifUHDK genes of Trichodesmium sp. IMS101 reveals stable nifD, nifDK and nifK transcripts. Microbiology 144:3359

Dore J, Letelier R, Church M, Lukas R, Karl D (2008) Summer phytoplankton blooms in the oligotrophic North Pacific Subtropical Gyre: historical perspective and recent observations. Progress in Oceanography 76:2-38

Duhamel S, Dyhrman ST, Karl DM (2010) Alkaline phosphatase activity and regulation in the North Pacific Subtropical Gyre. Limnol Oceanogr 55:1414-1425

Dyhrman S, Ammerman J, Van Mooy B (2007) Microbes and the marine phosphorus cycle. Oceanography 20:110-116.

Dyhrman ST, Chappell PD, Haley ST, Moffett JW, Orchard ED, Waterbury JB, Webb EA (2006) Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. Nature 439:68-70

Fong A, Karl D, Lukas R, Letelier R, Zehr J, Church M (2008) Nitrogen fixation in an anticyclonic eddy in the oligotrophic North Pacific Ocean. The ISME Journal 2:663-676

Goebel N, Edwards C, Carter B, Achilles K, Zehr J (2008) Growth and carbon content of three different-sized diazotrophic cyanobacteria observed in the subtropical North Pacific. Journal of Phycology 44:1212-1220

Grabowski M, Church M, Karl D (2008) Nitrogen fixation rates and controls at Stn ALOHA. Aquatic Microbial Ecology 52:175-183

Hutchins DA, Fu F, Zhang Y, Warner ME, Feng Y, Portune K, Bernhardt PW, Mulholland MR (2007) CO_2 control of *Trichodesmium* N_2 fixation, photosynthesis, growth rates, and elemental ratios: Implications for past, present, and future ocean biogeochemistry. Limnology and Oceanography 52:1293-1304.

Karl D, Björkman K, Dore J, Fujieki L, Hebel D, Houlihan T, Letelier R, Tupas L (2001a) Ecological nitrogen-to-phosphorus stoichiometry at station ALOHA. Deep-Sea Research Part II 48:1529-1566

Karl D, Letelier R, Tupas L, Dore J, Christian J, Hebel D (1997) The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. Nature 388:533-538

Karl DM (2002) Nutrient dynamics in the deep blue sea. Trends in Microbiology 10:410-418

Karl DM, Beversdorf L, Björkman KM, Church MJ, Martinez A, Delong EF (2008) Aerobic production of methane in the sea. Nature Geoscience 1:473-478

Karl DM, Bidigare RR, Letelier RM (2001b) Long-term changes in plankton community structure and productivity in the North Pacific Subtropical Gyre: The domain shift hypothesis. Deep Sea Research II 48:1449-1470

Karl DM, Björkman KM (2002) Dynamics of DOP. In: Hansell DA, Carlson CA (eds) Biogeochemistry of marine dissolved organic matter. Academic Press, London, p 249–366.

Karl DM, Michaels A, Bergman B, Capone DG, Carpenter EJ, Letelier RM, Lipschultz F, Paerl H, Sigman D, Stal L (2002) Dinitrogen fixation in the world's oceans. Biogeochemistry 57/58:47-98

Karl DM, Tien G (1992) MAGIC: a sensitive and precise method for measuring dissolved phosphorus in aquatic environments. Limnology and Oceanography 37:105-116

Kolowith LC, Ingall ED, Benner R (2001) Composition and cycling of marine organic phosphorus. Limnology and Oceanography 46:309-320

Legendre L, Gosselin M (1997) Estimation of N or C uptake rates by phytoplankton using ^{15}N or ^{13}C: revisiting the usual computation formulae. Journal of Plankton Research 19:263

Letelier RM, Dore J, Winn CD, Karl DM (1996) Seasonal and interannual variations in autotrophic carbon assimilation at Station ALOHA. Deep Sea Research II 43:467-490

Lohan M, Aguilar-Islas A, Franks R, Bruland K (2005) Determination of iron and copper in seawater at pH 1.7 with a new commercially available chelating resin, NTA Superflow. Analytica Chimica Acta 530:121-129

McCarthy JJ, Carpenter EJ (1979) Oscillatoria (Trichodesmium) thiebautii (Cyanophyta) in the central north Atlantic Ocean. J Phycol 15:75-82

Mills MM, Ridame C, Davey M, La Roche J, Geider RJ (2004) Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. Nature [Nature] 429:292-294

Montoya JP, Voss M, Kahler P, Capone DG (1996) A simple high-sensitivity tracer assay for N_2 fixation. Applied Environmental Microbiology 62:986-993

Perry M, Eppley R (1981) Phosphate uptake by phytoplankton in the central North Pacific Ocean. Deep Sea Research Part A Oceanographic Research Papers 28:39-49

Rees A, Law C, Woodward E (2006) High rates of nitrogen fixation during an in-situ phosphate release experiment in the Eastern Mediterranean Sea. Geophys Res Lett 33:L10607

Sañudo-Wilhelmy SA, Kustka AB, Gobler CJ, Hutchins DA, Yang M, Lwiza K, Burns J, Capone DG, Raven JA, Carpenter EJ (2001) Phosphorus limitation of nitrogen fixation by *Trichodesmium* in the central Atlantic Ocean. Nature 411:66-69

Sohm JA, Capone DG (2006) Phosphorus dynamics of the tropical and subtropical north Atlantic : *Trichodesmium* versus bulk plankton. Marine Ecology Progress Series 317:21-28

Strickland J, Parsons TR (1972) A practical handbook of seawater analysis, Vol 167. Fisheries Research Board of Canada

Valderrama JC (1981) The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Marine Chemistry 10:109-122

Van Mooy B, Devol A (2008) Assessing nutrient limitation of Prochlorococcus in the North Pacific subtropical gyre by using an RNA capture method. Limnology and Oceanography 53:78-88

Venrick E (1993) Phytoplankton seasonality in the central North Pacific: the endless summer reconsidered. Limnology and Oceanography 38:1135-1149

Vitousek P, Howarth R (1991) Nitrogen limitation on land and in the sea: how can it occur? Biogeochemistry 13:87-115

White AE, Karl DM, Björkman KM, Beversdorf L, Letelier RM (2010) Production of organic matter by *Trichodesmium* IMS101 as a function of phosphorus source. Limnol and Oceanogr 55:1755-1767

White AE, Spitz YH, Letelier RM (2007) What factors are driving summer phytoplankton blooms in the North Pacific Subtropical Gyre. Journal of Geophysical Research 112:C12006

Wu J, Sunda W, Boyle E, Karl D (2000) Phosphate depletion in the western North Atlantic Ocean. Science 289:759

Zehr JP, Ward BB (2002) Nitrogen Cycling in the Ocean: New Perspectives on Processes and Paradigms. Applied Environmental Microbiology 68:1015-1024

Zehr JP, Waterbury JB, Turner PJ, Montoya JP, Omoregie E, Steward GF, Hansen A, Karl DM (2001) Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. Nature 412:635-616

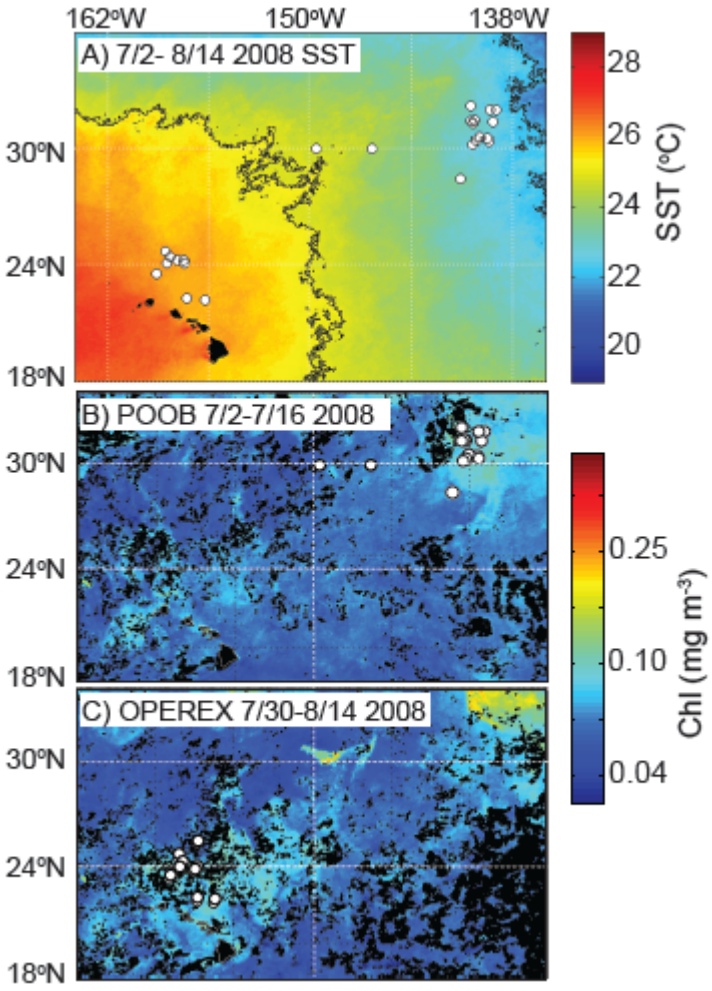

Figure 2-1. A) Mean (July-August 2008) MODIS SST (°C) the solid black contour denoting the 25°C isotherm and B) MODIS chlorophyll *a* (mg m^{-3}) averaged over the duration of POOB (July 2- July 16) and C) OPEREX (July 30- August 14). Station locations are shown as white circles.

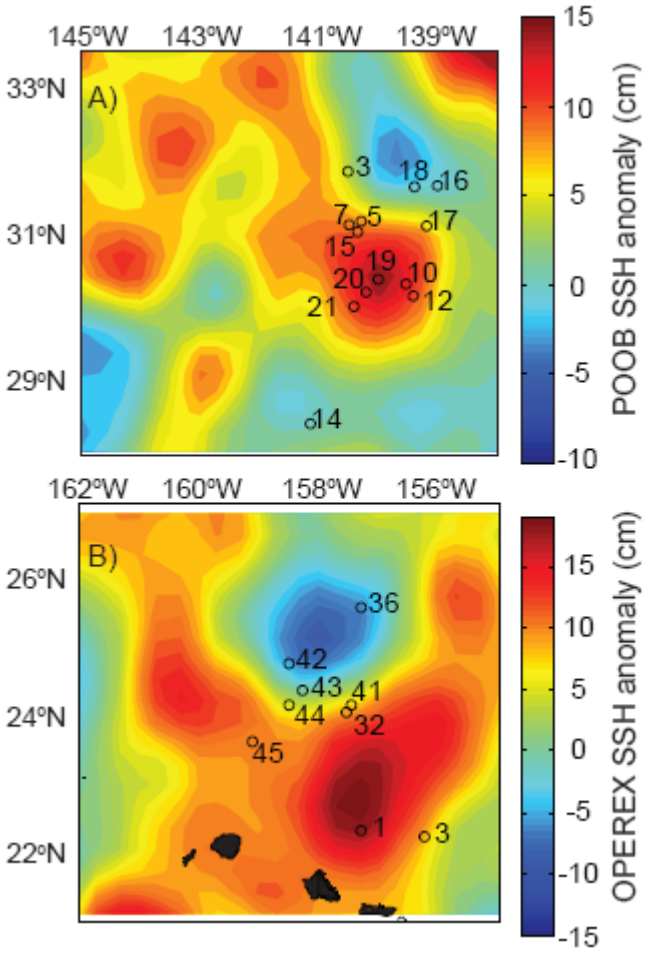

Figure 2-2. Weekly sea surface height anomaly field derived from AVISO for A) the week of July 9th, 2008 during the POOB cruise and B) the week of August 6, 2008 during the OPEREX cruise. All stations for which N fixation data were collected are shown with the exception of Station 1-2 for POOB which were located at 30°N, 145-150°W. Sampling efforts for OPEREX were focused where surface slicks of *Trichodesmium* were observed along a transition between a cyclonic and anticyclonic eddy (Stations 41-45).

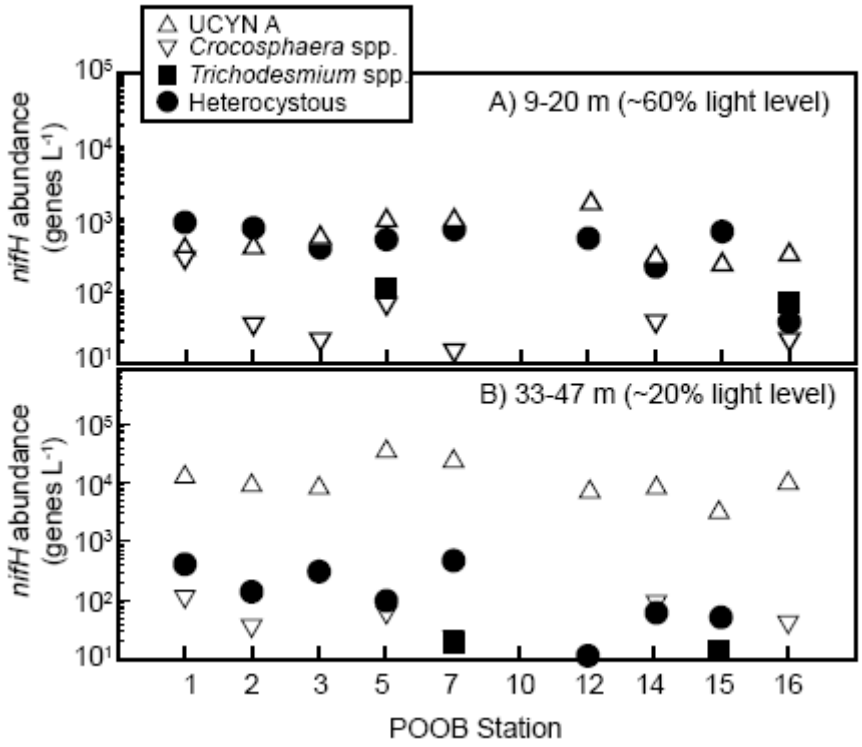

Figure 2-3. *nifH* gene abundances for selected diazotrophs for two vertical regions of the euphotic zone (60% and 20% surface irradiance isopleths, respectively) during the POOB cruise. Top panel presents gene abundances measured along the POOB cruise transect in the upper 9-20 m depth (60% surface irradiance); bottom panel depicts gene abundances measured at the 20% surface irradiance isopleths (33-47 m). Unicellular Group A phylotypes abbreviated as UCYN A; heterocystous includes sum of Het1, Het2, and Het3 cyanobacteria phylotypes (see text for details)

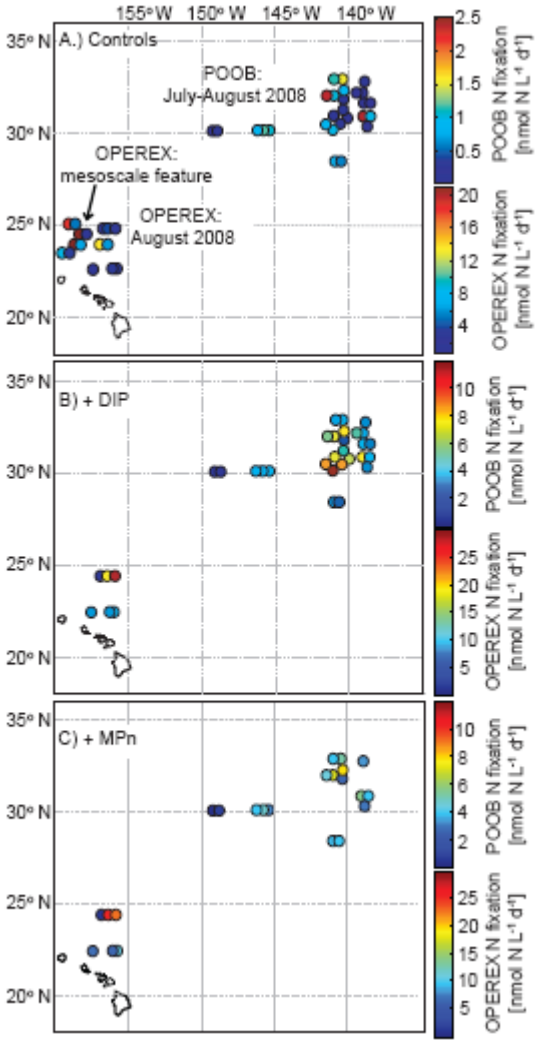

Figure 2-4. A) Bulk seawater N_2 fixation (no exogenous additions) measured during OPEREX and POOB. Note different scales for each cruise as OPEREX rates were notably higher than POOB and the P additions were markedly higher than controls. B) N_2-fixation rates in response to DIP additions and C) MPn additions for these same cruises. The color of each dot represents the magnitude of N fixation corresponding to the appropriate color bar. Values from duplicate incubations are shown.

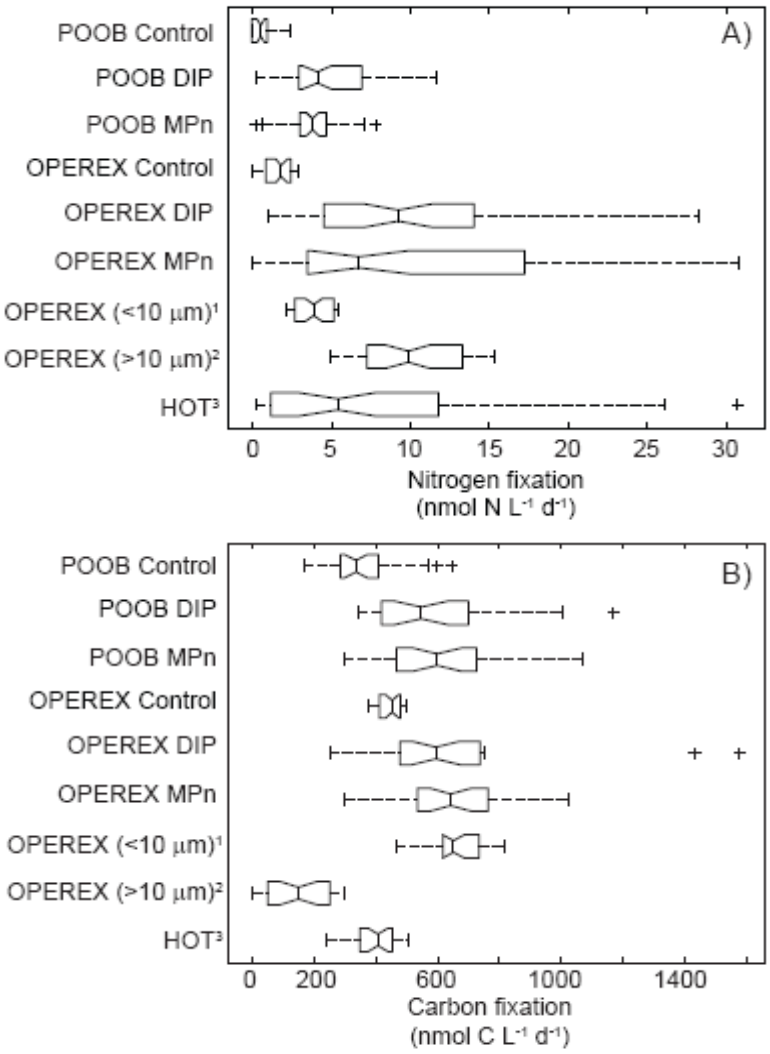

Figure 2-5. A) Summary of N_2 fixation and B) carbon fixation rates from both POOB and OPEREX control and manipulation experiments (manipulation less control) along with (1-2) size-fractionated rate measurements (<10 μm and > 10 μm fractions respectively) data from stations 41-45 of OPEREX and (3) historical data from the HOT time series data presented in Grabowski et al. (2008), Fong et al. (2008) and Church et al. (2009). Box edges represent the 25% and 75% percentile of data, the median is shown as a solid line within each box, the notches represent 95% confidence intervals, whiskers extend to +/- 2.7 standard deviations of each date set and outliers are denoted by plus signs.

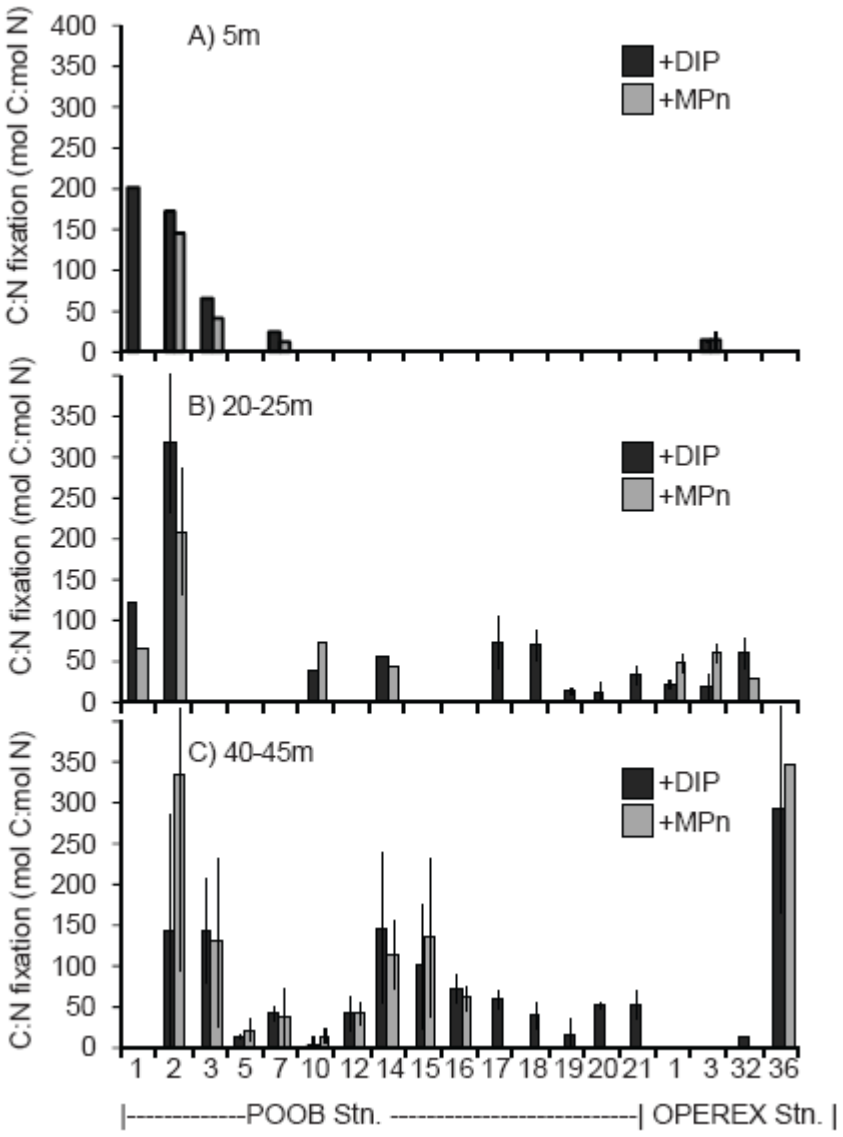

Figure 2-6. A) Summary of molar C:N fixation ratios calculated as rates less controls for A) the upper 5 m, B) the 20-25 m and C) the 40-45 m depth horizons.

Table 2-1. Summary of the magnitude of N and C fixation rates in control incubations (no added P) and the enhanced rates measured after MPn and DIP additions (less the values recorded in parallel controls). Values in parentheses are standard deviations of duplicate measures.

			^{15}N fixation [nmol N L^{-1} d^{-1}]			^{14}C fixation [nmol C L^{-1} d^{-1}]		
Cruise	Stn.	Depth	Control	MPn-Control	DIP-Control	Control	MPn-Control	DIP-Control
POOB	1	5	0.02	0.24	0.2	302	297(7)	343(40)
POOB	1	25	0.08	0.62	0.27	364	405(148)	397(18)
POOB	2	5	0.89	2.87	2.53	570	984	1002
POOB	2	25	1.16(0.45)	3.36(0.90)	2.53(0.46)	366(52)	1068(177)	1170(150)
POOB	2	45	1.04(0.40)	2.00(1.39)	3.10(1.11)	265(15)	936(121)	703(423)
POOB	3	5	1.10	4.11	3.18	433	600	636
POOB	3	44	1.49(0.02)	2.36(0.20)	1.85(0.20)	333(14)	639(242)	598(113)
POOB	5	45	0.92(0.06)	6.90(0.28)	6.63(1.79)	295(2)	444(104)	386(15)
POOB	7	5	2.32	4.75	4.71	599	647	706
POOB	7	42	1.02(0.02)	3.43(0.05)	4.81(0.60)	415(32)	545(120)	617(8)
POOB	10	25	2.37	3.17	4.96	428	654	615
POOB	10	45	0.98(0.03)	2.90(0.18)	3.48(0.52)	339(8)	382(24)	345(45)
POOB	12	45	0.04(0.01)	2.88(0.21)	2.91(0.28)	271(34)	393(10)	392(49)
POOB	14	25	0.76	2.94	2.15	377	506	495
POOB	14	45	0.59(0.03)	3.31(0.32)	1.51(2.45)	244(56)	621(118)	461(21)
POOB	15	45	0.24(0.03)	2.29(1.50)	1.77(0.59)	286(7)	595(83)	462(120)
POOB	16	45	0.07(0.1)	3.30(0.02)	3.49(0.52)	287(42)	490(23)	538(21)
POOB	17	20	NA	NA	4.08	189(0.2)	NA	486(133)
POOB	17	40	0.04(0.05)	NA	2.72(0.36)	226(11)	NA	391(16)
POOB	18	20	0.04(0.06)	NA	5.49(0.16)	167(94)	NA	547(1)
POOB	18	40	0.02(0.02)	NA	2.94(0.04)	291(6)	NA	410(46)
POOB	19	20	NA	NA	6.72	549(19)	NA	637(19)
POOB	19	40	0.09(0.13)	NA	5.31(0.45)	353(61)	NA	440(82)
POOB	20	20	0.09(0.03)	NA	8.58(0.63)	647(24)	NA	715(141)
POOB	20	40	0.21(0.18)	NA	6.83(0.23)	330(5)	NA	689(19)
POOB	21	20	0.97	NA	10.8	388(1)	NA	742(116)
POOB	21	40	0.85(0.06)	NA	7.90(0.49)	336(9)	NA	755(133)
OPEREX	1	25	1.83	6.69(1.18)	7.94(1.4)	461	489(23)	595(37)
OPEREX	3	5	2.06	10.54(5.51)	9.88(0.86)	500	691(37)	604(32)
OPEREX	3	25	1.89	6.42(0.84)	7.62(5.46)	377	619(43)	479(128)
OPEREX	32	25	1.74	22.97	18.36	445	646(128)	1433(269)
OPEREX	32	45	2.84	25.39(7.64)	28.27	1213	1024	1578
OPEREX	36	45	0.1	1.23	1.20(0.21)	249(6)	837(469)	571(125)
OPEREX	41	25	12.67	NA	NA	782	NA	NA
OPEREX	41	45	2.48	NA	NA	529	NA	NA
OPEREX	42	25	17.66	NA	NA	641	NA	NA
OPEREX	42	45	4.31	NA	NA	734	NA	NA
OPEREX	43	25	20.52	NA	NA	602	NA	NA
OPEREX	43	45	5.19	NA	NA	653	NA	NA
OPEREX	44	25	19.82	NA	NA	934	NA	NA
OPEREX	44	45	5.49	NA	NA	650	NA	NA
OPEREX	45	25	7.62	NA	NA	672	NA	NA
OPEREX	45	45	2.70	NA	NA	465	NA	NA

Table 2-2. Summary of the DIP, DOP and chlorophyll (Chl) concentrations measured at stations where N or C fixation rates were measured. The range, mean and standard deviations are presented. NA indicates that data are not available, as a sample was not collected. ND indicates gene abundances below the limit of detection (~30 gene copies L^{-1}).

Cruise	Stations	Depth range	DIP (nmol L^{-1})	DOP (nmol L^{-1})	Chl (µg L^{-1})	Unicellular N_2 fixers (*nifH* gene L^{-1})	Filamentous N_2 fixers (*nifH* genes L^{-1})
POOB	1-21	5-45	16.0 - 61.1	181-339	0.07-0.10	ND - 3.1×10^4	ND - 9.0×10^2
			29.7 ± 11.5	246 ± 38	0.08 ± 0.01	$5.8 \times 10^3 \pm 8.3 \times 10^3$	$3.1 \times 10^2 \pm 3.0 \times 10^2$
OPEREX	1,3,32-45	5-45	22.2 - 47.1	220-289	0.07-0.19	NA	NA
			37.7 ± 8.8	251 ± 30	0.11 ± 0.05	NA	NA

Intracellular phosphorus fractionation of natural assemblages of phytoplankton and *Trichodesmium*

Katie S. Watkins-Brandt and Angelicque E. White

In preparation for Marine Chemistry

3 Intracellular phosphorus fractionation of natural assemblages of phytoplankton and *Trichodesmium*

3.1 Abstract

We evaluated intracellular phosphorus (P) compartmentalization by both the bulk natural assemblage of plankton and isolated colonies of *Trichodesmium* from five cruises within the North and South Pacific subtropical Gyres (NPSG and SPSG, respectively). Using a trichloroacetic (TCA) acid extraction method, samples were analyzed for total and acid soluble fractions of particulate P. Field data suggest that natural populations of *Trichodesmium* demonstrate a unique internal composition of primarily inorganic phosphorus while the bulk plankton assemblage are composed of primarily organic P pools. For all five cruises, the mean intracellular P content of *Trichodesmium* biomass was 60% ± 10% particulate inorganic P (PIP) with the remaining fraction as 40% ± 10% particulate organic P (POP). In the bulk phytoplankton community, the ratios were primarily organic in composition with mean POP concentration of 61% ± 5% and a mean PIP concentration of 39% ± 5%. Variability of bulk PIP: POP ratios may be in part due to variable contributions of PIP rich organisms such as *Trichodesmium*. Results from these cruises suggest that the fractionation of total particulate phosphorus between PIP and POP in surface seawater is relatively constant. In sum, our results suggest that *Trichodesmium* may be unique in their allocation of P to primarily inorganic P pools.

3.2 Introduction

Phosphorus (P) is an essential element for all living organisms and is an important constituent of cellular material (phospholipids), nucleic acids (DNA and RNA) as well as a source of energy (ATP). As with all elemental constituents in the marine environment, P is formed, transformed and ultimately consumed by marine phytoplankton; all of which are intrinsically linked to the internal composition and fractionation of P within a cell. Studies investigating the role of the dissolved fractions (dissolved inorganic phosphorus- DIP and dissolved organic phosphorus- DOP) of the marine P pool suggest that P bioavailability may regulate primary production and in the case of diazotrophs, rates of nitrogen fixation (Sañudo-Wilhelmy et al. 2001; Grabowski et al. 2008; See Chapter 1). To enhance our present understanding of P regulation of growth, we have sought to determine the relationship between P content and intracellular P-compartmentalization within various phytoplankton species (Miyata and Hattori 1986). Comparing P-compartmentalization in natural populations of bulk phytoplankton assemblages to *Trichodesmium* colonies in the open ocean may provide insight into the physiological adaptations and stoichiometric flexibility of *Trichodesmium* and other phytoplankton and their capacity to thrive in P restricted environments.

Some phytoplankton have the capacity to consume P in excess of growth requirements and store these P residues for later use, in some cases in the form of polyphosphate (Kuenzler and Ketchum, 1962; White et al. 2006; Orchard et al. 2010). Studies of natural populations in Tokyo Bay have also linked the intracellular compartmentalization of P in natural assemblages to variability in ambient DIP concentrations, suggesting that internal P composition reflects response of microbial populations to fluctuations of the external nutrient field (Miyata and Hattori, 1986).

Moreover, *Trichodesmium* cultures maintained in P-deplete conditions demonstrated inorganic P uptake at nearly six times the rate of P-replete cultures following exogenous P additions, further resulting in the uncoupling of growth and P uptake rates (Fu et al. 2005). Another laboratory experiment under P-replete, P-

restricted and light controlled conditions, showed that the *Trichodesmium* strain IMS101 deviated six fold from the traditional Redfield ratio with growth capable at C:N:P ratios of $C_{585\pm56}$:$N_{90\pm10}$:P_1 (White et al. 2006). Isolated colonies of *Trichodesmium* from the Atlantic and Pacific maintained a low intracellular P quota in relation to C and N (Letelier et al. 1996). In the field, trichomes and colonies yielded PN:PP ratios three times higher than the estimated Redfield ratio, 45:1 to 16:1, respectively, providing further evidence that *Trichodesmium* in the system were P-limited and maintained a low P quota (Letelier and Karl, 1998). All of the above results provide evidence that *Trichodesmium* has a low cellular P quota relative to C and N and hence P deficient organic matter is not a reliable proxy for P-limitation.

This unique physiological capacity of *Trichodesmium* and potentially other diazotrophs, to modulate internal P quotas will ultimately affect the productivity on an organismal scale and affect the dynamics and availability of C, N and P of the overall system. By measuring the PIP and POP fractions within the TPP pool we may begin to understand how *Trichodesmium* allocates P resources under variable resource supply rate. During a cruise where the autotrophic community structure was dominated by a population of *Skeletonema costatum* Miyata and Hattori (1986) found that TPP was comprised of primarily inorganic P in the form of orthophosphate. Another study conducted in the North Pacific suggested that the TPP pool was primarily organic in composition ranging from 80 to 90% of the total (Yoshimura et al. 2007). While many studies have examined the fractionation of phosphorus in the bulk plankton community, none to our knowledge have differentiated and attempted to look at the fractionation of select organisms. In this study, we employed a simple fractionation method to determine the intracellular composition of phytoplankton assemblages within the North and South Pacific subtropical gyres and specifically with the marine diazotroph, *Trichodesmium*. Our goal was to gain an understanding of the intracellular allocation of P resources in *Trichodesmium* relative to the bulk phytoplankton community.

3.3 Materials and Methods

Field samples were collected during five cruises within the North and South Pacific Subtropical gyres and include (1) the Ocean Perturbation Experiment (OPEREX, 22°N to 26°N, July 30- August 14, 2008, further denoted N.Pac *Trichodesmium* Rich), (2) Pacific Open Ocean Bloom (POOB, 28°N to 32°N, July 2- July 16, 2008, further denoted N.Pac Front), (3) Bloom Ecological Reconnaissance (BLOOMER, 22°N to 26°N, August 9- August 21, 2007, further denoted N.Pac), (4) Biogeochemistry of the Upper Ocean: Latitudinal Assessment (BULA, 20ºN to 15ºS, April 15- April 26, 2007, further denoted Equ) and (5) KM0703 (SPEEDO, 12ºS to 30ºS, March 39-April 14, 2007, further denoted S.Pac).

Bulk water plankton samples were collected using a Sea-Bird CTD (conductivity, temperature and depth) rosette within the upper 50 m of the water column. A total of 4-6 L of seawater were filtered onto an acid washed combusted Whatman glass fiber filter (GF/F) for later analysis of intracellular P of the bulk phytoplankton assemblage. Using a 202-µm mesh equipped with a 64-µm mesh cod end and flow meter, horizontal surface net tows were performed in the upper 5- 10m of the water column for the collection of *Trichodesmium* colonies. Individual colonies were then isolated using a sterile transfer loop, placed in 0.2 µm filtered seawater and filtered onto an acid washed combusted GF/F filter for intracellular P fractionation. Following filtration, all samples were stored in liquid nitrogen until transported to a -80 freezer and further analyzed.

3.3.1 Phosphorus fractionation: Recovery and Sample analysis

Methods for phosphorus fractionation were modified from the trichloroacetic acid (TCA) extraction protocol of Miyata and Hattori (1986). In short, sample filters were placed in new 50 ml Falcon tubes and washed by streaming 17 ml of cold (-20ºC) 3% NaCl solution onto the filter to wash off cells. The sample was then vortexed, placed in an ice bath and sonicated at 0º C for 3 minutes in order to lyse

cells. Preliminary tests were done to show that P recovery via sonication was maximal after 3 minutes (Table 3-1). Following sonication the filter was removed and placed in an acid-washed combusted glass tube and covered with foil so residual particulate phosphorus attached to the filter could be analyzed. After removing the filter, tubes were centrifuged (0º C, 3000 rpm) for 30 minutes. 5 ml of the homogenate was transferred into an acid-washed combusted glass tube for determination of total acid insoluble phosphorus (further denoted TP_A) via the Valderramma (1981) persulfate oxidation method. In short, samples are oxidized via alkaline persulfate oxidation and further measured as total dissolved phosphorus (TDP) as described by Strickland and Parsons (1972). 5ml of homogenate was placed into a clean 15 ml Falcon tube to measure the acid insoluble inorganic phosphorus concentration (PiA). From the remaining volume, 5ml of the homogenate was then placed in a 15 ml Falcon tube and 5 ml of cold 10% trichloroacetic acid (TCA) was added. Samples were then vortexed and stored in the dark at 20ºC for 2 hours. Using a 5 mg/L DNA stock and *Trichodesmium* IMS101, nucleic acid solubilization was found to be minimal after 2 hours (data not shown).

Following acid extraction, a 5 ml aliquot of the acid soluble (PiB) fraction was transferred to a clean 15 ml Falcon tube where 5ml of DI-water was added to dilute the acid content. The sample was then vortexed and SRP was measured following the method of Strickland and Parsons (1972). From the remaining volume, a 5 ml aliquot was placed into a Teflon© digestion bomb, 5 ml of DI-water was added, the sample was inverted and TDP was measured via Valderramma persulfate oxidation (1981). DI-water was added following the acid extraction in order to ensure a proper pH range for formation of the molybdenophosphoric blue complex in the SRP reaction step. Rigorous testing of the recovery of different phosphorus compounds was tested via the methodology outlined above in the absence of filters where stocks were added directly to 3% NaCl at varying concentrations. A CARY-300 IU-VIS spectrophotometer was used to measure absorbance at 880 nm for TPP and PIP analyses. A summary of the fractionation method is depicted in Fig. 3-1.

3.4 Results

The fractionation of total particulate phosphorus (TPP) between particulate inorganic phosphorus (PIP) and particulate organic phosphorus (POP) was relatively constant throughout the surface waters in all cruises (Fig. 3-2). Figure 3-3 demonstrates the internal P content of the bulk phytoplankton assemblage for all samples during each cruise. Interestingly, all cruises show a relatively constant surface water POP composition ranging from 53% to 66% with the exception of stations in the N.Pac where high abundances of *Trichodesmium* were observed. We hypothesize that the observed enhancement in the PIP composition during this cruise may be a result of the significant presence of *Trichodesmium* observed at station 3 and crossing a mesoscale feature (Stations 41-45). A summary of *Trichodesmium* PIP:POP ratios for each cruise in Fig. 3-4 demonstrates a primarily inorganic internal P composition.

3.5 Discussion

In the event of phosphorus limitation, *Trichodesmium*, have demonstrated adaptations preceding those of other organisms undoubtedly contributing to their often dominant presence in late summer blooms within the NPSG. The ability to consume and store phosphorus for later use provides a competitive advantage in an environment where P concentrations are low (<100 nmol L^{-1}) relative to biological demand (Karl and Björkman, 2002). The use of PIP:POP ratios as a potential index of phosphorus limitation may prove to be a useful tool.

Ultimately, three major findings have arisen from this research: (1) for natural populations of *Trichodesmium,* PIP pools are consistently greater than POP pool, (2) the surface water bulk particulate phosphorus fraction is relatively constant and composed primarily of POP, (3) the modified method outlined in this paper acts as a simple and efficient way to analyze the components of the TPP pool. During the test of commercially available P compounds we found that the majority of the recovered inorganic fraction was in the form of orthophosphate. It should also be noted that we did not find tested polyphosphates to be TCA soluble.

The next step in understanding fractionation of phosphorus within marine organisms entails running samples from completed laboratory experiments examining the internal phosphorus allocation in cultures of *Thallassiosira pseudonana*, *Trichodesmium theibautti* (H-9) and *Trichodesmium* IMS101 and *Prochlorococcus marinus* grown on either inorganic and organic phosphorus compounds over a growth cycle. Further this data will be compared to the *in situ* bulk plankton samples along with natural populations of isolated *Trichodesmium* within the North and South Pacific Subtropical gyres.

Acknowledgements: Support for this project was provided by the National Science Foundation (OCE-0623596). We would like to thank chief scientist Mark Brzezinski of the POOB cruise (N.Pac Front); chief scientist Zbigniew Kolber, of the OPEREX cruise (N.Pac *Trichodesmium* Rich); chief scientist Ricardo Letelier, of the BLOOMer cruise (N.Pac); chief scientist, Jonathan Zehr of the SPEEDO (S.Pac) cruise and Matthew Church, chief scientist of the BULA (Equ) cruise; the Captain and crew of the Kilo Moana and numerous colleagues from the University of Hawaii, the Center for Microbial Oceanography: Research and Education (C-MORE) and Oregon State University Ocean Microbial Ecology Laboratory (OMEL).

3.6 Literature cited

Fu F, Zhang Y, Bell P, Hutchins DA (2005) Phosphate uptake and growth kinetics of *Trichodesmium* (cyanobacteria) isolates from the North Atlantic Ocean and the Great Barrier Reef, Australia. Journal of Phycology 41:62-73

Karl DM, Björkman KM (2002) Dynamics of DOP. In: Hansell DA, Carlson CA (eds) Biogeochemistry of marine dissolved organic matter. Academic Press, London, p 249–366.

Kuenzler E, Ketchum B (1962) Rate of phosphorus uptake by *Phaeodactylum tricornutum*. The Biological Bulletin 123:134

Letelier RM, Karl DM (1996) Role of Trichodesmium spp. in the productivity of the subtropical North Pacific Ocean. Marine ecology progress series 133:1-3

Letelier RM, Karl DM (1998) Trichodesmium spp. physiology and nutrient fluxes in the North Pacific subtropical gyre. Aquatic Microbial Ecology 15:265-276

Miyata K, Hattori A (1986) A simple fractionation method for determination of phosphorus components in phytoplankton: Application to natural populations of phytoplankton in summer surface waters of Tokyo Bay. Journal of Oceanography 42:255-265

Orchard ED, Benitez-Nelson CR, Pellechia, PJ, Lomas MW, Dyhrman ST (2010) Polyphosphate in *Trichodesmium* from the low phosphorus Sargasso Sea. Limnology and Oceanography 55:2161-2169

Overbeck J (1962) Studies on phosphate metabolism of green algae. III. Behavior of the cell fractions of *Scenedesmus quadricauda* (Turp.) Breb. in the daily cycle under different lighting conditions and using different phosphate compounds. Archiv f ¼r Mikrobiologie 41:11

Sañudo-Wilhelmy SA, Kustka AB, Gobler CJ, Hutchins DA, Yang M, Lwiza K, Burns J, Capone DG, Raven JA, Carpenter EJ (2001) Phosphorus limitation of nitrogen fixation by *Trichodesmium* in the central Atlantic Ocean. Nature 411:66-69

Strickland J, Parsons TR (1972) A practical handbook of seawater analysis, Vol. Fisheries Research Board of Canada

Valderrama JC (1981) The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Marine Chemistry 10:109-122

White AE, Spitz YH, Karl DM, Letelier RM (2006) Flexible elemental stoichiometry in *Trichodesmium* spp. and its ecological implications. Limnology and Oceanography 51:1777-1790

Yoshimura T, Nishioka J, Saito H, Takeda S, Tsuda A, Wells M (2007) Distributions of particulate and dissolved organic and inorganic phosphorus in North Pacific surface waters. Marine Chemistry 103:112-121

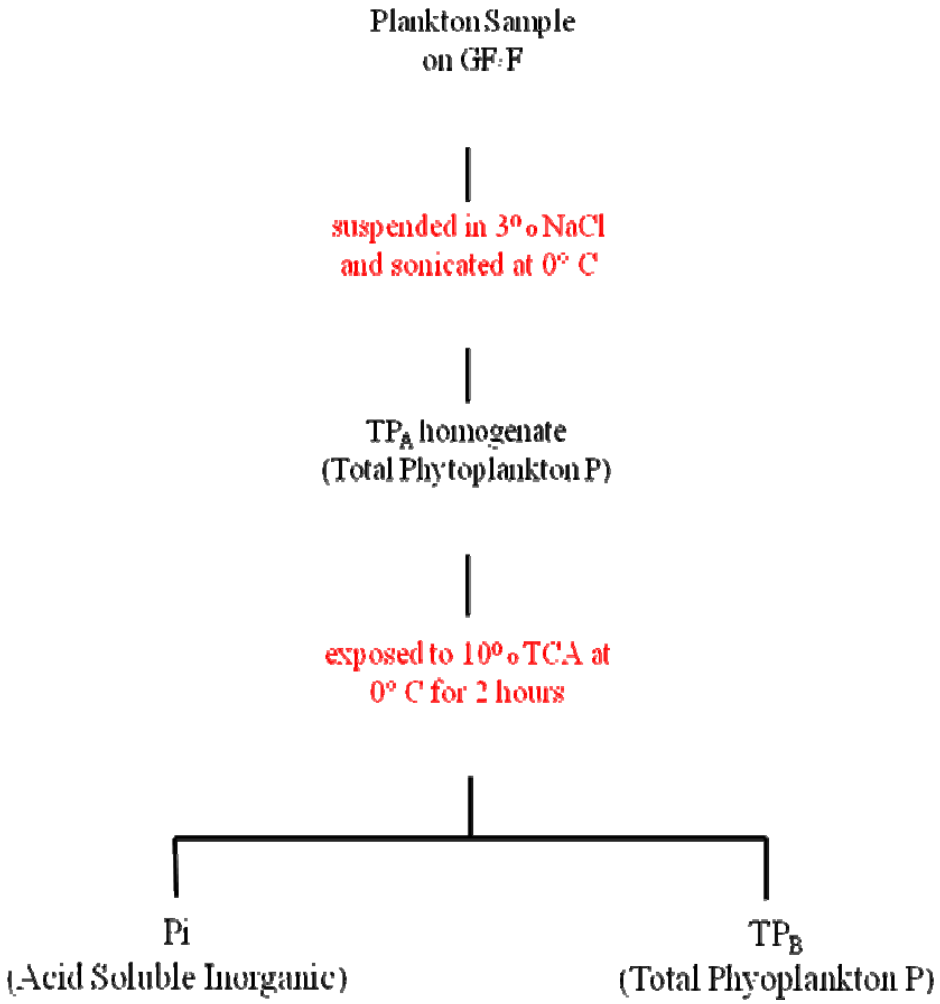

Figure 3-1. A simplified depiction of the phosphorus fractionation methodology.

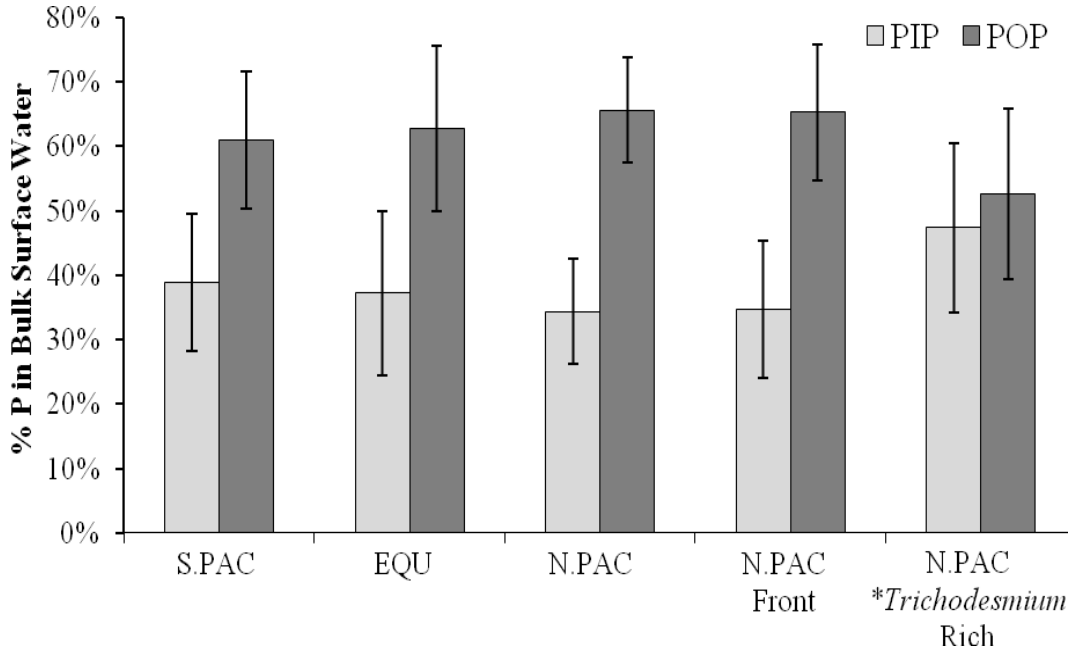

Figure 3-2. Percent of phosphorus in particulate inorganic P (PIP) and particulate organic P (POP) in bulk surface water samples.

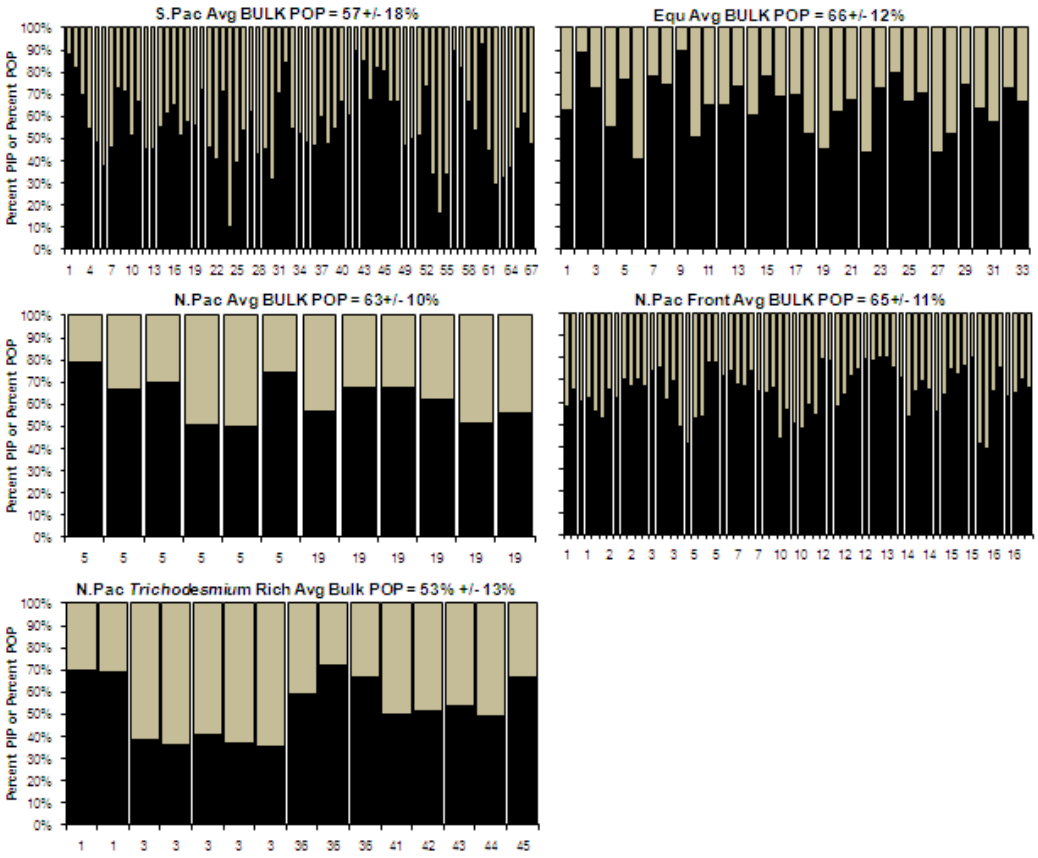

Figure 3-3. A summary of all samples for each cruise, demonstrating a primarily bulk POP composition with the exception of N.Pac *Trichodesmium* Rich stations 3 and 41-45 where *Trichodesmium* biomass was observed in significant concentrations. The x-axis represents the station number and includes multiple depths (ranging from 5 to 50m) while the y-axis represents the percentage of PIP or percentage of POP.

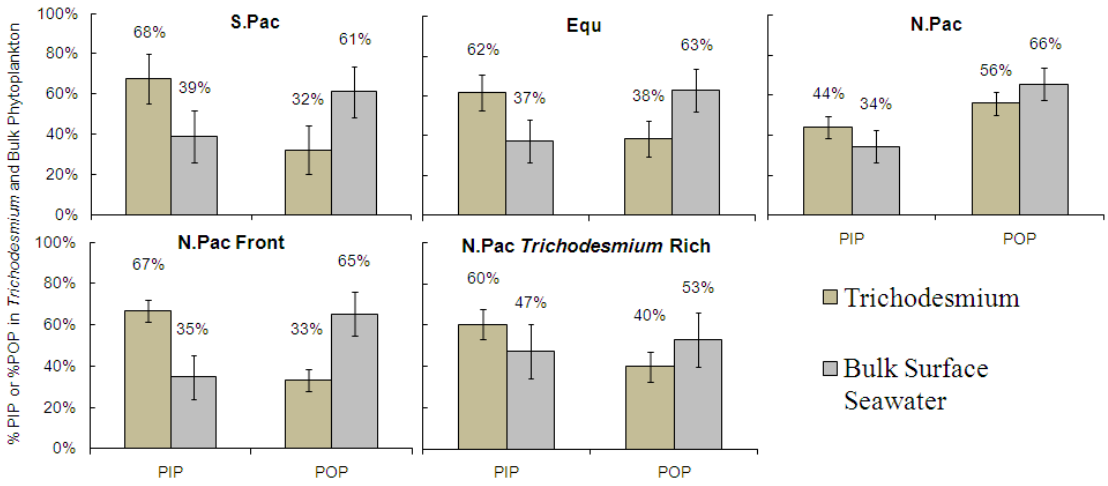

Figure 3-4. A summary of *Trichodesmium* PIP:POP ratios in comparison to the bulk surface seawater PIP:POP ratios.

Table 3-1. Sonication efficiency as a function of time.

treatment	number of samples	homogenate TDP [µM]	remaining volume and filter resuspended in 3% NaCl: TDP [µM]	% recovered
1 min. sonication	3	4.3 +/- 0.52	0.3 +/- 0.51	93%
3 min. sonication	3	5.2 +/- 1.84	2.1 +/- 3.13	71%
5 min. sonication	3	5.5 +/- 1.50	0.3 +/- 0.50	95%

4 Conclusions

The role of phosphorus in regulating productivity in the marine environment is undoubtedly important, especially in the regulation of diazotroph physiology. With the variability reported in estimations and measurements of nitrogen fixation rates it is important to not only understand the impact of phosphorus on rate processes but also to gain perspective into the spatial and temporal variability of these rate processes. We must also consider P cycling as significant on a small scale even at the level of intracellular compartmentalization. Organisms, such as *Trichodesmium*, who have the capacity to thrive in low-P environments and exhibit flexible elemental stoichiometry may provide insight into the flow of elements within a system. The two chapters that form the majority of this thesis barely scratch the surface of understanding how phosphorus availability, albeit inorganic or organic, regulate rate processes in the marine environment and how unique adaptations, such as those of *Trichodesmium*, control the flow of elements, specifically P, within a system.

Overall, several conclusions can be drawn from this research. Within chapter two we reported several significant findings. First, we have compiled a spatially extensive record of N_2 and carbon fixation rates measured within the subtropical gyre and subarctic fronts of the NPSG. Also, by adding exogenous dissolved organic phosphorus, specifically the compound MPn, we have shown that select marine organisms not only have the affinity for phosphonate utilization but utilization occurs at the same rate as inorganic P over a single photoperiod. Further, both MPn and DIP additions resulted in the stimulation of carbon fixation in excess of that which diazotrophs can be responsible for ultimately leading the enhancement of net primary productivity on short time-scales.

In chapter three, we presented several years worth of community and isolated *Trichodesmium* phosphorus fractionation data in terms of intracellular P comprised of inorganic or organic pools. The ratios of PIP:POP are relatively constant for the bulk plankton community within the surface waters in both the NPSG and the SPSG further suggesting that the results of isolated *Trichodesmium* colonies represent a unique,

primarily inorganic composition. These results suggest that internal storage of inorganic P in the form of orthophosphate may be a mechanism of coping in low-P environments. In sum, the above research has provided insight into the role of phosphorus availability, both inorganic and organic, on controlling rate processes and the unique intracellular P composition of the marine diazotroph, *Trichodesmium*.

4.1 Future Directions

Much effort has gone into understanding phosphorus physiology and the role phosphorus plays in regulating rate processes, however much is still unknown. Further investigations into the role of phosphorus in regulating production should be encouraged. The composition and bioavailability of the DOP pool is of great interest as we enter a period of new advances in methods and technology. While we have seen that natural populations of *Trichodesmium* are primarily composed of intracellular inorganic P it is crucial that we perform laboratory experiments designed to identify when inorganic phosphorus storage occurs under varying experimental conditions. The next step in research concerning the intracellular compartmentalization is to run the samples taken during growth experiments in different concentrations of phosphorus (low-P, P-deplete, P-enriched) using a variety of phytoplankton cultures to examine whether or not *Trichodesmium* are unique in their inorganic storage and when inorganic versus organic composition is dominant over a growth cycle.

5 Bibliography

Aspila KI, Agemian H, Chau ASY (1976) A semi-automated method for determination of inorganic, organic and total phosphate in sediments. Analyst 101:187-197

Baldwin D (1998) Reactive "organic" phosphorus revisited. Water Research 32:2265-2270

Bates NR, Hansell DA (2004) Temporal variability of excess nitrate in the subtropical mode water of the North Atlantic Ocean. Marine Chemistry 84:225-241

Benitez-Nelson C (2000) The biogeochemical cycling of phosphorus in marine systems. Earth Science Reviews 51:109-135

Berman-Frank I, Lundgren P, Chen Y-B, Kuepper H, Kolber Z, Bergman B, Falkowski P (2001) Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. Science 294:1534-1537

Björkman KM, Karl DM (2003) Bioavailability of dissolved organic phosphorus in the euphotic zone at Station ALOHA, North Pacific Subtropical Gyre. Limnology and Oceanography 48:1049-1057

Björkman KM, Thomson-Bulldis AL, Karl DM (2000) Phosphorus dynamics in the North Pacific subtropical gyre. Aquatic Microbial Ecology 22:185-198

Boyle E, Bergquist BA, Kayser RA, Mahowald N (2005) Iron, Manganese, and Lead at Hawaii Ocean Timeseries Station ALOHA: Temporal Variability and an Intermediate Water Hydrothermal Plume. Geochimica et Cosmochimica Acta 69:933-95.

Capone DG (2001) Marine nitrogen fixation: What's the fuss? Current Opinions in Microbiology 4:341-348

Carpenter EJ (1983) Physiology and ecology of marine *Oscillatoria* (*Trichodesmium*). Marine Biology Letters 4:69-85

Carpenter EJ, Capone DG, Rueter JG (1992) Marine pelagic cyanobacteria: *Trichodesmium* and other diazotrophs. NATO Ser CV 362 Kluwer Acdemic Publishing, Dordrecht The Netherlands

Cavender-Bares K, Karl D, Chisholm S (2001) Nutrient gradients in the western North Atlantic Ocean: Relationship to microbial community structure and comparison to patterns in the Pacific Ocean. Deep-Sea Research Part I 48:2373-2395

Cembella AD, Antia NJ, Harrison PJ (1982) The utilization of inorganic and organic phosphorous compounds as nutrients by eukaryotic microalgae: a multidisciplinary perspective: Part I. Critical Reviews in Microbiology 10:317-391

Church M, Björkman K, Karl D, Saito M, Zehr J (2008) Regional distributions of nitrogen-fixing bacteria in the Pacific Ocean. Limnology and Oceanography 53:63

Church M, Mahaffey C, Letelier R, Lukas R, Zehr J, Karl D (2009) Physical forcing of nitrogen fixation and diazotroph community structure in the North Pacific subtropical gyre. Global Biogeochemical Cycles 23:3418

Church MJ, Jenkins BD, Karl DM, Zehr JP (2005) Vertical distributions of nitrogen-fixing phylotypes at Stn ALOHA in the oligotrophic North Pacific Ocean. Aquatic Microbial Ecology 38:3-14

Codispoti L (1989) Phosphorus vs. nitrogen limitation of new and export production. John Wiley & Sons, p 377

Davis CS, McGillicuddy DJ (2006) Transatlantic abundance of the N_2-fixing colonial cyanobacterium *Trichodesmium*. Science 312:1517-1520

Dore J, Brum J, Tupas L, Karl DM (2002) Seasonal and interannual variability in sources of nitrogen supporting export in the oligotrophic subtropical North Pacific Ocean. Limnology and Oceanography 47:1595-1607

Dore J, Letelier R, Church M, Lukas R, Karl D (2008) Summer phytoplankton blooms in the oligotrophic North Pacific Subtropical Gyre: historical perspective and recent observations. Progress in Oceanography 76:2-38

Dominic B, Chen Y, Zehr J (1998) Cloning and transcriptional analysis of the nifUHDK genes of Trichodesmium sp. IMS101 reveals stable nifD, nifDK and nifK transcripts. Microbiology 144:3359

Duhamel S, Karl DM (2010) Alkaline phosphatase activity and regulation in the North Pacific Subtropical Gyre. Limnol Oceanogr 55:1414-1425

Dyhrman S, Ammerman J, Van Mooy B (2007) Microbes and the marine phosphorus cycle. Oceanography 20:110-116.

Dyhrman ST, Chappell PD, Haley ST, Moffett JW, Orchard ED, Waterbury JB, Webb EA (2006) Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. Nature 439:68-70

Dyhrman ST, Haley ST (2006) Phosphorus scavenging in the unicellular marine diazotroph *Crocosphaera watsonii*. Applied and Environmental Microbiology 72:1452-1458

Fong A, Karl D, Lukas R, Letelier R, Zehr J, Church M (2008) Nitrogen fixation in an anticyclonic eddy in the oligotrophic North Pacific Ocean. The ISME Journal 2:663-676

Fu F, Zhang Y, Bell P, Hutchins DA (2005) Phosphate uptake and growth kinetics of *Trichodesmium* (cyanobacteria) isolates from the North Atlantic Ocean and the Great Barrier Reef, Australia. Journal of Phycology 41:62-73

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA, Karl DM, Michaels AF, Porter JH, Townsend AR, Vöosmarty CJ (2004) Nitrogen Cycles: Past, Present, and Future. Biogeochemistry 70:153-226

Goebel N, Edwards C, Carter B, Achilles K, Zehr J (2008) Growth and carbon content of three different-sized diazotrophic cyanobacteria observed in the subtropical North Pacific. Journal of Phycology 44:1212-1220

Griffith E, Beeton A, Spencer J, Mitchell D Environmental Phosphorus Handbook, Vol. John Wiley & Sons.

Grabowski M, Church M, Karl D (2008) Nitrogen fixation rates and controls at Stn ALOHA. Aquatic Microbial Ecology 52:175-183

Hutchins DA, Fu F, Zhang Y, Warner ME, Feng Y, Portune K, Bernhardt PW, Mulholland MR (2007) CO_2 control of *Trichodesmium* N_2 fixation, photosynthesis, growth rates, and elemental ratios: Implications for past, present, and future ocean biogeochemistry. Limnology and Oceanography 52:1293-1304.

Karl D, Björkman K, Dore J, Fujieki L, Hebel D, Houlihan T, Letelier R, Tupas L (2001a) Ecological nitrogen-to-phosphorus stoichiometry at station ALOHA. Deep-Sea Research Part II 48:1529-1566

Karl D, Letelier R (2008) Nitrogen fixation-enhanced carbon sequestration in low nitrate, low chlorophyll seascapes. Marine Ecology Progress Series 364:257-268

Karl D, Letelier R, Tupas L, Dore J, Christian J, Hebel D (1997) The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. Nature 388:533-538

Karl DM (2007) The marine phosphorus cycle. In: Hurst CJ, Crawford RL, Garland JL, Lipson DA, Mills AL, Stetzenbach LD (eds) Manual of environmental microbiology. American Society of Microbiology, 523-539.

Karl DM, Michaels A, Bergman B, Capone DG, Carpenter EJ, Letelier RM, Lipschultz F, Paerl H, Sigman D, Stal L (2002) Dinitrogen fixation in the world's oceans. Biogeochemistry 57/58:47-98

Karl DM (2002) Nutrient dynamics in the deep blue sea. Trends in Microbiology 10:410-418

Karl DM, Björkman KM (2002) Dynamics of DOP. In: Hansell DA, Carlson CA (eds) Biogeochemistry of marine dissolved organic matter. Academic Press, London, p 249–366.

Karl DM, Bidigare RR, Letelier RM (2001b) Long-term changes in plankton community structure and productivity in the North Pacific Subtropical Gyre: The domain shift hypothesis. Deep Sea Research II 48:1449-1470

Karl DM, Michaels A, Bergman B, Capone DG, Carpenter EJ, Letelier RM, Lipschultz F, Paerl H, Sigman D, Stal L (2002) Dinitrogen fixation in the world's oceans. Biogeochemistry 57/58:47-98

Karl DM, Tien G (1992) MAGIC: a sensitive and precise method for measuring dissolved phosphorus in aquatic environments. Limnology and Oceanography 37:105-116

Karl DM, Beversdorf L, Björkman KM, Church MJ, Martinez A, Delong EF (2008) Aerobic production of methane in the sea. Nature Geoscience 1:473-478

Kolowith LC, Ingall ED, Benner R (2001) Composition and cycling of marine organic phosphorus. Limnology and Oceanography 46:309-320

Kuenzler E, Ketchum B (1962) Rate of phosphorus uptake by Phaeodactylum tricornutum. The Biological Bulletin 123:134

Legendre L, Gosselin M (1997) Estimation of N or C uptake rates by phytoplankton using ^{15}N or ^{13}C: revisiting the usual computation formulae. Journal of Plankton Research 19:263

Letelier RM, Dore J, Winn CD, Karl DM (1996) Seasonal and interannual variations in autotrophic carbon assimilation at Station ALOHA. Deep Sea Research II 43:467-490

Letelier RM, Karl DM (1996) Role of *Trichodesmium* spp. in the productivity of the subtropical North Pacific Ocean. Marine ecology progress series Oldendorf 133:1-3

Letelier RM, Karl DM (1998) *Trichodesmium* spp. physiology and nutrient fluxes in the North Pacific subtropical gyre. Aquatic Microbial Ecology 15:265-276

Lohan M, Aguilar-Islas A, Franks R, Bruland K (2005) Determination of iron and copper in seawater at pH 1.7 with a new commercially available chelating resin, NTA Superflow. Analytica Chimica Acta 530:121-129

McCarthy JJ, Carpenter EJ (1979) Oscillatoria (*Trichodesmium*) *thiebautii* (Cyanophyta) in the central north Atlantic Ocean. J Phycol 15:75-82

Mills MM, Ridame C, Davey M, La Roche J, Geider RJ (2004) Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. Nature 429:292-294

Miyata K, Hattori A (1986) A simple fractionation method for determination of phosphorus components in phytoplankton: Application to natural populations of phytoplankton in summer surface waters of Tokyo Bay. Journal of Oceanography 42:255-265

Moisander PH, Beinart RA, Hewson I, White AE, Johnson KS, Carlson CA, Montoya JP (2010) Unicellular cyanobacterial distributions broaden the ocean N_2 fixation domain. Science 327:1512-1514

Montoya J, Voss M, Kahler P, Capone D (1996) A Simple, High-Precision, High-Sensitivity Tracer Assay for N_2 Fixation. Applied and Environmental Microbiology 62:986

Montoya JP, Holl CM, Zehr JP, Hansen A, Villareal TA, Capone DG (2004) High rates of N_2 fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. Nature 430:1027-1032

Mulholland MR, Floge S, Carpenter EJ, Capone DG (2002) Phosphorus dynamics in cultures and natural populations of *Trichodesmium* spp. Marine Ecology Progress Series 239:45-55

Murphy J, Riley JP (1962) A modified single solution method for determination of phosphate in natural waters. Analytical Chimica Acta 24:31-36

Orchard ED (2010) Phosphorus physiology of the marine cyanobacterium *Trichodesimum*. Woods Hole Oceanographic Institution

Orchard ED, Benitez-Nelson CR, Pellechia, PJ, Lomas MW, Dyhrman ST (2010) Polyphosphate in *Trichodesmium* from the low phosphorus Sargasso Sea. Limnology and Oceanography 55:2161-2169

Overbeck J (1962) Studies on phosphate metabolism of green algae. III. Behavior of the cell fractions of *Scenedesmus quadricauda* (Turp.) Breb. in the daily cycle

under different lighting conditions and using different phosphate compounds. Archiv en Mikrobiologie 41:11

Perry M, Eppley R (1981) Phosphate uptake by phytoplankton in the central North Pacific Ocean. Deep Sea Research Part A Oceanographic Research Papers 28:39-49

Rees A, Law C, Woodward E (2006) High rates of nitrogen fixation during an in-situ phosphate release experiment in the Eastern Mediterranean Sea. Geophys Res Lett 33:10607

Rigler F (1968) Further observations inconsistent with the hypothesis that the molybdenum blue method measures orthophosphate in lake water. Limnology and Oceanography 13:7-13

Romans KM, Carpenter EJ, Bergman B (1994) Buoyancy regulation in the colonial diazotrophic cyanobacterium *Trichodesmium tenue*: Ultrastructure and storage of carbohydrates, polyphosphate and nitrogen. Journal of Phycology 30:935-942

Sañudo-Wilhelmy SA, Kustka AB, Gobler CJ, Hutchins DA, Yang M, Lwiza K, Burns J, Capone DG, Raven JA, Carpenter EJ (2001) Phosphorus limitation of nitrogen fixation by *Trichodesmium* in the central Atlantic Ocean. Nature 411:66-69

Sohm JA, Capone DG (2006) Phosphorus dynamics of the tropical and subtropical north Atlantic : *Trichodesmium* versus bulk plankton. Marine Ecology Progress Series 317:21-28

Solorzano L, Sharp JH (1980) Determination of total dissolved phosphorus and particulate phosphorus in natural waters. Limnology and Oceanography 25:754-758

Strickland J, Parsons TR (1972) A practical handbook of seawater analysis, Vol. Fisheries Research Board of Canada

Thomson-Bulldis A, Karl D (1998) Application of a novel method for phosphorus determinations in the oligotrophic North Pacific Ocean. Limnology and Oceanography 43:1565-1577

Tyrrell T (1999) The relative influences of nitrogen and phosphorus on oceanic primary production. Nature 400:525-531

Valderrama JC (1981) The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Marine Chemistry 10:109-122

Van Mooy B, Devol A (2008) Assessing nutrient limitation of *Prochlorococcus* in the North Pacific subtropical gyre by using an RNA capture method. Limnology and Oceanography 53:78-88

Venrick E (1993) Phytoplankton seasonality in the central North Pacific: the endless summer reconsidered. Limnology and Oceanography 38:1135-1149

Villareal TA, Carpenter, EJ (2003) Buoyancy regulation and the potential for vertical migration in the oceanic cyanobacterium *Trichodesmium*. Microbial Ecology 45:1-10

Vitousek P, Howarth R (1991) Nitrogen limitation on land and in the sea: how can it occur? Biogeochem 13:87-115

White AE, Karl D, Björkman K, Beversdorf L, Letelier R (2010) Production of organic matter by *Trichodesmium* IMS101 as a function of phosphorus source. Limnology and Oceanography in press

White AE, Spitz YH, Karl DM, Letelier RM (2006) Flexible elemental stoichiometry in *Trichodesmium* spp. and its ecological implications. Limnology and Oceanography 51:1777-1790

White AE, Spitz YH, Letelier RM (2007) What factors are driving summer phytoplankton blooms in the North Pacific Subtropical Gyre. Journal of Geophysical Research 112:12006

Wu J, Sunda W, Boyle E, Karl D (2000) Phosphate depletion in the western North Atlantic Ocean. Science 289:759

Yoshimura T, Nishioka J, Saito H, Takeda S, Tsuda A, Wells M (2007) Distributions of particulate and dissolved organic and inorganic phosphorus in North Pacific surface waters. Marine Chemistry 103:112-121

Zehr JP, Ward BB (2002) Nitrogen Cycling in the Ocean: New Perspectives on Processes and Paradigms. Applied Environmental Microbiology 68:1015-1024

Zehr JP, Waterbury JB, Turner PJ, Montoya JP, Omoregie E, Steward GF, Hansen A, Karl DM (2001) Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. Nature 412:635-616