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Determining the Ecological and Physiological Factors Contributing to the Competitive Success of Prochlorococcus in the Oligotrophic Ocean

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To the Graduate Council:

I am submitting herewith a dissertation written by Benjamin C. Calfee entitled "Determining the Ecological and Physiological Factors Contributing to the Competitive Success of Prochlorococcus in the Oligotrophic Ocean." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Erik R. Zinser, Major Professor

We have read this dissertation and recommend its acceptance:

Erik R. Zinser, Steven W. Wilhelm, Elizabeth M. Fozo, Jen Schweitzer

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Determining the Ecological and Physiological Factors Contributing to the Competitive Success of Prochlorococcus in the Oligotrophic Ocean

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Benjamin Carter Calfee

December 2021

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As this document will later show, interactions are key to success. I am no exception to this rule, and so I would like to acknowledge and thank all those who provided assistance along the way. First, I would like to thank my advisor, Erik Zinser, and committee members Elizabeth Fozo, Steven Wilhelm, Jeffery Morris, and Jen Schweitzer for their unending support prior to, during, and likely after my graduate studies. In addition to those above, faculty and graduate students of the Aquatic Microbial Ecology Research Group within the Department of Microbiology created a truly positive environment to conduct science and largely influenced my decision to continue into graduate studies. Namely among the AMERG I must thank Gary LeCleir, Alison Buchan, and Steven Wilhelm, as well as the captain and crew of the R/V Atlantic Explorer, for making my first oceanographic research experience delightful, despite the myriad equipment failures and experimental setbacks. Outside the Department of Microbiology, I must give special thanks to the late Frank Vogt of the University of Tennessee Department of Chemistry for his generosity with both laboratory resources and equipment at a time in which they were crucial to my further success and progress. While those previously mentioned assisted and guided my research and development, Sherry Roberts of the Biology Business Office and Rachelle Allen of the Microbiology Office staff assisted with innumerable aspects of graduate life and for their constant and willing aid I am thankful. Finally, I would like to give special thanks to Elizabeth McPherson, whose teaching practices and constructive critiques have heavily influenced my abilities as an educator.

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ABSTRACT

Prochlorococcus is a genus of extremely successful marine cyanobacteria. This success is realized through its pervasive biogeographical range and presence in almost all open ocean environments where it usually it the dominant phytoplankton. Limited capabilities of culturing and genetic manipulation of this organism have resulted in assumptions about this success overwhelmingly based on field observations. These studies have assumed adaptations for resource uptake and utilization in nutrient limited environments to cause dominance of *Prochlorococcus* over other photosynthetic microbes. In an attempt to definitively explain this through laboratory culture, we developed a culturing system to assay questions of nutrient limitation effects upon *Prochlorococcus* and its competitive ability based on nitrogen limited populations within the North Pacific Subtropical Gyre. We determined that competition for nitrogen did explain the population dynamics of *Prochlorococcus* and another cyanobacterium, *Synechococcus*, and were able to recreate their observed abundances in nitrogen limited laboratory culture. Interestingly this outcome could only be achieved through the inclusion of a heterotrophic bacterium, which facilitated the success of *Prochlorococcus* through complex crossfeeding interactions. In an effort to further explore the important microbial interactions influencing this dominant cyanobacterium, we developed an additional culturing system to determine if rival phytoplankton (*Synechococcus* and picoeukaryotes), with whom *Prochlorococcus* competes for nutrients, could protect *Prochlorococcus* from oxidative stress from hydrogen peroxide. These rivals successfully protected *Prochlorococcus* from mortality when cocultured at ecologically relevant abundances. Lastly we determined the affect of seasonal change on *Prochlorococcus* ecotypes and potential relationships between high-light and low-light adapted ecotypes and between ecotypes and abundant microbial phyla that varied seasonally. Herein we speculate on the overall importance of microbial interactions based on nutrient transformation and environmental conditioning to the ecology and biogeography of *Prochlorococcus*.

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Prochlorococcus and the Oligotrophic Ocean

Microbial Fitness and Success

Global oceans represent the majority of habitable space on the planet's surface, and within these large gyre systems microbes carryout roughly half of global primary production (Falkowski, 1994). The factors that control abundance, activity, and diversity of phytoplankton are important to research due to their influence of vast biogeochemical cycles. Concentrations of nutrients have long been considered a controlling factor of phytoplankton community structure (Hutchinson, 1957, Hutchinson, 1967, Whipple, 1899), and studies have implicated the importance of nitrogen, phosphorus, and other trace compounds (Dillon and Rigler, 1974, Graziano et al., 1996, O'Brien, 1974, Schindler, 1977, Huntsman and Sunda, 1980). While early studies were limited to correlative analyses of nutrient influences, later deterministic studies were able to demonstrate or even predict an organism's response to limited nutrients (Rothhaupt, 1988, Tilman, 1977, Tilman, 1981). Thus, competitive ability, defined by resource competition theory as the efficient uptake and utilization of nutrients compared to other organisms, and abundance became the gold standard and metric for gauging fitness and success (Huston and DeAngelis, 1994, Tilman, 1976, Tilman, 1990). Since its discovery in 1988, the cyanobacterium *Prochlorococcus* has been recognized as the most abundant photosynthetic organism on the planet (Chisholm et al., 1988, Partensky et al., 1999). Due to its global importance, understanding the causes of this abundance and success are a significant area of research.

In this review chapter we will discuss biotic and abiotic aspects of the oligotrophic ocean that directly influence the ecology and potentially contribute to the competitive success of *Prochlorococcus*. We will first discuss influences of physical aspects of the euphotic, or light exposed, portion of surface oceans, namely temperature, light availability, nutrient concentrations and reactive oxygen species, and consequences of these upon *Prochlorococcus* ecology and distribution. After reviewing abiotic influences, we will discuss biotic factors including interactions, synergy, and competition between *Prochlorococcus* and members of the microbial community. In conclusion, and to transition into this dissertation, we will discuss how these factors can be considered to shape a population of *Prochlorococcus* in an environment crucial to biogeochemical cycles and global processes, the North Pacific Subtropical Gyre, and describe the aims and objectives of this work.

Abiotic Influences on *Prochlorococcus* Niche

Cyanobacteria of the genus *Prochlorococcus* are ubiquitous in the euphotic zone (0-200m) of open ocean environments between 40 °N and 40 °S (Biller et al., 2015, Johnson et al., 2006, Partensky et al., 1999), and often outnumber photosynthetic microbes within the euphotic zone by up to an order of magnitude (Flombaum et al., 2013, Visintini et al., 2021). Physical aspects of the marine environment outside of this described range prevent dominance but also often even survival or detection of this organism. Still, it is significant that a single organism should hold sway and dominate over such an expansive spatial niche. Much research has been devoted to exploring this phenomenon and has determined the genus *Prochlorococcus* is a collection of distinct subgroups. These groups, termed ecotypes, are phylogenetically distinct sub-genera classifications defined by combined comparisons of the inter-transcribed spacer (ITS) region found between 16S and 23S rRNA genes and physiology (Ahlgren et al., 2006, Rocap et al., 2002, Zinser et al., 2006). Environmental observations of ecotype distributions have determined each display optima for physical parameters such as temperature, light availability, and nutrients (Johnson et al., 2006, Malmstrom et al., 2010, Zinser et al., 2007), likely contributing to their biogeographical range and allowing established populations through the euphotic zone.

Temperature and Light

Temperatures within the latitudinal range previously described and the attenuation of sunlight at depths below the euphotic represent major biogeographical constraints on the distribution of *Prochlorococcus* (Johnson et al., 2006, Malmstrom et al., 2010, Zinser et al., 2007). Major divisions in *Prochlorococcus* ecotypes readily differentiate based on their preference for light availability. Those able to withstand exposure to surface sunlight and thrive within the mixed layer make up the High-Light (HL) ecotypes, while those unable to grow under these high light conditions are generally restricted to below the mixed layer and were designated Low-Light (LL) ecotypes (Moore et al., 1998, Urbach et al., 1998, West and Scanlan, 1999). Evolutionary trajectory of these groups has varied to the extent that genomic and physiological aspects of the cell vary between the groups (Rocap et al., 2003). Both genera are predicted to have evolved from a common ancestral origin (Dufresne et al., 2005), LL ecotypes retain a greater degree of similarity than HL ecotypes to the closely related cyanobacterial genus of

Synechococcus (Rocap et al., 2002, Zhaxybayeva et al., 2009). LL ecotypes are typically larger in cell and genome size than HL ecotypes (Biller et al., 2015, Rocap et al., 2003), possess higher concentrations and slightly altered profiles of chlorophyll (Moore and Chisholm, 1999, Moore et al., 1995), and retain cellular capabilities (nutrient utilization and stress response systems) previously lost in the other group (Mann et al., 2002, Moore et al., 2002). Temperature represents less of a controlling factor on LL ecotypes, as water temperatures below the surface mixed layer are routinely colder than those at the surface, but LL ecotypes demonstrate similar temperature preferences optima to HL ecotypes in laboratory culture and nature (Smith et al., 2021, Johnson et al., 2006, Zinser et al., 2007). The ecology of LL ecotypes and how it is shaped by physical aspects of the ocean remains understudied compared to HL ecotypes, usually because of their lower abundance and presence at greater depths. However recent studies have determined that significant LL diversity exists, even within ecotype groups, and that this diversity allows adaptation to yet unknown controlling environmental factors (Thompson et al., 2021) highlighting the need for robust analysis of LL ecotypes as well.

HL ecotypes of *Prochlorococcus* are the opposite of the LL groups in almost every aspect. Cells in these groups are smaller in both cell and genome size, survive and thrive in greater light intensity, and display distributions tightly correlated with temperature (Biller et al., 2015, Johnson et al., 2006, Zinser et al., 2007). The latitudinal distribution of two important and abundant HL ecotypes (HLI – eMED4 [e = ecotype, MED4 = type strain] and HLII – eMIT9312) is determined by temperature, with eMIT9312 dominant in equatorial and tropical waters and eMED4 dominant throughout mid latitude and subtropical regions (Johnson et al., 2006, Zinser et al., 2007). While the inflection point of dominance between these two ecotypes varies somewhat with season, work has shown that it always occurs between 19-21 °C (Chandler et al., 2016). And while the effects of temperature on these ecotypes are well documented, recent work has highlighted that, similar to the LL ecotypes, immense diversity exists within each group that likely assists in adaptation and acclimation to changing environmental conditions (Larkin et al., 2016, Smith et al., 2021, Yan et al., 2020, Kent et al., 2016).

Other phytoplankton groups (*Synechococcus* and picoeukaryotic phytoplankton) display growth trends tightly linked with water temperature (Chen et al., 2014), and *Synechococcus* ecotypes also differentiate into major oceanic dominance regimes based on temperature trends

(Sohm et al., 2016). Heterotrophic bacteria within microbial communities show influence by temperature as well, but are influenced most in their metabolic capabilities (Hall et al., 2008). Therefore, while temperature and light exert vast influence over *Prochlorococcus* diversity and distribution, surrounding microbial communities are influenced to a similar extent, which will affect interactions to be discussed below.

Resource Competition and Nutrient Limitation

Open ocean environments where *Prochlorococcus* dominate are extremely nutrient limited, or oligotrophic. Largely lacking input of terrestrially derived resources, these regions are dependent on nutrient regeneration through the microbial loop (Bratbak et al., 1994, Caron, 1994), particle deposition (Christodoulaki et al., 2013, Jickells et al., 2005, Krom et al., 2010), and rainfall events (Willey et al., 2004) to supply the macro and trace nutrients necessary for growth. Microorganisms living in oligotrophic oceans experience limitation or colimitation typically for nitrogen, phosphorus, or iron, however the nature of this limitation is variable by ocean basin and latitude (Browning et al., 2017, Cunningham and John, 2017, Mann and Chisholm, 2000, Moore et al., 2013, Ustick et al., 2021). Because of the nutrient variability in oceanic regions, evolutionary strategies have developed in response to nutrient stress. In regions of limited phosphorus organisms may possess high affinity uptake mechanisms (Bracken et al., 2015, Moore et al., 2008, Moore et al., 2005) or substitute sulfolipids to reduce the amount of phosphorus invested in cell membrane biosynthesis (Van Mooy et al., 2009). Evolutionary responses to nitrogen or iron limitation include high affinity uptake systems or more efficient use within the cell (Bragg, 2011, Gilbert and Fagan, 2011, Rusch et al., 2010, Thompson et al., 2011, Tolonen et al., 2006); additional physiological adaptations to nitrogen limitation will be discussed below.

Aside from adaptations to acquire limiting nutrients, organisms dominant in oligotrophic environments have reduced cell quotas for those limiting nutrients. They are typically small in size with a genomic content that could be considered minimal compared to surrounding microbes (García-Fernández et al., 2004, Giovannoni et al., 2005, Sabath et al., 2013, Swan et al., 2013). Modern streamline theory describes the evolutionary process in free-living microbes that selects for a reduced cell size and complexity to better accommodate growth under limitation (Giovannoni et al., 2014). The cyanobacterium *Prochlorococcus* or marine heterotroph

Pelagibacter represent the auto- and heterotrophic models for this process, respectively (Biller et al., 2015, Giovannoni et al., 2005, Partensky and Garczarek, 2010). Reductions in cell size lower replication costs and allow higher surface to volume ratios, which reduce internal nutrient quotas and increase uptake affinity (Button, 1991, Moya et al., 2009, Sowell et al., 2009). These adaptations allow for steady state enzyme kinetics and maximal growth in oligotrophic environments, but also have the added benefit of reduced self-shading for photosynthetic microbes (Dufresne et al., 2005). Reductions in genome size are usually due to accrued mutations in and eventual loss of genes selected against by oligotrophic environments. The benefit of this process depends on what, as opposed to how much genetic content, is lost. Stable environments like the oligotrophic ocean change little over time and do not commonly experience large inputs of nutrients, so adapted organisms lose mechanisms to sense environmental conditions, respond quickly to nutrient fluctuations, or mediate external stressors (Giovannoni et al., 2014, Morris et al., 2012, Swan et al., 2013).

These aspects of streamline theory have been evidenced in *Prochlorococcus*, specifically by observations of small cell size (Ting et al., 2007), reduced genome lacking robust regulatory mechanisms and stress responses (Dufresne et al., 2005, Kettler et al., 2007), and efficient utilization of nutrients (Bragg, 2011, Gilbert and Fagan, 2011, Van Mooy et al., 2009). *In situ Prochlorococcus* growth rates have been observed near maximum (Kirchman, 2016, Liu et al., 1997) and at rates greater than rival cyanobacteria *Synechococcus*, but only in oligotrophic waters (Liu et al., 1995, Liu et al., 1998, Moore et al., 1995, Worden and Binder, 2003). However aside from environmental observations of growth rate and recognition of adaptations for success in the oligotroph, laboratory-based evidence of competitive success is lacking and an understanding of how these adaptations influence the abundance or growth of *Prochlorococcus* remains incomplete.

Hydrogen Peroxide Dynamics

Organisms within sunlit, aquatic environments are often exposed to reactive oxygen species (ROS) produced biotically as metabolic by-products of respiratory and photosynthetic processes as well as for communication purposes (Bond et al., 2020, Collén et al., 1995, Diaz et al., 2013, Diaz et al., 2018, Gonzalez-Flecha and Demple, 1995, Palenik et al., 1987, Hansel et al., 2016). These ROS compounds and molecules are also abiotically produced through

photooxidation of organic substances (Cooper and Zika, 1983, Draper and Crosby, 1983, Gerringa et al., 2004, Mopper et al., 2015, Zhang et al., 2012) and interactions between various oxygenic molecules and metals called the Fenton reaction (Enami et al., 2014, Kornweitz et al., 2015, Lloyd et al., 1997, Southworth and Voelker, 2003). Finally, rainfall events serve as a major source of ROS in the form of hydrogen peroxide (H_2O_2) and ROS-reactive iron (Cooper et al., 1987, Hanson et al., 2001, Willey et al., 2004, Yuan and Shiller, 2000, Zika et al., 1982, Zika et al., 1985). The consequences of this process are significant as concentrations of H_2O_2 in rainwater can be up to two or three orders of magnitude greater than marine surface waters and adds temporal and spatial variation to H_2O_2 dynamics. H_2O_2 makes up a significant proportion of total ROS in aquatic environments and is consistently detected across oceanic basins (Yuan and Shiller, 2001, Yuan and Shiller, 2005, Zika et al., 1985). Biotic production of H_2O_2 varies drastically, as organisms within trophic levels serve as sinks or sources due to differences in metabolism and physiology (Bond et al., 2020, Collén et al., 1995, Palenik et al., 1987, Schneider et al., 2016). Total daily abiotic production at the surface layer of the oligotrophic ocean has been estimated at approximately 800 nM, but the activity of microbes' H_2O_2-degrading enzymes, catalase and peroxidase, maintain concentrations between 100-200 nM (Morris et al., 2016). Exposure to high concentrations of H_2O_2, such as from rainfall, is lethal to organisms lacking these enzymes but even those that possess them can experience cellular damage (Farmer and Mueller, 2013, Imlay, 2003, Imlay, 2013). All *Prochlorococcus* isolates lack homologs of known catalase genes (Morris et al., 2008, Scanlan et al., 2009), and are extremely sensitive to oxidative stress by H_2O_2. A study by Morris et al. (2011) found that *Prochlorococcus* growth in monoculture from a low starting inoculum (1,000 cells mL^{-1}) could be significantly reduced or completely hindered by addition of 100 nM or 200 nM H_2O_2, respectively (Morris et al., 2011). Such a sensitivity should preclude an organism from growth in an environment where 800 nM H_2O_2 is produced daily, and vastly greater concentrations are introduced with every rainfall, yet *Prochlorococcus* dominates in almost all open ocean environment from subtropic to subtropic. The interactions that allow this success with be discussed below.

Microbial Interactions

As described above, the physical aspects of an environment can influence the ecology and shape the distribution of most organisms. While it remains insightful to examine the response or adaptation of a particular organism, seldom do any appear in nature isolated from contact or influence by a community of coexisting organisms. Within these communities, microbes participate in a multitude of net positive or negative and neutral interactions that span a diverse array of activities. Net negative interactions involve environmental conditioning through production of secondary metabolites (toxin production) and other compounds (acidic metabolic by-products) or through predator-prey interactions (phage infection or grazing) (Chevallereau et al., 2021, Paz-Yepes et al., 2013). Net positive and neutral interactions also involve environmental conditioning, but through the degradation of toxic compounds (ROS), and nutrient transformation and metabolic crossfeeding (D'Souza et al., 2018, Morris et al., 2011, Roth-Rosenberg et al., 2020). Many studies have shown that the ecological success of an organism is not due solely to mediation of physical and chemical environmental changes, but to complex community level interactions that ultimately influence its fate. Here we will review biological interactions involving *Prochlorococcus* that mediate or exacerbate the influence of abiotic factors previously discussed.

Temperature and Light Interactions

As described earlier, temperature exerts tight control over *Prochlorococcus* populations and especially the distribution of HL ecotypes sequestered within the surface mixed layer. Exposure to multiple environmental stressors can sometimes provide a synergistic or compounding effect that increases or decreases the adaptive ability of microorganisms in response to that stress. In plants, exposure to high light conditions increases adaptive ability to high temperature stress by facilitating the activity and repair of photosystems (Allakhverdiev et al., 2008). However in photosynthetic microbes exposure to high heat and sunlight usually has a compounding detrimental effect (Wohlers-Zollner et al., 2011), while exposure to sunlight acts as a prerequisite to successful mediation of oxidative stress (Blot et al., 2011).

Just as exposure to one environmental stressor can mediate or exacerbate the stress of another, interactions between certain microbes can have a similar effect. Of importance to this dissertation are positive microbial interactions that bolster against environmental stress, within

eukaryotic plant studies these interactions are termed ‘facilitation’ (Callaway et al., 2002, Callaway and Walker, 1997). Previous work has highlighted the ability of cocultured bacteria to mediate thermal stress in a eukaryotic phytoplankton by supplying necessary cofactors (Xie et al., 2013). Coculture studies involving *Prochlorococcus* have determined the presence of the marine heterotrophic bacteria *Alteromonas macleodii*, an often cooccurring microbe in natural environments, effectively mediates temperature stress at the upper and lower extents of the permissible range (Ma et al., 2018). This interaction was due to increased growth rate at temperature extremes by reducing damage from both temperature and ROS. Additionally, coculture with this heterotroph facilitated the survival of *Prochlorococcus* exposed to light after periods of extended darkness (Coe et al., 2016). These examples of microbial facilitation highlight the importance of community interactions for the survival and success of *Prochlorococcus*.

Resource Interactions

Just as microbial interactions mediate environmental stress from temperature and light, so too are they influential in survival of nutrient stress. Inter-trophic coculture of cyanobacteria and heterotrophic bacteria have produced stable, long-term communities that facilitate nutrient cycling and regeneration in a laboratory setting (Christie-Oleza et al., 2017). Additionally, recovery of nutrient starved *Prochlorococcus* cultures was facilitated through coculturing with heterotrophic bacteria, without which recovery never occurred (Roth-Rosenberg et al., 2020). Further evidence of potential metabolic cross-feeding comes from the supplement of trace metals and cofactors to eukaryotic phytoplankton from cooccurring heterotrophic bacteria (Cooper et al., 2019, Xie et al., 2013). Even when cocultured in nutrient replete conditions, heterotrophic bacteria have a significant impact on gene expression in cyanobacteria, including *Prochlorococcus* (Beliaev et al., 2014, Christie-Oleza et al., 2015, Kaur et al., 2018, Zheng et al., 2020, Biller et al., 2016, Sher et al., 2011). Given the widespread and ubiquitous nature of metabolic interactions or cross-feeding in survival in oligotrophic environments (D'Souza et al., 2018), it is possible that by influencing survival these interactions are influencing the competitive abilities and success of numerically dominant organisms like *Prochlorococcus*. However, studies attempting to observe the influence of cross-feeding on competitive outcomes are lacking and represent an interesting area of research.

Hydrogen Peroxide Interactions

As stated previously, *Prochlorococcus* cells are extremely sensitive to oxidative stress, mainly by H_2O_2, due to loss of the *katG* catalase-peroxidase gene. Isolation and study of this organism were expedited when this deficiency was realized (Morris et al., 2008), and again the importance of coculture interactions with heterotrophic bacteria was evident. Specifically heterotrophs capable of degrading H_2O_2 conditioned the surrounding environment to allow survival of *Prochlorococcus* and reduce oxidative damage, allowing robust growth of this organisms in laboratory culture (Morris et al., 2011). This evolutionary loss was termed the Black Queen Hypothesis, which defines the beneficial loss of a function which other members of microbial communities can fulfill due to its "leaky" nature (Morris et al., 2012). Again, more evidence was added supporting the crucial function of microbial interactions for survival in the oligotrophic ocean, however coculture interactions involving the beneficial degradation of H_2O_2 have been restricted to *Prochlorococcus* and heterotrophic organisms, and while environmental data suggests a significant role (Morris et al., 2016), the contribution of photosynthetic microbes to the protection of *Prochlorococcus* remains unknown.

Prochlorococcus in the North Pacific Subtropical Gyre

The North Pacific Subtropical Gyre (NPSG) is the largest of the oceanic gyres which are estimated to contribute roughly half of global primary production (Falkowski, 1994). Due to its size, this gyre represents the largest contiguous biome on the planet and is crucial to global biogeochemical cycling (Karl, 1999). *Prochlorococcus* is the dominant photosynthetic microorganism throughout this region, and its spatial abundance dynamics and diversity have been well studied (Campbell et al., 1997, Campbell et al., 1994, Chandler et al., 2016, Kashtan et al., 2017, Larkin et al., 2016, Malmstrom et al., 2010, Martiny et al., 2009). While its biogeography across the gyre has been the subject of numerous studies, these have lacked seasonal analysis of both HL and LL ecotypes throughout the entire euphotic zone from samples covering large portions of the gyre. A study by Van Mooy and Devol (2008) determined total abundance and activity of *Prochlorococcus* in this region is controlled by nitrogen availability by measuring how specific nutrient amendments affected RNA transcription (Van Mooy and Devol, 2008). Under conditions of extreme nitrogen limitation, *Prochlorococcus* remains numerically dominant over rival phytoplankton such as the cyanobacterium *Synechococcus* or picoeukaryotic

phytoplankton *Micromonas* and *Ostreococcus*, presumably through competition for nitrogen (Flombaum et al., 2013, Visintini et al., 2021). However, the nature of numerical dominance and competitive success of *Prochlorococcus* over photosynthetic members of the microbial community has not been assessed in a laboratory setting. And finally, as seen in other field observations and laboratory studies, *Prochlorococcus* populations of the NPSG are dependent on surrounding microbes for the detoxification of H_2O_2. Heterotrophic bacteria from this region and others have been shown to facilitate the growth of *Prochlorococcus* in laboratory culture by performing in this capacity (Morris et al., 2011, Morris et al., 2008), however both hetero- and autotrophic prokaryotes and eukaryotes from the NPSG have been shown to express genes for H_2O_2 degradation (Morris et al., 2016). The specific contributions from either heterotrophic bacteria or phytoplankton to H_2O_2 degradation, and the ultimate effect of this upon *Prochlorococcus* abundance, ecology, and competitive success, remains unknown. In order to address this knowledge gap, I determined to utilize the NPSG as a model system to address the following questions: what ecological and physiological factors most influence the competitive success of *Prochlorococcus*, competition for nitrogen, microbial interactions, or both; do prokaryotic and eukaryotic phytoplankton directly contribute to the survival of *Prochlorococcus* by degrading H_2O_2, and what impact does this have on their overall ecology and coexistence; and finally what biotic and abiotic forces and microbial interactions shape HL and LL ecotype distribution and abundance in the euphotic zone on a seasonal scale throughout the NPSG. These questions will be addressed in the following chapters and general findings are summarized below.

Chapter Summaries

In Chapter 2 I describe experiments detailing the nature of competition of *Prochlorococcus* and *Synechococcus* for limited nitrogen. I determined that competition for this resource did decide the difference in observed environmental abundances of these organisms, but to recreate the dominance of *Prochlorococcus* over *Synechococcus* in nitrogen limited laboratory culture required the inclusion of a heterotrophic bacterium. Without the addition of a heterotrophic bacteria both cyanobacteria coexisted equally in extended coculture growth. The decline of *Synechococcus* in tripartite (three member) culture was determined to be a result of

competition for nitrate with the heterotroph, which would only significantly drawdown this resource when fueled by photosynthetic exudate from *Prochlorococcus*. Finally, the effect of *Prochlorococcus*, *Synechococcus*, and heterotroph strain variability on tripartite outcomes and the ultimate influence these interactions have upon their environmental abundance and success are discussed.

In Chapter 3 I describe experiments detailing the contribution of photosynthetic microorganisms to protection of *Prochlorococcus* from oxidative stress. Specifically, we show that coculture of *Prochlorococcus* with *Synechococcus*, *Micromonas*, or *Ostreococcus* at ecologically relevant abundances was sufficient to reduce H_2O_2-mediated mortality. This protection was consistent over a variety of concentrations and exposure regimes that simulated a number of environmental conditions and occurrences. Finally, through comparisons with cocultures with heterotrophic bacteria, estimations of both heterotrophic and autotrophic microorganisms' contribution to H_2O_2 degradation by the entire microbial community were made and the ecological and competitive consequences of this activity are discussed.

In Chapter 4 I describe environmental observations that highlight the different biotic and abiotic forces acting upon HL and LL ecotype abundance and diversity seasonally across the NPSG. Differences in ecotype abundance between winter and summer were best described by changes in the magnitude of vertical mixing (mixed layer depth) and the abundance of rival phytoplankton, *Synechococcus* and picoeukaryotes. Increase in stratification during the summer shifted dominance of *Prochlorococcus* community to eMED4 even though water temperatures were greater than in winter, a result that could have implications for the dominant *Prochlorococcus* ecotype in future oceans predicted to be warmer and more stratified. The potential for HL-LL ecotype and ecotype-phyla interactions was determined through comparison of ecotype and prokaryote OTU (operational taxonomic unit) relative abundance, connections that have not been previously analyzed.

In Chapter 5 I summarize the major results and conclusions of this dissertation and address the overall ability of these findings to fill the knowledge gap and answer questions posed in this introductory chapter. Finally, I will detail future studies to continue this work and better describe the ecology of *Prochlorococcus*.

CHAPTER 2

Prochlorococcus Exudate Stimulates Heterotrophic Bacterial Competition with Rival Phytoplankton for Available Nitrogen

Publication Note

This chapter has been submitted to and is currently under review the journal mBio. Authors are listed below.

Contributions

Experiments were designed by Benjamin Calfee and Erik Zinser; all experiments were performed by Benjamin Calfee; *Alteromonas* mutant was characterized by Liz Glasgo; and Benjamin Calfee and Erik Zinser crafted manuscript.

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Abstract

The marine cyanobacterium *Prochlorococcus* numerically dominates the phytoplankton community of the nutrient-limited open ocean, establishing itself as the most abundant photosynthetic organism on Earth. This ecological success has been attributed to lower cell quotas for limiting nutrients, superior resource acquisition, and other advantages associated with cell size reduction and genome streamlining. In this study we tested the prediction that *Prochlorococcus* outcompetes its rivals for scarce nutrients, and that this advantage leads to its numerical success in nutrient-limited waters. Strains of *Prochlorococcus* and its sister genus *Synechococcus* grew well in both mono- and co-culture when nutrients were replete. However, in nitrogen-limited medium *Prochlorococcus* outgrew *Synechococcus*, but only when heterotrophic bacteria were also present. In the nitrogen-limited medium, the heterotroph *Alteromonas macleodii* outcompeted *Synechococcus* for nitrogen, but only if stimulated by exudate released by *Prochlorococcus*, or if a proxy organic carbon source was provided. Analysis of a nitrate reductase mutant *Alteromonas* suggested that *Alteromonas* outcompetes *Synechococcus* for nitrate, during which co-cultured *Prochlorococcus* grows on ammonia or other available nitrogen species. We propose that *Prochlorococcus* can stimulate antagonism between heterotrophic bacteria and potential phytoplankton competitors through a metabolic cross-feeding interaction, and this stimulation could contribute to the numerical success of *Prochlorococcus* in the nutrient-limited regions of the ocean.

Introduction

The phytoplankton community occupying the vast majority of the sunlit ocean experiences chronic nutrient limitation (DiTullio et al., 1993, Graziano et al., 1996, Saito et al., 2014, Moore et al., 2013) . Depending on the location, the limiting nutrient(s) include nitrogen, phosphorus, iron, and other metals. While the diversity of phytoplankton in these regions can be quite high, numerical superiority is often achieved by a single genus of cyanobacteria, *Prochlorococcus*. The most abundant photosynthetic organism in the ocean, *Prochlorococcus* can grow to populations that exceed 100,000 cells ml^{-1}, besting their competitors by orders of magnitude in many instances (Biller et al., 2015, Flombaum et al., 2013, Visintini et al., 2021, Campbell et al., 1997).

The reasons underpinning the numerical dominance of *Prochlorococcus* in nutrient-limited waters have not been fully elucidated, but several distinguishing features of this unusual cyanobacterium have been implicated. *Prochlorococcus* has the smallest cell and genome size for a photoautotroph, which collectively lowers the cell quota for nitrogen, iron, and phosphorus (Dufresne et al., 2005, Garcia-Fernandez and Diez, 2004, Gilbert and Fagan, 2011, Morris et al., 2011). Phosphorus quota is further reduced by the replacement of phospholipids with sulfolipids as the predominant membrane lipids (Van Mooy et al., 2009, Van Mooy et al., 2006). Additional means of economy (Grzymski and Dussaq, 2012, Bragg, 2011, Garcia-Fernandez and Diez, 2004, Moore et al., 1995) may further contribute to the ability of *Prochlorococcus* to reproduce at lower cost relative to its competitors under nutrient-limited conditions.

Reduction in cell size is thought to provide *Prochlorococcus* with the additional advantage of superior nutrient acquisition (Ting et al., 2007). Lomas et al. noted that when normalized to cell quota, *Prochlorococcus* had a higher affinity to phosphorus relative to *Synechococcus* and picoeukaryotic phytoplankton (Lomas et al., 2014). Notably, resource competition theory applied to global ocean simulations predicted the numerical domination of the oligotrophic regions by analogs of *Prochlorococcus*, which could draw nutrients down to concentrations that cannot be accessed by their competitors (Barton et al., 2010, Dutkiewicz et al., 2009, Follows et al., 2007).

Despite the net loss of genes through streamlining, the genetic diversity within *Prochlorococcus* is high and believed to contribute to the numerical dominance of

Prochlorococcus by facilitating niche expansion. Phylogenetically distinct clades, termed ecotypes, exist within the genus and have demonstrated different optima for temperature, light intensities, and nutrient utilization that correlate with their environmental distributions (Johnson et al., 2006, Martiny et al., 2009, Zinser et al., 2007, Moore and Chisholm, 1999, Moore et al., 2005, Moore et al., 1998, Berube et al., 2016, Zwirglmaier et al., 2008, West and Scanlan, 1999). Notably, within these ecotypes, sub-ecotypes have been found with their own distinct ecologies, suggesting that the open ocean niche is finely partitioned through environmental influences on *Prochlorococcus* evolution (Kashtan et al., 2017, Kashtan et al., 2014, Larkin et al., 2016).

A final contributor to the ecological success of *Prochlorococcus* may be the help it receives from the microbial community. All known genomes of *Prochlorococcus* lack the gene encoding the hydrogen peroxide scavenger, catalase (Morris et al., 2008, Morris et al., 2012, Scanlan et al., 2009). Loss of catalase is believed to improve growth efficiency by reducing cell quotas for iron and/or nitrogen, but it leaves cells highly susceptible to oxidative damage from environmental sources of hydrogen peroxide (Morris et al., 2011, Morris et al., 2012, Ma et al., 2018). *Prochlorococcus* survives this threat because it is cross-protected by co-occurring catalase-positive "helpers" such as *Alteromonas macleodii*, a heterotroph frequently co-isolated with *Prochlorococcus* (Kearney et al., 2021, Morris et al., 2011, Morris et al., 2008). *Alteromonas macleodii* rapidly scavenges extracellular H_2O_2, causing changes in gene expression and promoting the growth of co-cultured *Prochlorococcus* in conditions that would otherwise be lethal (Biller et al., 2016, Morris et al., 2008, Sher et al., 2011, Hennon et al., 2018).

The physiological and genetic evidence all predict the success of *Prochlorococcus* over its competitors in the nutrient-limited ocean. In this work we sought direct evidence that *Prochlorococcus* could achieve numerical success over a key rival, *Synechococcus*. We focused our study on nitrogen-limiting conditions simulating the North Pacific Subtropical Gyre (NPSG) (Van Mooy and Devol, 2008) where *Prochlorococcus* outnumbers *Synechococcus* and other rival phytoplankton by an order or magnitude or more (Campbell et al., 1994, Campbell et al., 1997, Flombaum et al., 2013). We found that competition for nitrogen explained the differences in *Prochlorococcus* and *Synechococcus* abundance, but only through the presence and specific activity of marine heterotrophic bacteria fed by *Prochlorococcus*-derived carbon. As these outcomes matched prior predictions of *Prochlorococcus* success, we argue that conditions such

as the ones examined could provide important insight into the global ecology of *Prochlorococcus*.

Materials and Methods

Strains and Culturing

Axenic cultures of *Prochlorococcus* strains MIT9215, MIT9312, and MED4, and *Synechococcus* strains WH7803, CC9605, and WH8102 were used in this study. Stock cultures of cyanobacteria were initially maintained in an artificial seawater medium, AMP-A (Jeffrey Morris and Zinser, 2013, Moore et al., 2007, Morris et al., 2011), and were inoculated and serially maintained (for up to two years) in AMP-MN (this study, described below) to prevent introduction of excess nitrogen (N). Axenicity of cyanobacterial stocks and experimental cultures was tested routinely by diluting a small volume of culture into 1/10X ProAC and YTSS media and incubating these cultures in the dark at room temperature for up to six weeks to monitor for any increase in turbidity indicating presence of heterotrophic bacteria (Morris et al., 2008). All experiments were carried out at 24 °C in Percival I36VLX incubators (Percival, Boone, IA) with modified controllers that allowed for gradual increase and decrease of cool white light to simulate sunrise and sunset with peak midday light intensity of 150 mmol quanta $m^{-2}s^{-1}$ on a 14 hr:10 hr light:dark cycle (Zinser et al., 2009). Ammonium (NH_4^+) was the N amendment in all experiments, unless otherwise stated, as it can be used by all strains in this study. Experiments that included different NH_4^+ concentrations were performed with NH_4^+ amendments to the AMP-A derivative, AMP-MN (Minus Nitrogen), which is identical to AMP-A except that no N source is included. Stepwise amendments of NH_4^+ to AMP-MN indicated that the residual N bioavailable to *Prochlorococcus* and *Synechococcus* was approximately 0.4 mM (Figure S1).

Axenic heterotrophic bacteria utilized were *Alteromonas macleodii* strain EZ55 (Morris et al., 2008), *Vibrio fischeri* strain ES114 (Soto et al., 2009), and *Phaeobacter* sp. strain Y3F (Buchan et al., 2000). Overnight cultures of heterotrophs were inoculated from cryo-preserved stocks prior to each experiment (-80°C in YTSS + 10% glycerol) into 5 mL volumes of YTSS (Sobecky et al., 1996) incubated shaking at 140 RPM at 24°C. Before inoculation into cyanobacterial cultures, heterotrophs were washed three times in 1.5 mL microcentrifuge tubes

by centrifugation at 8,000 RPM for two minutes in a tabletop microcentrifuge and resuspension in 1mL AMP-MN.

While all culture media was sterilized by autoclaving, sterilized spent or *Prochlorococcus*-conditioned media was generated by culturing *Prochlorococcus* strain MIT9215 in large volumes of AMP-MN (~300 mL). At stationary phase these cells were removed by gentle filtration (-7 inHg) in a 1 L filter tower (Nalgene) using 0.2 µm pore size GTTP Isopore Membrane Filters (MilliporeSigma, Burlington, MA). Prior studies indicated low pressure filtration does not cause detectable rupture of *Prochlorococcus* cells during filtration (Morris et al., 2011). Sterility of this conditioned media was determined by flow cytometry alongside the experiments it was utilized in, in addition to the purity assay detailed above.

Quantification of Cyanobacteria and Heterotroph Abundances

Abundances of cyanobacteria were quantified by flow cytometry using a Guava EasyCyte 8HT flow cytometer (Millipore, Burlington, MA) with populations of *Prochlorococcus* and *Synechococcus* differentiated in co-cultures by their red and red / yellow fluorescence, respectively (Cavender-Bares et al., 1998, Morris et al., 2008). Heterotrophs in mono and coculture experiments were quantified by viable counting with serial dilutions on YTSS 1.5% agar plates incubated at 24 °C.

Transposon Mutagenesis

Mutants of *Alteromonas macleodii* strain EZ55 incapable of growing on nitrate (NO_3^-) as a sole N source were generated by transposon mutagenesis using a mini-Himar1 *Mariner* transposon carrying a kanamycin resistance selectable marker (Bouhenni et al., 2005). The RB1 plasmid vector containing the transposon was propagated in *Escherichia coli* strain WM3064, a pir^+ and 2,6-diaminopimelic acid (DAP) auxotroph donor strain (Saltikov and Newman, 2003). Overnight cultures of donor strain were inoculated from cryopreserved stocks (-80°C in LB + 10% glycerol) into 5 mL LB amended with 10 µg/mL kanamycin and 150 µL of 100 mM DAP (Alfa Aesar, Haverhill, MA) and incubated shaking at 37 °C. Conjugations with EZ55 were performed by plating both donor and recipient onto YTSS agar plates for 8 hours. Ex-conjugants were selected on YTSS + 10 µg/mL kanamycin agar plates. Selected colonies were screened for NO_3^- utilization by replica plating (Lederberg and Lederberg, 1952) on AMP-A agar with 1.5%

Noble agar (Difco) amended with 500 µM sodium pyruvate (Sigma-Aldrich) and either 400 µM NH_4^+ or 882 µM NO_3^- as the nitrogen source. Replica plated colonies growing solely on plates containing NH_4^+ were transferred again into tubes of AMP-A with excess carbon and either nitrogen source to confirm the mutants were unable to grow on nitrate. Insertion location of the Mariner transposon within the *nirB* gene was verified by Arbitrary PCR (Saavedra et al., 2017), Sanger sequencing, and BLAST comparisons with the EZ55 genome (accession number 2785510739).

Results

Prochlorococcus outcompetes Synechococcus in the presence of heterotrophs

Cyanobacterial growth in mono- and co-cultures was assessed in low-nitrogen medium (AMP-MN), an artificial seawater medium lacking N amendment, and containing approximately 0.4 µM residual bioavailable N (Fig. 2.1). *Prochlorococcus* strain MIT9215 reached a higher maximum abundance in monoculture than when in coculture with *Synechococcus* strain WH7803, suggesting that competition in coculture caused a slight but significant reduction in MIT9215 cell yield (Fig. 2.2A) ($p < 0.0001$). Inversely, WH7803 maximum abundance was lower in monoculture than when in coculture with MIT9215, however this difference was not significant (Fig. 2.2A) ($p = 0.2754$).

Addition of the marine heterotrophic bacterium, *Alteromonas macleodii* strain EZ55, dramatically changed the outcome for the *Synechococcus-Prochlorococcus* cocultures (Fig. 2.2B). While MIT9215 growth rate declined moderately, addition of EZ55 to the coculture resulted in a near total loss of growth for WH7803 ($p = 0.0018$). In this AMP-MN medium, the EZ55 heterotroph grew rapidly to ~10^6 cells mL^{-1}, regardless of whether cyanobacteria were present (data shown in Fig. 2.4 and 2.7), indicating growth on trace contaminating organic carbon in the medium. The presence of the heterotroph in this nitrogen-limited medium thus shifted the phytoplankton community structure to one resembling open ocean communities, with *Prochlorococcus* numerically-dominant over rival *Synechococcus*.

The dynamics of resource competition were further investigated by challenging the cyanobacterial strains to invade established populations of their competitors when rare. At day

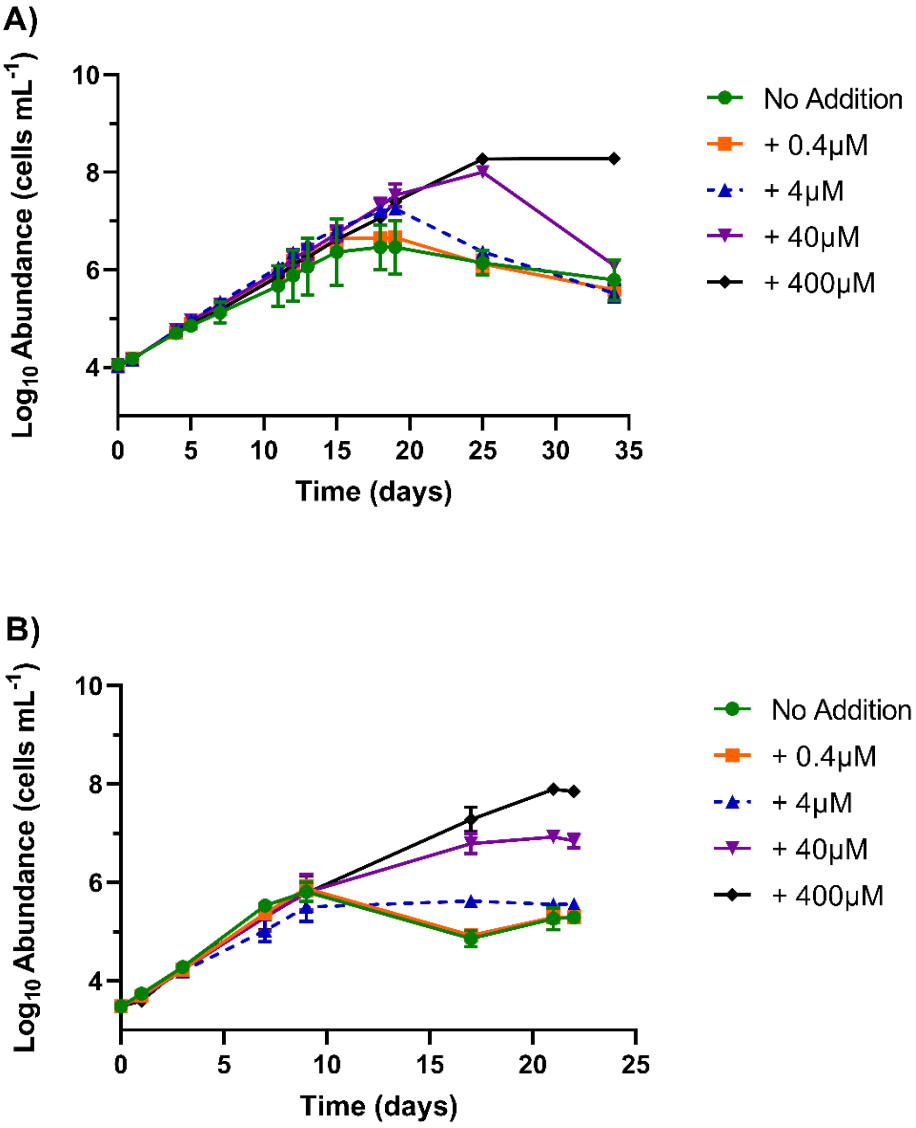


Fig. 2.1. Bioavailable Nitrogen in AMP-MN: Growth of *Prochlorococcus* strain MIT9215 (A) and *Synechococcus* strain WH7803 (B) in AMP-MN artificial seawater medium amended with varying concentrations of ammonium. Error bars represent one standard deviation of the geometric mean (n=3).

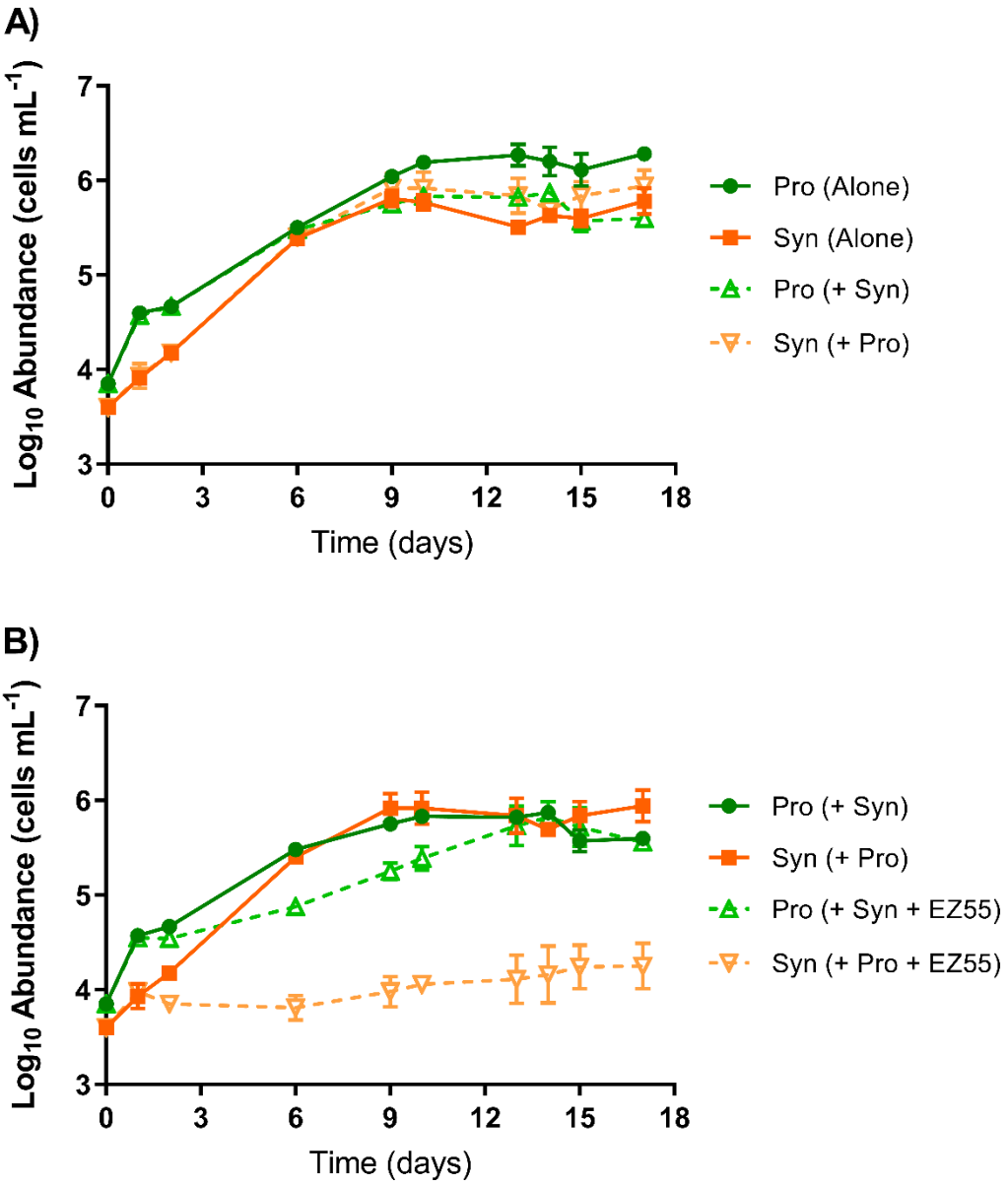


Fig. 2.2. Tripartite Competition in AMP-MN: Growth of *Prochlorococcus* strain MIT9215 and *Synechococcus* strain WH7803 in AMP-MN artificial seawater medium in monoculture (A), cyanobacteria coculture (A and B), and tripartite culture with *Alteromonas macleodii* strain EZ55 (B). Error bars represent one standard deviation of the geometric mean (n=3).

32 of growth in AMP-MN, a small inoculum (~3.00e^3 cell mL^{-1}) from WH7803 monocultures was added to cultures of MIT9215 ± EZ55; reciprocally, MIT9215 monocultures were inoculated into WH7803 ± EZ55 cultures. WH7803 were able to invade MIT9215 monocultures after a few days lag and reach an almost equal abundance over the next 17 days (Fig. 2.3A). However, WH7803 failed to grow in MIT9215 cultures when EZ55 was present, dropping below the limit of detection shortly after inoculation (Fig. 2.3B).

In the reciprocal invasion assay, MIT9215 rapidly grew when inoculated in the WH7803 monoculture, with both organisms co-existing at equal abundances (Fig. 2.3C). In the presence of EZ55, MIT9215 was still able to invade a culture of WH7803 (Fig. 2.3D). Interestingly, with EZ55 present, the MIT9215 population displaced WH7803 as the majority phytoplankter in the culture: WH7803 exhibited a dramatic decline in abundance (Fig. 2.3D) that was not observed when EZ55 was absent (Fig. 2.3C). Thus, independent of the starting ratios or cell concentration, the presence of the EZ55 heterotroph favored the growth of *Prochlorococcus* over *Synechococcus* when nitrogen was scarce.

Prochlorococcus exudate drives heterotroph N competition with Synechococcus

Critically, the inhibitory effect of EZ55 on WH7803 growth was absent if the *Prochlorococcus* MIT9215 strain was not included. WH7803 growth showed no significant difference in growth between mono- and coculture with EZ55 in AMP-MN (Fig. 2.4A) ($p = 0.91$). This outcome suggested that *Prochlorococcus* may be secreting a factor(s) that stimulates the competition of EZ55 for resource(s) shared by WH7803. To test this, EZ55 and WH7803 were placed in co-culture competition in media pre-conditioned by MIT9215. Whether MIT9215 remained (Fig. 2.5A and B) or were removed (via filtration) prior to competition (Fig. 2.4F), the outcome was the same: in contrast to unconditioned medium (Fig. 2.4A), WH7803 was inhibited in MIT9215-conditioned medium.

We next considered two hypotheses for the *Prochlorococcus*-driven loss of WH7803 growth in the presence of EZ55: either *Prochlorococcus* is driving EZ55 to compete for limited resources, or to produce a factor that is toxic to WH7803. Carbon and nitrogen amendment studies favored the former over the latter hypothesis.

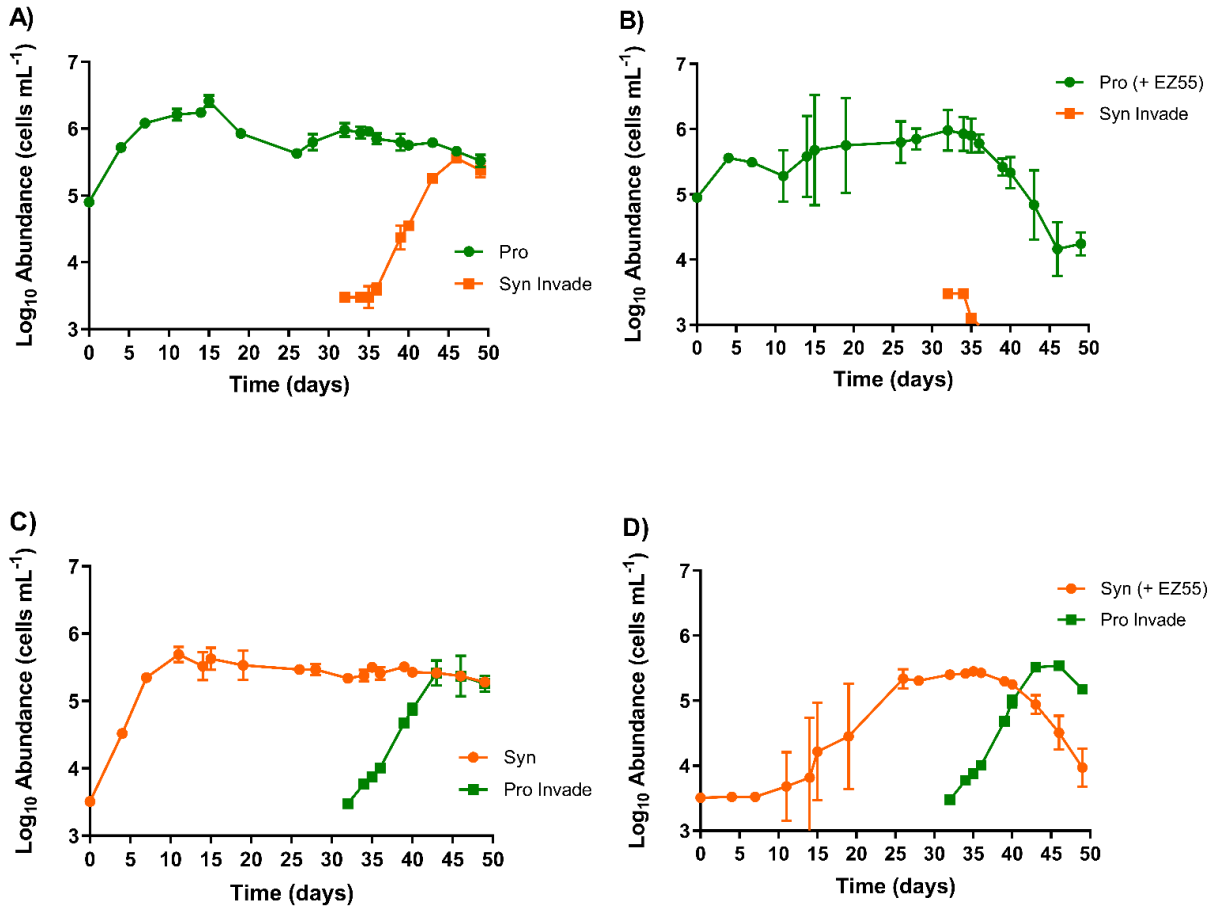


Fig. 2.3. Invasion Assay: Growth of *Prochlorococcus* strain MIT9215 (A-B) and *Synechococcus* strain WH7803 (C-D) in AMP-MN artificial seawater medium with and without *Alteromonas macleodii* strain EZ55. On day 32, cultures of the cyanobacteria without *Alteromonas* were inoculated as minority into the cultures of the rival cyanobacterium with and without *Alteromonas* to assess ability to invade. Error bars represent one standard deviation of the geometric mean (n=3).

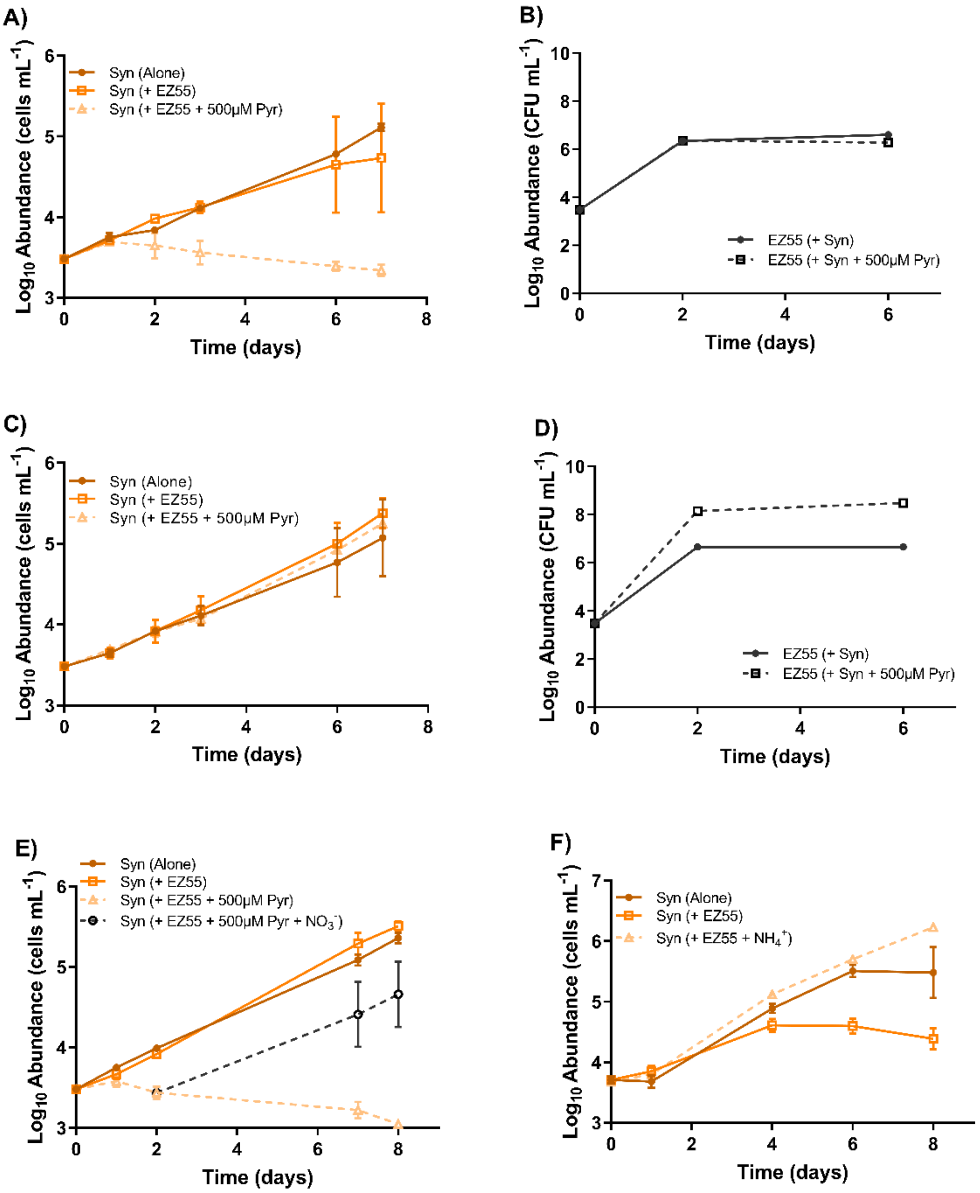


Fig. 2.4. Characterizing *Synechococcus-Alteromonas* Interactions: Growth of *Synechococcus* strain WH7803 (A, C, E, and F) and *Alteromonas macleodii* strain EZ55 (B and D) in AMP-MN (A, B, and E) and AMP-A (C and D) artificial seawater media in monoculture, coculture, and coculture with the addition of 500 µM sodium pyruvate (A-E). Cocultures were also amended with 500 µM sodium pyruvate and 800 µM sodium nitrate to demonstrate growth rescue by nutrient addition (E). WH7803 was also cultured in *Prochlorococcus*-conditioned AMP-MN alone and with EZ55 ± 400 µM NH_4^+ (F). Error bars represent one standard deviation of the geometric mean (n=3).

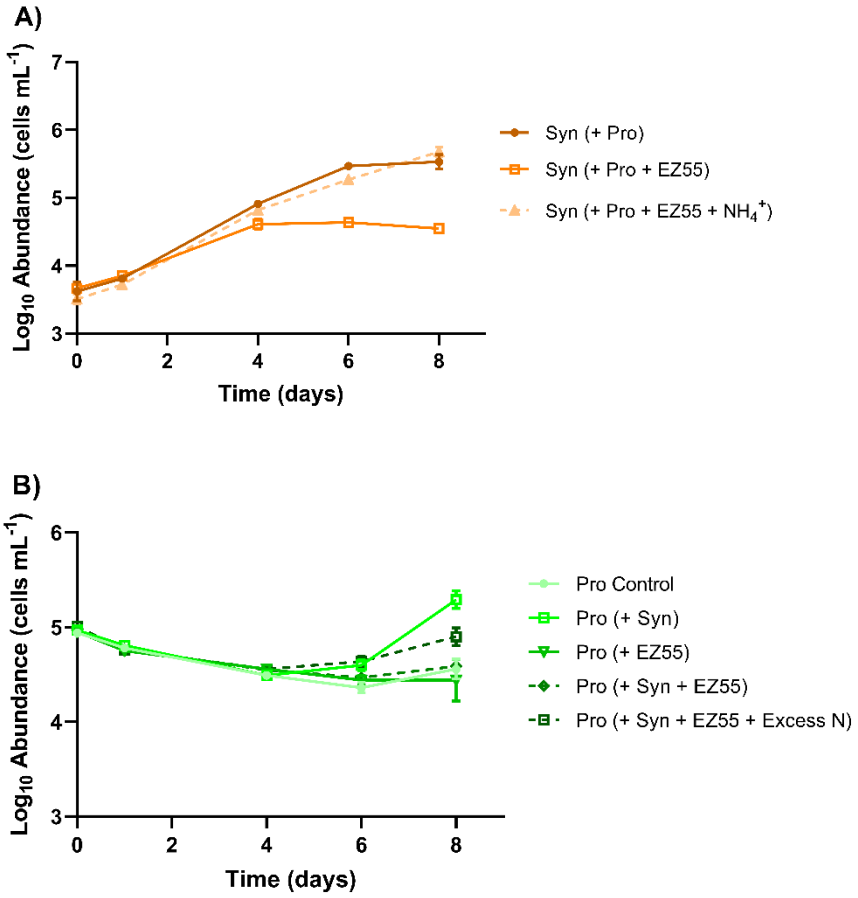


Fig. 2.5. Competition in *Prochlorococcus* Conditioned Media: Growth of *Synechococcus* strain WH7803 in AMP-MN artificial seawater medium pre-conditioned by *Prochlorococcus* strain MIT9215, which was still present during experiment. *Synechococcus* was inoculated alone and with *Alteromonas macleodii* strain EZ55 ± 400 µM NH_4^+ (A). Growth of *Prochlorococcus* strain MIT9215 in pre-conditioned AMP-MN artificial medium after inoculation of WH7803 and EZ55 (B). Error bars represent one standard deviation of the geometric mean (n=3).

Prochlorococcus releases a large fraction of fixed carbon as dissolved organic carbon during nitrogen-limited growth (Szul et al., 2019), so we reasoned that this excess source of carbon and energy could be energizing EZ55 to compete with *Synechococcus* for nitrogen in this nitrogen-limited medium. Pyruvate was examined as a proxy for *Prochlorococcus* exudate, and like the exudate, allowed EZ55 to prevent the growth of WH7803 (Fig. 2.4A). Notably, in tripartite cultures, addition of pyruvate (Fig. 2.6) further contributed to WH7803 reduction without apparent effect on MIT9215.

In the AMP-MN medium, nitrogen is the limiting resource for both *Prochlorococcus* and *Synechococcus* (Fig. 2.1A and B); other nutrients were provided in excess. As such, we reasoned that if EZ55 was restricting growth of WH7803, it was likely via competition for nitrogen. Consistently, addition of excess nitrogen to the medium as either NH_4^+ or NO_3^- restored the ability of WH7803 to grow in the presence of pyruvate or exudate-stimulated EZ55, whether at the onset of co-cultivation (Fig. 2.5A, 2.4C, and 2.4F) or after WH7803 has ceased growth for several days (Fig. 2.4E). Notably, in these co-culture studies, pyruvate additions enabled EZ55 to grow to several orders of magnitude higher when nitrogen was in excess (Fig. 2.4D) but not when limiting (Fig. 2.4B), suggesting the inhibition by EZ55 requires excess carbon relative to nitrogen.

Nitrogen Competition in Three-Member Cocultures

While the concentration of total bioavailable N in AMP-MN has been established (Fig 2.1), the constituent N species are not known. We hypothesized that while *Prochlorococcus* strain consumes the NH_4^+, the *Synechococcus* and heterotroph strains compete for a residual N resource *Prochlorococcus* cannot utilize but the other two can, namely NO_3^- (Moore et al., 2002). To test this hypothesis we generated a transposon insertion mutant of EZ55 that lacks the nitrate reductase enzyme encoded by *nirB*. The *nirB* mutant cannot utilize nitrate as a nitrogen source, and, unlike wild type (Fig. 2.7A), cannot prevent growth of WH7803 in tripartite cultures with MIT9215 (Fig. 2.7B). The *nirB* mutation did not impact growth of the *Alteromonas* strain (Fig. 2.7C and D), suggesting this mutation prevented nitrogen competition without impacting overall growth. The inability of the nitrate utilization-deficient strain of EZ55 to restrict the growth of WH7803 suggests that NO_3^- was present in AMP-MN, and that wild type EZ55 is able to outcompete WH7803 for this resource (when activated by *Prochlorococcus* exudate).

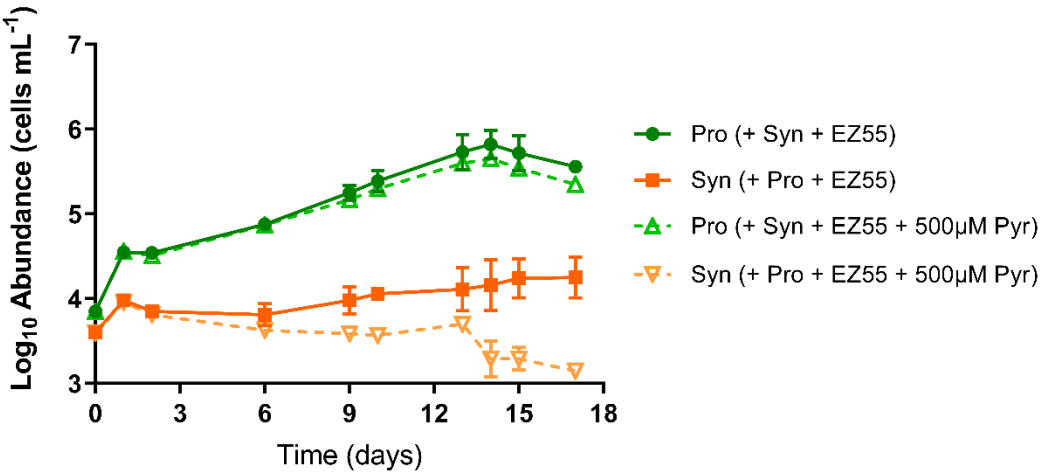
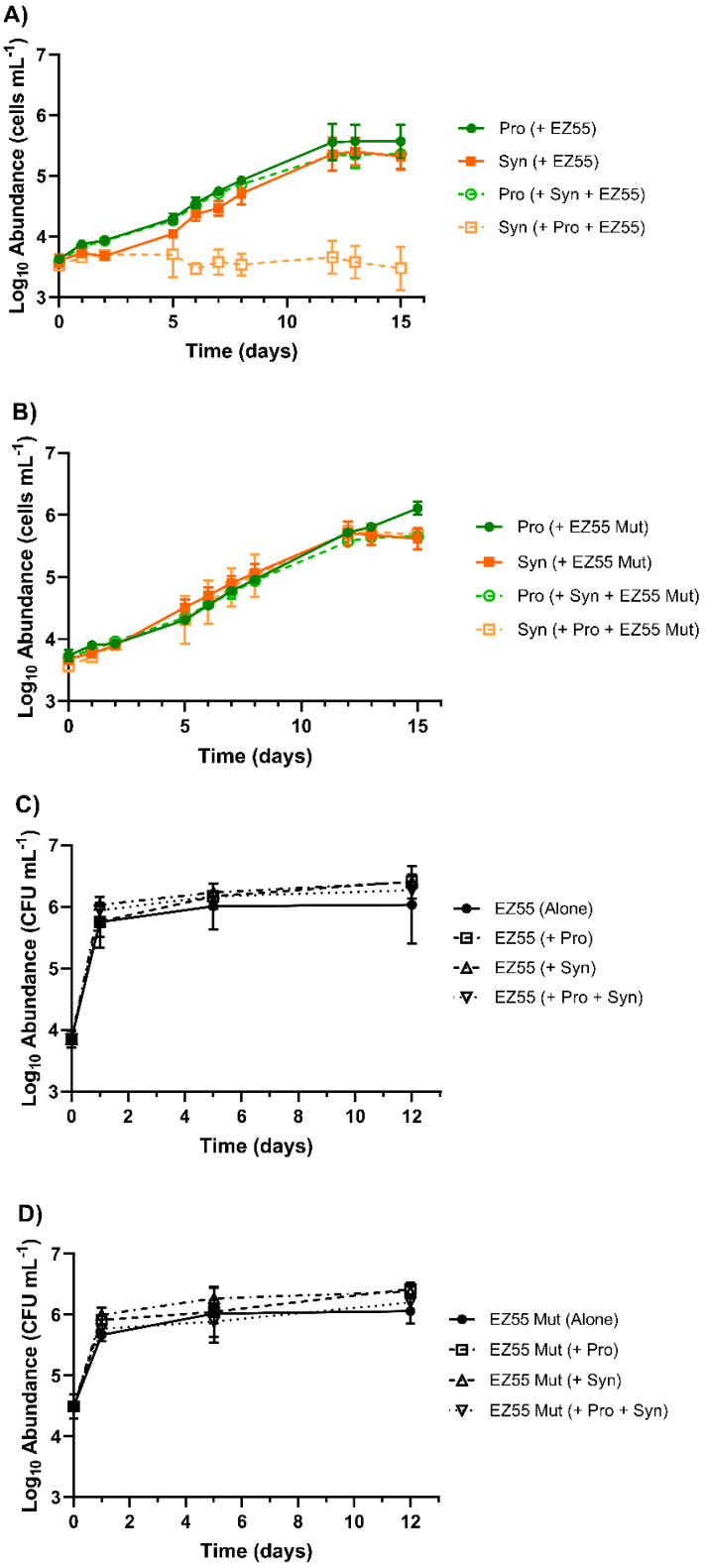


Fig. 2.6. Effect of Excess Carbon on Tripartite Competition: Growth of *Prochlorococcus* strain MIT9215, *Synechococcus* strain WH7803, and *Alteromonas macleodii* strain EZ55 in tripartite culture in AMP-MN artificial seawater medium with and without the addition of 500µM sodium pyruvate. Error bars represent one standard deviation of the geometric mean (n=3).

Fig. 2.7. Tripartite Competition with WT and Mutant *Alteromonas*: Growth of *Prochlorococcus* strain MIT9215 and *Synechococcus* strain WH7803 in AMP-MN artificial seawater medium in coculture and tripartite culture with *Alteromonas macleodii* strain EZ55 WT (A) or *Alteromonas macleodii* strain EZ55 mutant (B). Abundance of heterotroph in each treatment is shown for WT (C) and mutant (D). Error bars represent one standard deviation of the geometric mean (n=3).


A)
Log10 Abundance (cells mL-1)
Time (days)
Pro (+ EZ55)
Syn (+ EZ55)
Pro (+ Syn + EZ55)
Syn (+ Pro + EZ55)
B)
Log10 Abundance (cells mL-1)
Time (days)
Pro (+ EZ55 Mut)
Syn (+ EZ55 Mut)
Pro (+ Syn + EZ55 Mut)
Syn (+ Pro + EZ55 Mut)
C)
Log10 Abundance (CFU mL-1)
Time (days)
EZ55 (Alone)
EZ55 (+ Pro)
EZ55 (+ Syn)
EZ55 (+ Pro + Syn)
D)
Log10 Abundance (CFU mL-1)
Time (days)
EZ55 Mut (Alone)
EZ55 Mut (+ Pro)
EZ55 Mut (+ Syn)
EZ55 Mut (+ Pro + Syn)

Competition outcomes are robust with regard to genotype

To determine the extent to which strain genotype impacts the outcomes of co-cultivation, we modified the mixed culture experiments by substituting MIT9215, WH7803, or EZ55 with different strains of *Prochlorococcus*, *Synechococcus*, or heterotrophic bacterium, respectively. Like MIT9215, high light-adapted *Prochlorococcus* strains MIT9312 or MED4 outcompeted WH7803 in the presence of EZ55 (Fig. 2.8A). And, like WH7803, *Synechococcus* strains CC9605 and WH8102 were outcompeted by MIT9215 in the presence of EZ55 (Fig. 2.8B).

As a final constraint on the *Synechococcus* – heterotroph coculture outcomes, different marine heterotrophic bacteria were substituted for EZ55: *Phaeobacter* sp. strain Y3F and *Vibrio fischeri* strain ES114. When grown in N-replete AMP-A +/- pyruvate, or N-limited AMP-MN without pyruvate, coculturing with any of the three heterotrophs did not cause any significant deviation of WH7803 maximal abundance compared to the monoculture control (Fig. 2.9A-C). However, as with EZ55, the addition of pyruvate to AMP-MN caused a reduction in WH7803 maximal abundance when in co-culture with YF3 or ES114 compared to either the monoculture control (Fig. 2.9D) ($p < 0.0001$) or cocultures in AMP-MN without pyruvate (Fig. 2.8C) ($p < 0.0001$). With the exception of ES114, all heterotrophs maintained steady long-term populations in AMP-MN regardless of amendments; ES114 declined steadily and only maintained its starting abundance with pyruvate addition (Fig. 2.9E-G).

Discussion

The numerical dominance of *Prochlorococcus* over rival phytoplankton in the oligotrophic ocean has been recognized since its discovery in 1988 (Chisholm et al., 1988). In this study we describe conditions in which such dominance is reproduced in culture. Importantly, we observed that *Prochlorococcus* outgrows *Synechococcus* in low-nitrogen conditions, simulating the North Pacific Subtropical Gyre, and only in the presence of heterotrophic bacteria, simulating the multi-trophic mixed community of the ocean.

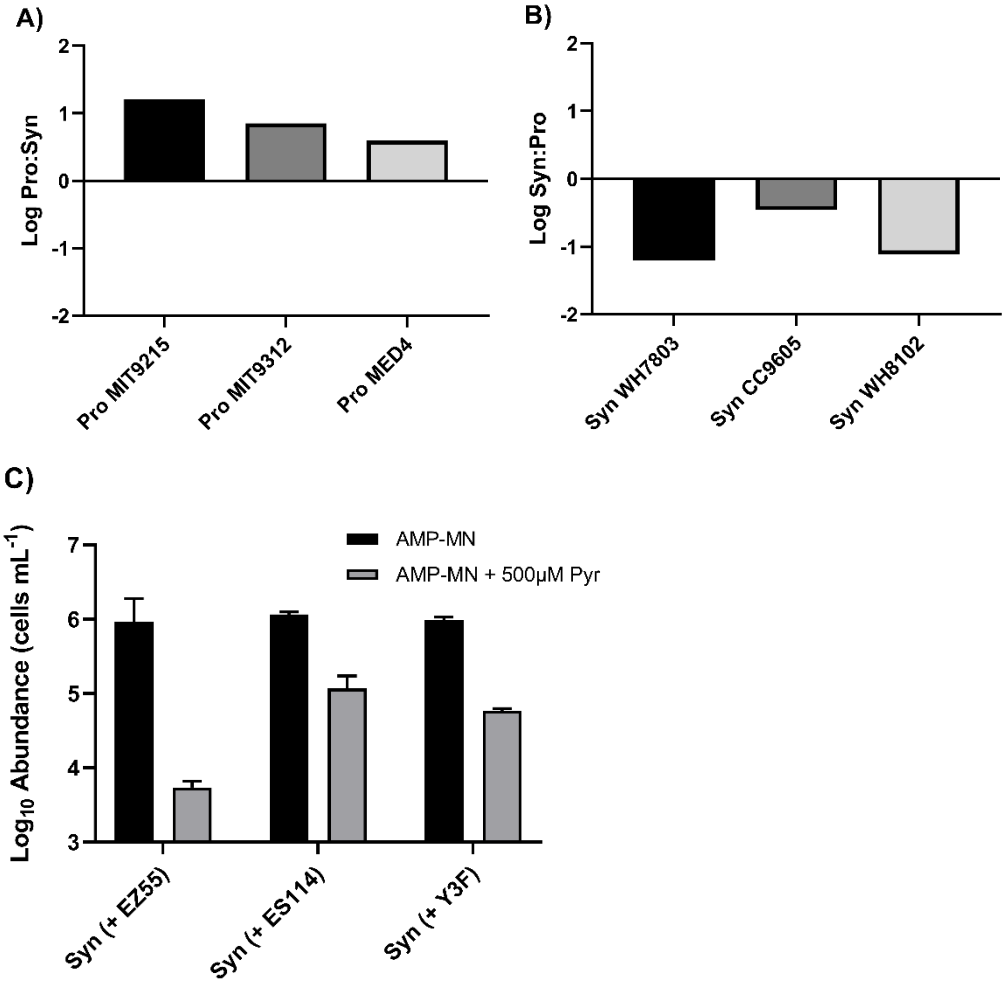
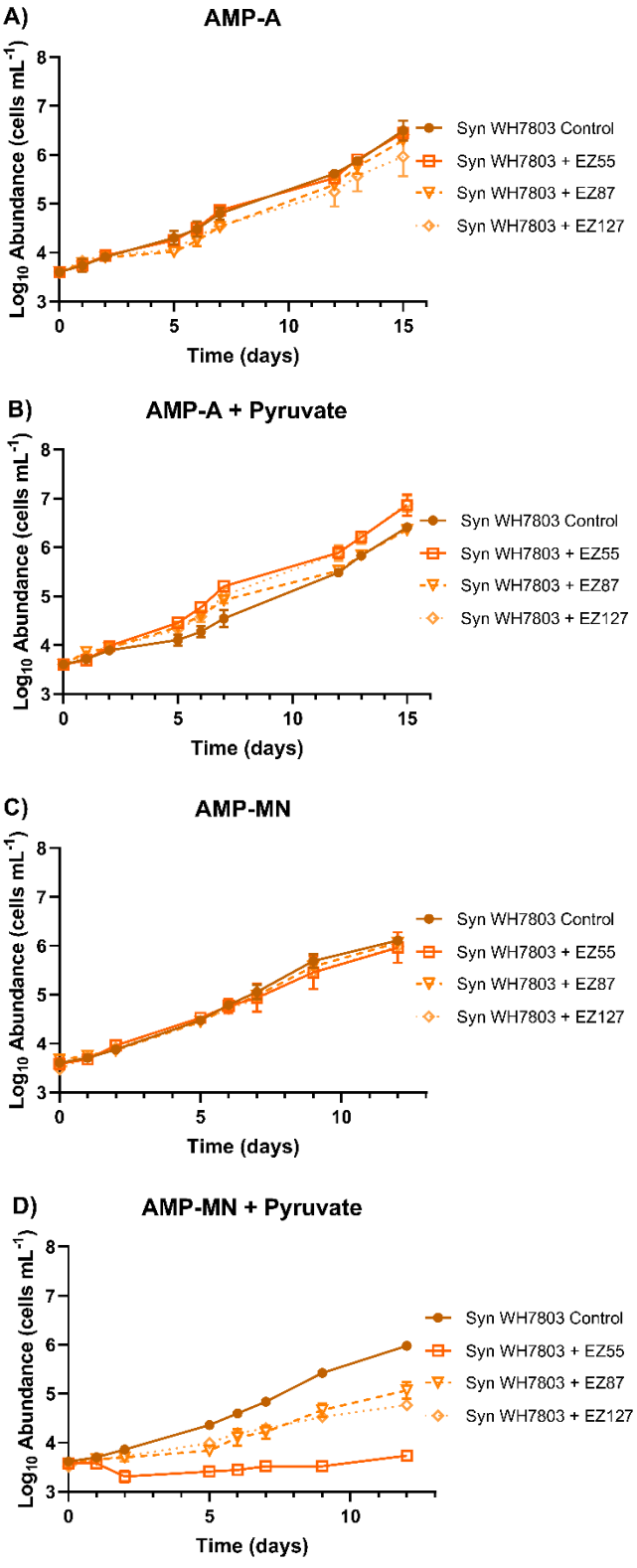


Fig. 2.8. Consistency of Prochlorococcus and Synechococcus Tripartite Outcomes: Log ratio of *Prochlorococcus* and *Synechococcus* strains maximal abundances in tripartite culture with *Alteromonas macleodii* strain EZ55 in AMP-MN artificial seawater medium. *Prochlorococcus* strains (A) were cultured with *Synechococcus* strain WH7803 and EZ55 and *Synechococcus* strains (B) were cultured with *Prochlorococcus* strain MIT9215 and EZ55. Maximum abundances of Synechococcus strain WH7803 were observed when cultured in AMP-MN or AMP-MN + 500 µM sodium pyruvate with different marine heterotrophic bacteria (C). Error bars represent one standard deviation of the geometric mean (n=3).

Fig. 2.9. Competition of Synechococcus with Other Marine Heterotrophs: Growth of *Synechococcus* strain WH7803 in AMP-A and AMP-MN artificial seawater media with and without 500 µM sodium pyruvate in monoculture and coculture with individual heterotrophs (A-D): *Alteromonas macleodii* strain EZ55, *Vibrio fischeri* strain ES114 (EZ87), and *Phaeobacter* sp. strain Y3F (EZ127). Heterotroph abundances in all coculture treatments are shown (E-G). Error bars represent one standard deviation of the geometric mean (n=3).


A)
AMP-A
B)
AMP-A + Pyruvate
C)
AMP-MN
D)
AMP-MN + Pyruvate
Log10 Abundance (cells mL-1)
Time (days)
Syn WH7803 Control
Syn WH7803 + EZ55
Syn WH7803 + EZ87
Syn WH7803 + EZ127

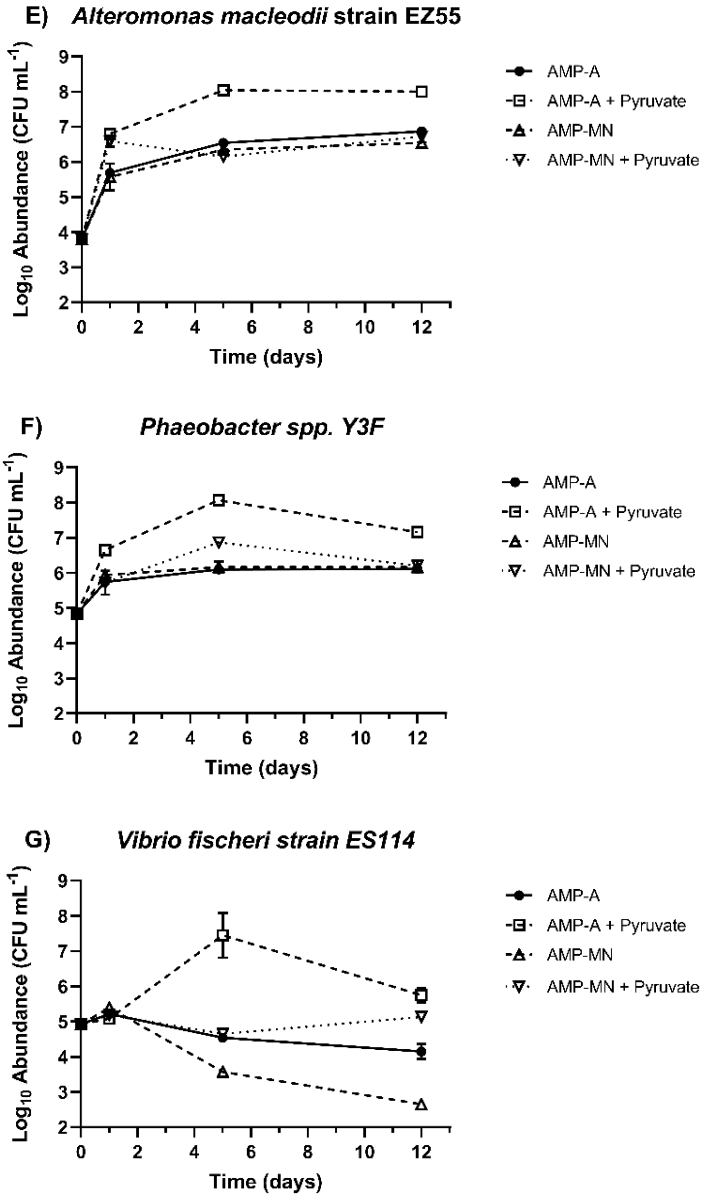


Fig. 2.9. Continued.

In the NPSG, where nitrogen is thought to limit growth (Moore et al., 2013, Saito et al., 2014, Van Mooy et al., 2009, Ustick et al., 2021), *Prochlorococcus* can outnumber *Synechococcus* (and other members of the phytoplankton community) by several orders of magnitude (Campbell et al., 1997, Flombaum et al., 2013, Campbell et al., 1994). In these nitrogen-limited waters heterotrophic bacteria can grow to between 300,000 and 500,000 cells ml^{-1} and outnumber phytoplankton (Church et al., 2002, Li, 1998, Johnson, 2013). Our low-nitrogen culture medium recapitulated these trends: heterotrophs grew to an only slightly greater abundance of 10^6 cells ml^{-1}, and in tripartite cultures the dynamics of the picocyanobacteria favored *Prochlorococcus* over *Synechococcus*, regardless of the relative starting abundances. When co-inoculated with *Alteromonas*, *Prochlorococcus* could grow while *Synechococcus* could not. In absence of *Prochlorococcus*, *Synechococcus* could grow and stably coexist with *Alteromonas* for weeks. However, once introduced into this culture, *Prochlorococcus* could invade rapidly and ultimately displace the *Synechococcus* population.

Our results suggest that *Prochlorococcus* acts indirectly, through a heterotroph intermediate, to dictate the growth outcome of its rival *Synechococcus* in low-nitrogen environments. In low nitrogen, low organic carbon medium, *Prochlorococcus* scavenges a residual source(s) of nitrogen, apparently with superior capability relative to *Alteromonas* and *Synechococcus*. *Alteromonas* can grow on residual organic carbon until it becomes growth arrested by a lack of carbon and energy. In this state it is poised to compete for nitrogen but lacks the carbon and energy resources to do so, unless fed by *Prochlorococcus*. Once fed, *Alteromonas* can begin to compete with *Synechococcus* for alternative nitrogen source(s). The inability of a mutant *Alteromonas* lacking the nitrate reductase enzyme to arrest the growth of *Synechococcus* suggests that the competition involves nitrate, a resource both *Synechococcus* and wild type *Alteromonas* can utilize, but the strains of *Prochlorococcus* examined in this study cannot. Nitrate-utilizing strains of *Prochlorococcus* have been recently isolated (Berube et al., 2015), and future studies in tripartite cultures with these strains could prove informative. In the paragraphs that follow we unpack this model to discuss the key supporting evidence and identify unanswered questions.

Our study implicates the release of organic carbon by *Prochlorococcus* for the stimulation of *Alteromonas* to outcompete *Synechococcus* for nitrogen. Neither

Prochlorococcus nor *Alteromonas* acting alone was sufficient to diminish the growth of *Synechococcus*, but when together in a tripartite community diminished *Synechococcus* growth.

Importantly this effect was observed only when nitrogen was limiting in the medium; addition of excess nitrogen was all that was needed to retore *Synechococcus* growth. This latter result also argues against the production of a growth-limiting substance by *Alteromonas* as the explanation of the growth arrest of *Synechococcus*.

Prochlorococcus exudate was sufficient to stimulate the N-competition by *Alteromonas*, as was a proxy form of *Prochlorococcus* exudate, pyruvate. *Prochlorococcus* exudes a large fraction of fixed carbon as dissolved organic matter (Becker et al., 2014, Bertilsson et al., 2005, Lopez-Sandoval et al., 2013) much of which is bioavailable to heterotrophic bacteria (Sarmento and Gasol, 2012, Sharma et al., 2014). Recently it was observed that *Prochlorococcus* can also release membrane vesicles (Biller et al., 2014), which may serve as complex nutrients for co-occurring heterotrophs. Critically, under nitrogen limitation the release of dissolved organic matter by *Prochlorococcus* is exacerbated (Roth-Rosenberg et al., 2021, Szul et al., 2019). The specific form(s) of released organic carbon that stimulated *Alteromonas* competition for nitrogen in this study is not known, but it is rather curious that *Synechococcus* exudate was not sufficient for this effect: bipartite cultures of *Alteromonas* and *Synechococcus* stably co-existed in low-N medium. *Synechococcus* is known to release organic carbon, and this release increases under nutrient limitation (Christie-Oleza et al., 2017), so this distinction between *Prochlorococcus* and *Synechococcus* exudate warrants further investigation.

As with carbon, the nitrogen species involved in the tripartite interactions are not yet completely identified. Our artificial seawater medium lacked nitrogen amendment, but trace amounts of nitrogen from unknown sources were capable of supporting microbial growth to 10^6 cells ml^{-1}. Due to the volatility of ammonia and reported cases of ammonia contamination in other systems (Behera et al., 2013), we suspect that it serves as a major component of the unamended medium. As the preferred nitrogen source for *Prochlorococcus* and most microbes, we suspect that ammonia is the primary nitrogen source consumed by *Prochlorococcus*, whether in mono- or mixed-cultures. However, strain MIT9215 has the genetic potential to utilize urea as well (Moore et al., 2002, Scanlan et al., 2009), so this species cannot be ruled out. Nitrate is a likely component of the medium, as the ability of *Alteromonas* to prevent *Synechococcus* growth

was eliminated when the nitrate reductase of the heterotroph was knocked out. While some strains of *Prochlorococcus* can utilize nitrate (Berube et al., 2015), the ones assayed in this study could not. Whether or not the nitrate-utilizing *Prochlorococcus* strains can also compete with *Synechococcus* for this resource could be resolved in future studies.

In the ocean, *Prochlorococcus* and *Synechococcus* compete for a variety of nitrogen sources, including organic forms such as amino acids (Aldunate et al., 2019, Berthelot et al., 2018, Berube et al., 2016, Duhamel et al., 2018, Fawcett et al., 2011, Zubkov et al., 2003). In a 2018 study, Berthelot et al. observed that co-occurring populations of *Prochlorococcus*, *Synechococcus*, and the photosynthetic picoeukaryotes in the N-limited North Pacific Subtropical Gyre all utilize ammonia, urea, and nitrate, although to different extents (Berthelot et al., 2018).

While capable of sourcing their nitrogen from organic carbon molecules like amino acids, marine heterotrophs have been shown to also compete with phytoplankton for inorganic nitrogen in the form of ammonia or nitrate (Horrigan et al., 1988, Wheeler and Kirchman, 1986, Jacquet et al., 2002, Deng et al., 2021). Heterotrophs can account for 30% or more of inorganic nitrogen uptake at some locations (Bradley et al., 2010, Kirchman and Wheeler, 1998), and in some studies, inorganic nitrogen accounted for half or more of the total nitrogen acquired by heterotrophs (Jørgensen et al., 1994, Keil and Kirchman, 1991).

Importantly, the ability of heterotrophs to compete for inorganic nitrogen scavenging appears to be stimulated by organic carbon. Several studies by the Kirchman group and others noted the necessity of sufficient carbon for inorganic N uptake by bacteria (Kirchman et al., 1990, Keil and Kirchman, 1991, Kirchman et al., 1989, Kroer et al., 1994, Wheeler and Kirchman, 1986, Jacquet et al., 2002). The physiological basis for this stimulation is not yet understood, however, studies with laboratory cultures provide some clues. For *Escherichia coli*, carbon limitation depletes the TCA cycle intermediate and key substrate for inorganic nitrogen assimilation, α-ketoglutarate (2-oxo-glutarate) (Schumacher et al., 2013). Consequently, C-starved cells have diminished rates of ammonium assimilation and potentially other N utilization pathways, even when cells are N limited (Schumacher et al., 2013). Notably, a recent study found that *Alteromonas* significantly reduced expression of genes involved in nitrogen metabolic pathways under carbon and iron colimitation (Manck et al., 2020).

The stimulation of inorganic nitrogen uptake in these studies is entirely consistent with our observations of *Alteromonas* and other marine heterotrophs in the N-limited medium. Like *E. coli*, carbon-limited *Alteromonas* may be deprived of the necessary a-ketoglutarate for assimilation of ammonia or nitrate. Alternatively, or in addition, carbon limitation may deprive the cells of the energy needed to drive transport of these substrates. In either case, the provision of organic carbon by *Prochlorococcus* appears to satisfy the requirements for enhanced inorganic nitrogen uptake and assimilation by these heterotrophs, out-competing *Synechococcus* in the process.

Prior studies have highlighted the beneficial effects of heterotroph interactions with picocyanobacteria (Becker et al., 2019, Biller et al., 2016, Christie-Oleza et al., 2017, Hennon et al., 2018, Sher et al., 2011, Tai et al., 2009, Zhang et al., 2021, Zheng et al., 2020). Previously we described how heterotrophic bacteria protect *Prochlorococcus* from oxidative stress (Ma et al., 2018, Morris et al., 2011). Coe et al. (2016) and Roth-Rosenberg et al. (2020) have shown that heterotrophs promote the survival of *Prochlorococcus* during long-term light and nutrient (N or P) deprivation, respectively. Christie-Oleza et al. (2017) found a similar relation between *Synechococcus* and a marine roseobacter. In that study, long-term co-existence under nutrient limitation was facilitated by an exchange of resources between the phototroph and heterotroph.

Interactions between picocyanobacteria have been less well characterized, but a recent study from Knight and Morris (2020) showed that *Synechococcus* could aid the growth of *Prochlorococcus* under conditions simulating ocean acidification. The mechanism of this help was not identified, but because these co-cultures were grown in the presence of *Alteromonas* sp. EZ55, the authors speculated that *Synechococcus* could help *Prochlorococcus* indirectly by stimulating EZ55. The potential for allelopathic interactions between picocyanobacteria has also been noted (Cubillos-Ruiz et al., 2017, Li et al., 2010, Paz-Yepes et al., 2013).

Our study provides a new dimension to the picocyanobacteria-heterotroph and picocyanobacteria-picocyanobacteria interactions: the ability of one phototroph (*Prochlorococcus*) to drive a shift from co-existence to competition between a second phototroph (*Synechococcus*) and a heterotroph. Christie-Oleza et al. (2017) found that *Synechococcus* and heterotroph strains co-exist during prolonged coculture in unamended seawater, and that upon N addition, cross-feeding could occur by the conversion of N substrates unusable by the other

microbe: the heterotroph strain could convert organic nitrogen (peptone) to ammonia, while WH7803 could convert nitrate to dissolved organic nitrogen. In our study, both heterotroph and phototroph could utilize nitrate, and unless the former was mutated in its nitrate reductase, the heterotroph could apparently outcompete the *Synechococcus* strain for this resource when fed organic carbon by *Prochlorococcus*.

This study demonstrates that metabolic interactions between trophic groups can influence relative abundances within trophic groups. The prediction that *Prochlorococcus* outcompetes rival phytoplankton including *Synechococcus* under nutrient limitation is largely confirmed, but this outcome may require the ability of *Prochlorococcus* to energize heterotrophic bacteria to outcompete their photosynthetic rivals for resources that they themselves do not use. If our results can be extrapolated to the natural environment, it highlights an important connection between carbon and nitrogen availability, and suggests complex microbial interactions can benefit streamlined, efficient genera such as *Prochlorococcus* to the detriment of their competition.

CHAPTER 3

Prochlorococcus is protected from hydrogen peroxide-mediated mortality by rival marine phytoplankton *Synechococcus*, *Micromonas*, and *Ostreococcus*

Publication Note

This chapter is being prepared for publication. Authors are listed below.

Contributions

Experiments were designed by Benjamin Calfee and Erik Zinser; all experiments were performed by Benjamin Calfee; and Benjamin Calfee and Erik Zinser crafted manuscript.

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Abstract

The marine cyanobacterium *Prochlorococcus* is the most abundant photoautotrophic microorganism on the planet and numerically dominates the photosynthetic portion of microbial communities in all lower latitude, open ocean environments. *Prochlorococcus*, having lost the catalase gene in most strains, is entirely dependent on its surrounding microbial community for protection from oxidative stress caused by exposure to hydrogen peroxide (H_2O_2). Protection of this kind by heterotrophic 'helper' bacteria has been well characterized and shown to be a necessity in both environmental and laboratory studies. While the potential contribution of photosynthetic microbes to the protection of *Prochlorococcus* from H_2O_2 has been suggested through environmental observation, this interaction has not been fully explored in a laboratory setting. In this study we assessed the ability of relevant marine photoautotrophs, the cyanobacterium *Synechococcus* and picoeukaryotic phytoplankton *Micromonas* and *Ostreococcus*, to protect *Prochlorococcus* from H_2O_2 exposure when cocultured at ecologically relevant abundances. We determined all three phytoplankton groups provided sufficient protection and mortality reduction under a variety of H_2O_2 addition regimes that simulated both a rainfall event and the upper limit of daily abiotic production (800 nM). While the degradative ability of each helper was directly proportional to its abundance, we showed that *Synechococcus* consistently displayed faster degradation rates and could degrade lower concentrations of H_2O_2 than the picoeukaryotes. Based on our data we estimate that these phytoplankton groups contribute significantly to total community degradation in the open ocean, which has interesting implications on the competition and coexistence between *Prochlorococcus* and its rival phytoplankton.

Introduction

Organisms within sunlit, aquatic environments are often exposed to reactive oxygen species (ROS), produced biotically as metabolic by-products (Bond et al., 2020, Collén et al., 1995, Diaz et al., 2013, Diaz et al., 2018, Gonzalez-Flecha and Demple, 1995, Palenik et al., 1987, Hansel et al., 2016) and abiotically through photooxidation of organic substances (Cooper and Zika, 1983, Draper and Crosby, 1983, Gerringa et al., 2004, Mopper et al., 2015, Zhang et al., 2012) and rainfall events (Cooper et al., 1987, Hanson et al., 2001, Willey et al., 2004, Yuan and Shiller, 2000). Hydrogen peroxide (H_2O_2) makes up a significant proportion of total ROS in aquatic environments and is consistently detected across oceanic basins (Yuan and Shiller, 2001, Yuan and Shiller, 2005, Zika et al., 1985). Biotic production of H_2O_2 varies drastically, as organisms within trophic levels serve as sinks or sources due to differences in metabolism and physiology (Bond et al., 2020, Collén et al., 1995, Palenik et al., 1987, Schneider et al., 2016). Total daily abiotic production at the surface layer of the oligotrophic ocean has been estimated at approximately 800 nM, but the activity of microbes' H_2O_2-degrading enzymes, catalase and peroxidase, maintain concentrations between 100-200 nM (Morris et al., 2016). While exposure to high concentrations of H_2O_2 is lethal to organisms lacking these enzymes, even those that possess them can experience cellular damage (Farmer and Mueller, 2013, Imlay, 2003, Imlay, 2013) and alterations in physiological processes (Baltar et al., 2013, Drabkova et al., 2007b, Tolar et al., 2016).

Cyanobacteria of the genus *Prochlorococcus*, the most abundant photoautotroph on Earth, thrive in almost all open ocean environments and are numerically dominant over eukaryotic phytoplankton and other cyanobacteria, such as *Synechococcus* species, and small (<2 μm) eukaryotic phytoplankton, *Micromonas* and *Ostreococcus* (Biller et al., 2015, Campbell et al., 1997, Flombaum et al., 2013, Visintini et al., 2021). The numerical dominance of *Prochlorococcus* is often attributed to the streamlined nature of their genome and physiology, which has manifested as a higher growth efficiency at the cost of diminished stress response and fewer DNA repair mechanisms (Dufresne et al., 2005, Kettler et al., 2007, Giovannoni et al., 2014, Swan et al., 2013), amongst other losses in physiological capability.

Due to the evolutionary loss of a functioning catalase enzyme, *Prochlorococcus* are completely dependent upon other members of the microbial community for the detoxification of

ROS - specifically hydrogen peroxide (H_2O_2) – in the surface mixed layer (Morris et al., 2011, Morris et al., 2012). Prior research has demonstrated certain heterotrophic "helper" organisms benefit the growth of *Prochlorococcus* in natural environments and laboratory cultures by degrading H_2O_2 (Morris et al., 2008, Ma et al., 2018). Strains of the heterotrophic bacteria *Alteromonas macleodii* are often co-isolated alongside *Prochlorococcus* and have been shown to both provide efficient protection against H_2O_2 and cause significant changes in gene expression when in coculture with *Prochlorococcus* (Biller et al., 2016, Morris et al., 2008, Sher et al., 2011). Thus while evidence of intimate interactions with and protection by heterotrophic bacteria abounds, the potential contributions of members of the photosynthetic community toward protection of *Prochlorococcus* from H_2O_2 have only been suggested in field observations (Morris et al., 2016), and the degradative abilities of phytoplankton has only been assessed in monoculture (Drabkova et al., 2007a, Leunert et al., 2013, Petasne and Zika, 1997, Wong et al., 2003).

To fill this knowledge gap, we strove to constrain the potential contributions of photosynthetic microbes to H_2O_2 degradation and protection of *Prochlorococcus* populations under ecologically relevant conditions, as confirmation of this interaction would have implications in competition, coexistence, and the overall ecology of these organisms. In this study we determined that prominent open ocean strains of the cyanobacterium *Synechococcus* and picoeukaryotic phytoplankton *Micromonas* and *Ostreococcus* could protect *Prochlorococcus* from H_2O_2-mediated mortality when cocultured at abundances that resembled their population dynamics in the oligotrophic ocean.

Methods

Strains and Culturing

Axenic cultures of strains of picocyanobacteria *Prochlorococcus* (MIT9215) and *Synechococcus* (WH7803 and CC9605), picoeukaryotic phytoplankton *Micromonas* (RCC299 and CCMP) and *Ostreococcus* (CCMP2972A), and marine heterotroph *Alteromonas macleodii* (EZ55) were used in this study. All cyanobacterial stock cultures were maintained in an artificial seawater medium, AMP-A (Jeffrey Morris and Zinser, 2013, Moore et al., 2007, Morris et al.,

2011), however picoeukaryote stock cultures were maintained and all experiments were performed using an AMP-A derivative, AMP-PE (for Pico-Eukaryotes, this study), which allowed for efficient and consistent growth of all photosynthetic microbes in mono- and coculture. This media has an identical recipe and preparation as AMP-A except for the following alterations: 10x addition of trace metal working stock, $1.06e^{-4}$ M silica, $2.96e^{-7}$ M thiamine, $2.05e^{-9}$ M biotin, and $3.69e^{-10}$ M cyanocobalamin. Stocks of these last three nutrients were filter sterilized and added after sterilization of the base saltwater medium. Axenic heterotrophic bacteria *Alteromonas macleodii* strain EZ55 (Morris et al., 2008) was inoculated from cryo-preserved stocks prior to each experiment (-80°C in YTSS + 10% glycerol) into 5 mL YTSS (Sobecky et al., 1996) and incubated shaking at 140 RPM at 24°C overnight. Before inoculation into experimental cultures, the heterotroph was washed three times in 1.5 mL microcentrifuge tubes by centrifugation at 8,000 RPM for two minutes in a tabletop microcentrifuge and resuspension in 1mL AMP-A. All experiments were carried out at 24 °C in Percival I36VLX incubators (Percival, Boone, IA) that allowed for gradual increase and decrease of cool white light to simulate sunrise and sunset with peak midday light intensity of 150 μmol quanta $m^{-2}s^{-1}$ on a 14 hr:10 hr light:dark cycle (Zinser et al., 2009). Purity tests to determine the axenicity of cyanobacteria and picoeukaryote stock and experimental cultures were routinely performed as previously described (Morris et al., 2008).

Cell Abundance Quantification

Abundances of cyanobacteria were quantified by flow cytometry using a CytoFLEX S flow cytometer (Beckman Coulter, Brea, CA) with populations of *Prochlorococcus* and *Synechococcus* differentiated in co-cultures by their red (675 nm) and red / yellow (675 nm / 578 nm) fluorescence, respectively (Cavender-Bares et al., 1998, Morris et al., 2008). Picoeukaryotes were quantified by red (675 nm) and far red (770 nm) fluorescence. Detection of red and yellow fluorescence was achieved after excitation with a blue (488 nm) laser, while detection of far red fluorescence required excitation by a yellow (565 nm) laser. Quantification of *Prochlorococcus* in coculture was achieved by observing events determined by red fluorescence after events that corresponded to the fluorescence properties of either *Synechococcus* (red / yellow) or picoeukaryotes (red / far red) were removed from abundance calculation. Heterotrophs in

coculture experiments were quantified by viable counting with serial dilutions on YTSS 1.5% agar plates incubated at 24 °C.

Hydrogen Peroxide Quantification and Addition

The concentration of HOOH in the medium and cultures was measured on an Orion L Microplate Luminometer (Titertek Instruments Inc, Berthold Detection Systems, Pforzheim, Germany) using an acridinium ester (Cayman Chemical Company, Ann Arbor, MI) chemiluminescence method (Morris et al., 2011). Concentrations in cultures were adjusted via both instantaneous and incremental (over 14 hour period) addition to achieve specific exposure conditions.

Results

To explore the interactions between *Prochlorococcus* and other photosynthetic marine microbes under conditions simulating natural exposures to hydrogen peroxide (H_2O_2), we conducted coculture experiments utilizing *Prochlorococcus* strain MIT9215 and three strains of *Synechococcus* at ecologically relevant abundances. In monoculture, MIT9215 grew at a steady rate in the presence of low (<100 nM) H_2O_2 but experienced several orders of magnitude drop in cell counts when transferred to media containing 350 nM H_2O_2 (Fig. 3.1A). Growth resumed after several days even though the *Prochlorococcus* monocultures did not degrade the H_2O_2 (Fig 3.1B).

To assess the ability of rival phytoplankton to help *Prochlorococcus*, the same exposures were performed and growth and H_2O_2 kinetics were measured. In contrast to *Prochlorococcus*, growth of catalase-positive *Synechococcus* strains WH7803 and CC9605 was unaffected by the higher H_2O_2 exposures (Fig 3.2A and B). In both high and low H_2O_2 conditions growth was concomitant with a decay in H_2O_2 concentration in the medium (Table 3.1), and by 8 days incubation the concentration was near or below the limit of detection (~10 nM) (Fig 3.2C and D). Like the *Synechococcus* strains, growth of the picoeukaryotic phytoplankton *Micromonas commoda* strain RCC299, *Micromonas pusilla* strain CCMP 1545, and *Ostreococcus lucimarinus* strain CCMP2972A was unaffected by the high H_2O_2 concentration (Fig. 3.3A-C) and under the high H_2O_2 conditions reduced H_2O_2 concentrations at a similar rate (Table 3.1) by

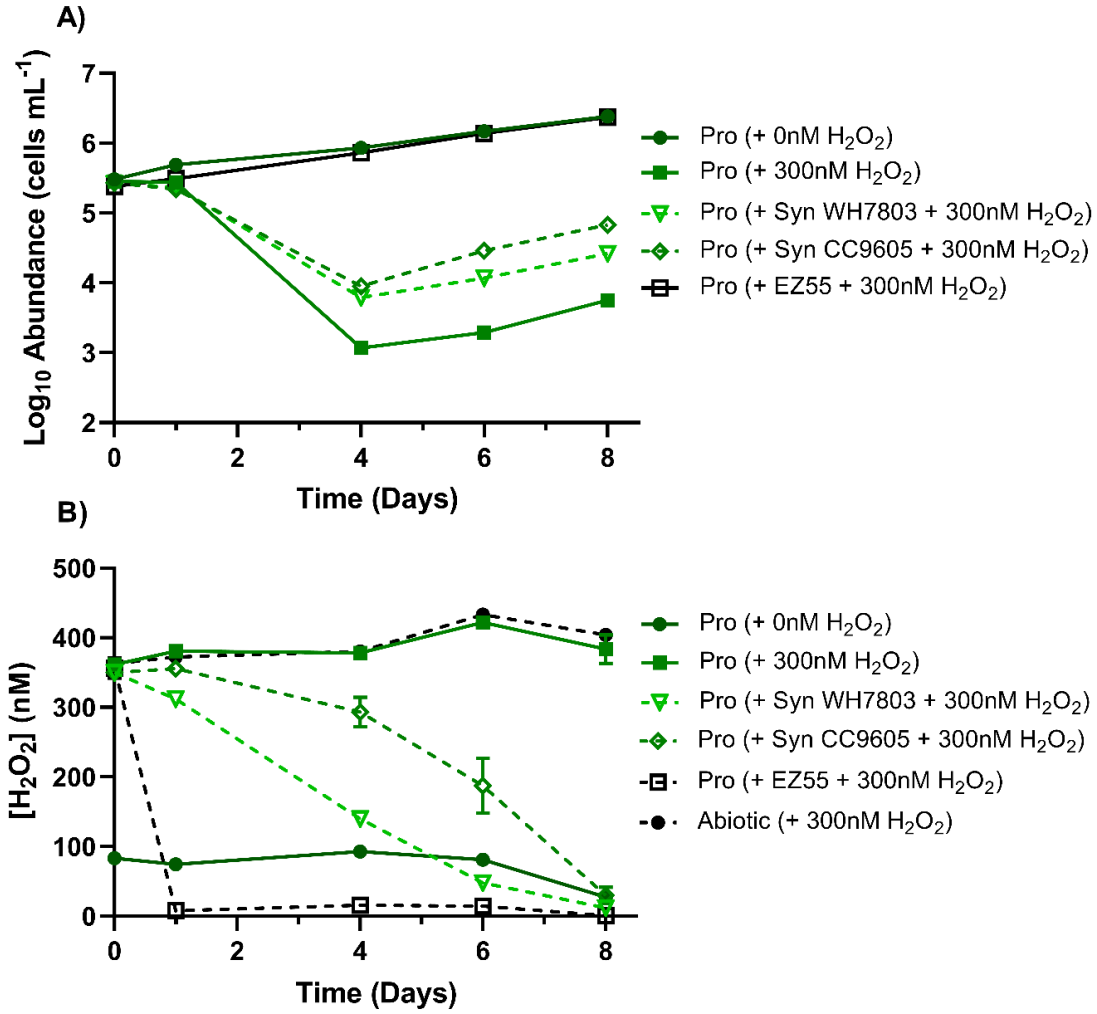

Fig. 3.1. Survival of *Prochlorococcus* with 300 nM H_2O_2: Growth of *Prochlorococcus* strain MIT9215 in mono- and coculture with *Synechococcus* strains WH7803 or CC9605; or *Alteromonas macleodii* strain EZ55 in AMP-A artificial seawater medium exposed to either 0 nM or 300 nM instantaneous addition of H_2O_2 (A). Concentrations of H_2O_2 in these treatments were quantified, including an abiotic control, over the course of the experiment (B). In the figure legend for this and subsequent figures, information within the parentheses describes addition(s) of a particular strain / treatment. Error bars represent one standard deviation of the geometric mean (n=3).

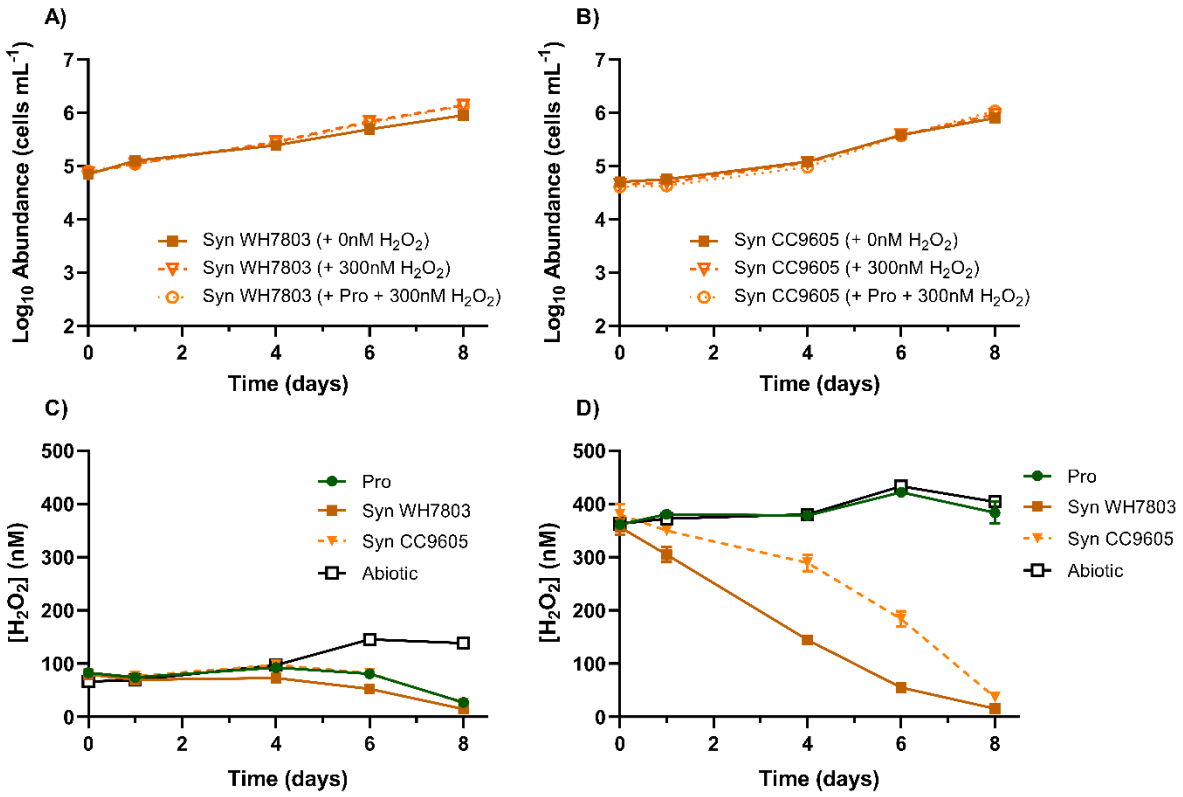

Fig. 3.2. Survival of *Synechococcus* with 300 nM H_2O_2: Growth of *Synechococcus* strains WH7803 (A) and CC9605 (B) in monoculture and coculture with *Prochlorococcus* MIT9215 in AMP-A artificial seawater medium after addition of 0 nM or 300 nM H_2O_2. Concentrations of H_2O_2 quantified for monocultures of all strains and an abiotic control with addition of 0 nM (D) or 300 nM (E) H_2O_2. Error bars represent one standard deviation of the geometric mean (n=3).

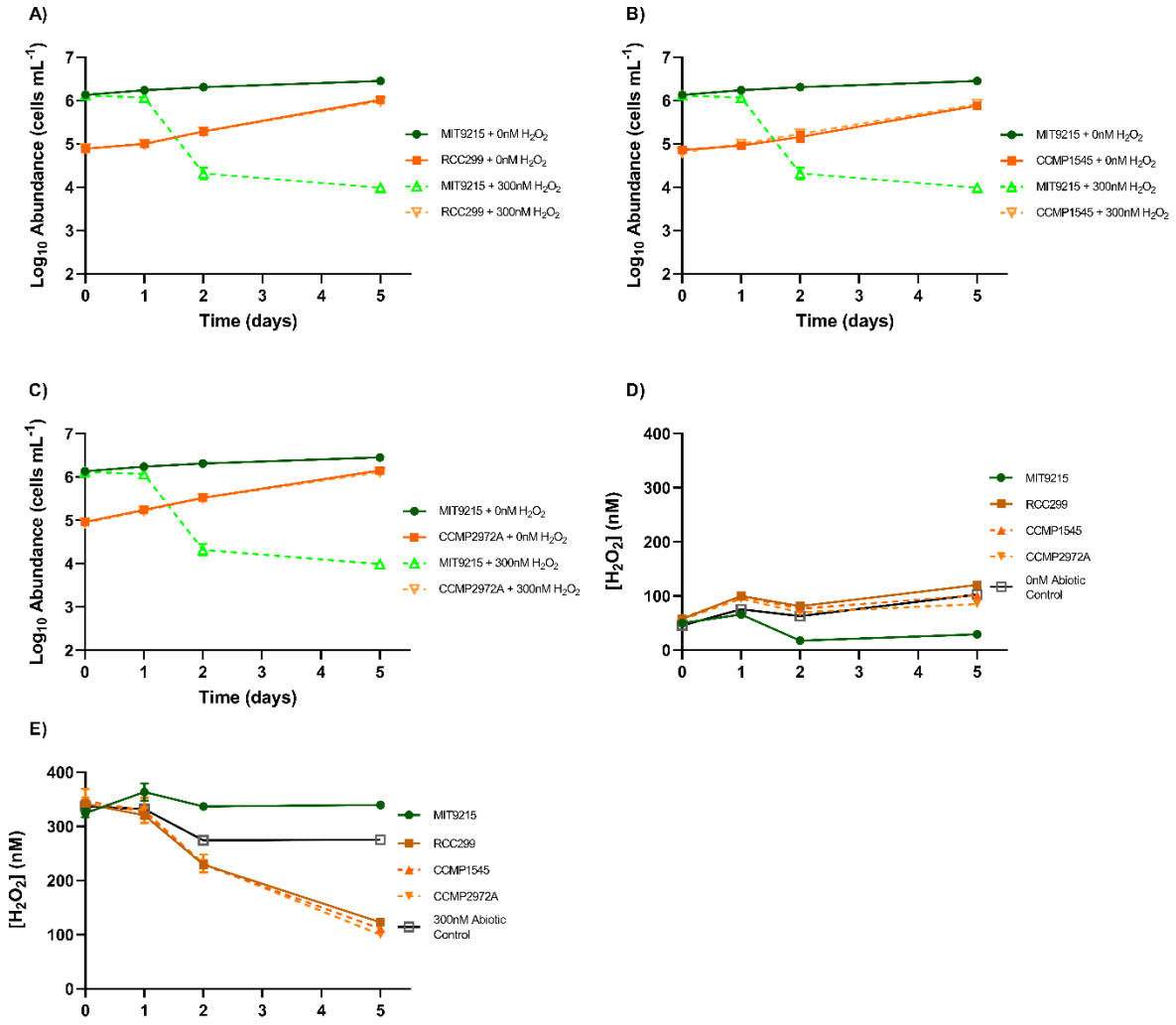

Fig. 3.3 Exposure of *Prochlorococcus* and Picoeukaryotes to 300 nM H_2O_2: Growth of *Prochlorococcus* strain MIT9215 (all panels), *Micromonas commoda* strain RCC299 (A), *Micromonas pusilla* strain CCMP1545 (B), and *Ostreococcus lucimarinus* strain CCMP2972A (C) in monoculture in AMP-A artificial seawater medium after addition of 0 nM or 300 nM H_2O_2. Concentrations of H_2O_2 quantified for monocultures of all strains and an abiotic control with addition of 0 nM (D) or 300 nM (E) H_2O_2. Error bars represent one standard deviation of the geometric mean (n=3).

Table 3.1 H_2O_2 Degradation Rates: Degradation rates (nM day^{-1}) of H_2O_2 by *Synechococcus*, *Micromonas*, and *Ostreococcus* strains when in coculture with *Prochlorococcus*, exposed to either instantaneous or incremental addition of H_2O_2. Rates were calculated as the slope of a linear regression using at least three time points.

	Treatment			
Strain	**+ 300 nM (instantaneous) Monoculture**	**+ 800 nM (incremental) Coculture**	**+ 800 nM (instantaneous) Coculture**	**+ 800 nM (instantaneous) (10X Abundance) Coculture**
***Synechococcus* WH7803**	45.06 ± 4.088	191.8 ± 11.94	112.0 ± 23.02	363.2 ± 135.7
Synechococcus CC9605	38.63 ± 7.929	192.5 ± 19.91		
***Micromonas commoda* RCC299**	45.44 ± 6.234	99.29 ± 8.424	58.83 ± 16.75	248.7 ± 19.25
***Ostreococcus lucimarinus* CCMP2972A**	51.72 ± 6.486	75.46 ± 0.102	80.60 ± 12.85	277.4 ± 27.68

200 nM (~60%) (Fig. 3.3E). However, in contrast to *Synechococcus*, these picoeukaryotes did not degrade H_2O_2 when it was less concentrated (<100 nM) (Fig. 3.3D).

In coculture with *Prochlorococcus* the growth and H_2O_2 decomposition kinetics for the two *Synechococcus* strains was essentially the same as in monoculture. Critically, the mortality of *Prochlorococcus* caused by the high H_2O_2 exposure was reduced by an order of magnitude when cocultured with ecologically-relevant concentrations of either WH7803 ($p = 0.0007$) or CC9605 ($p < 0.0005$) (Fig. 3.1A). As expected, coculture of *Prochlorococcus* with *Alteromonas macleodii* strain EZ55 mitigated all negative effects of H_2O_2 exposure by degrading below the level of detection by day one (Fig. 3.1A). H_2O_2 concentrations in abiotic controls changed little over the course of the experiment, suggesting that abiotic production and degradation of H_2O_2 were negligible (Fig. 3.1B, 3.2C and D).

The experiments just described simulate rapid influxes of H_2O_2 that can occur during rainfall events (Cooper et al., 1987, Hanson et al., 2001, Yuan and Shiller, 2000). To simulate the gradual progression of H_2O_2 increase over the photoperiod, H_2O_2 was increased incrementally over the experimental day to 800 nM, the mean value for H_2O_2 production in abiotic surface waters of the oligotrophic (Fig 3.4B) (Morris et al., 2016). Monocultures of *Prochlorococcus* strain MIT9215 exposed to this H_2O_2 regime showed over 99% mortality and reached their lowest abundance by day four (Fig. 3.4A). Coculturing with *Synechococcus* strains WH7803 or CC9605 reduced the mortality of *Prochlorococcus* to one order of magnitude ($p = 0.0003$ and 0.0002, respectively) (Fig. 3.4A), with H_2O_2 decreasing to a sublethal concentration by day four (Fig. 3.4B). No significant difference in degradation was observed between *Synechococcus* strains, but both reduced H_2O_2 at least four times faster than when exposed to 300 nM at comparable cell concentrations (Table 3.1) with no noticeable effect upon their growth (Fig. 3.5A). All negative effects of this H_2O_2 regime were mitigated for *Prochlorococcus* by cocultures of EZ55, with MIT9215 final abundances in coculture matching the monoculture controls lacking the H_2O_2 additions (Fig. 3.4A). Whereas the cyanobacteria provided marginal H_2O_2 degradation by day one, EZ55 provided substantial degradation within the first 12 hours, and could maintain concentrations below 400 nM at all times with total degradation by the end of the day (Fig. 3.4B).

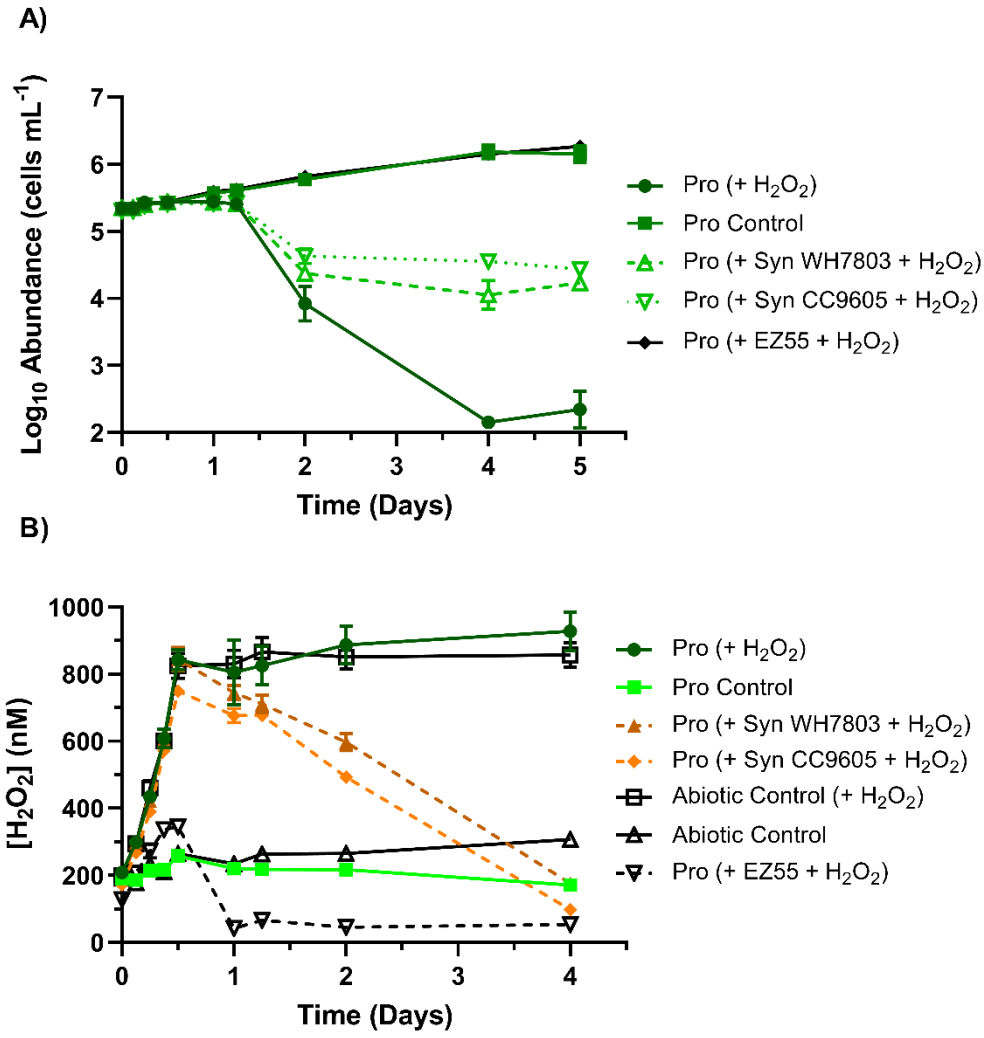

Fig. 3.4. Survival of *Prochlorococcus* with *Synechococcus* After H_2O_2 Diel: Growth of *Prochlorococcus* strain MIT9215 in mono- and coculture with *Synechococcus* strains: WH7803 and CC9605; or *Alteromonas macleodii* strain EZ55 in AMP-A artificial seawater medium exposed to an incremental addition of 800 nM H_2O_2 over the course of the daylight portion of a single diel (A). Concentrations of H_2O_2 in these treatments were quantified, including abiotic controls, over the course of the experiment (B). Error bars represent one standard deviation of the geometric mean (n=3).

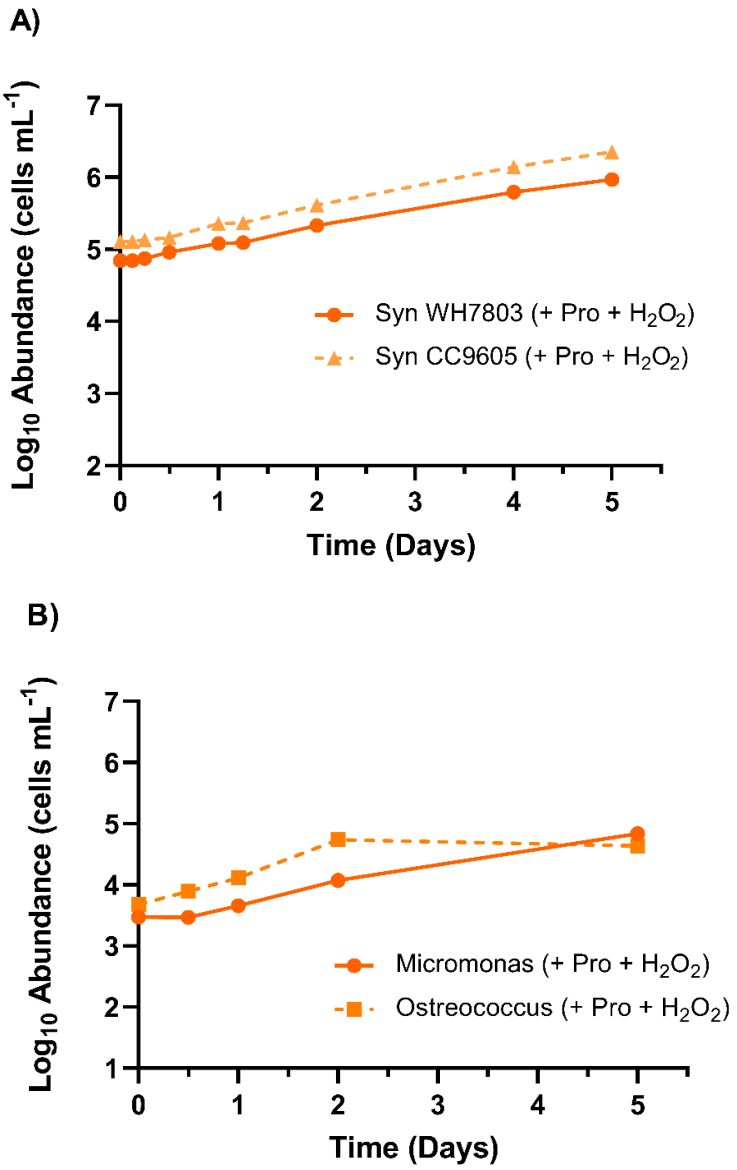

Fig. 3.5. Growth of *Synechococcus* and Picoeukaryotes After H_2O_2 Diel: Growth of *Synechococcus* strains WH7803 and CC9605 (A), *Micromonas commoda* strain RCC299 (B), *Ostreococcus lucimarinus* strain CCMP2972A (B) in coculture with *Prochlorococcus* strain MIT9215 in AMP-A artificial seawater medium exposed to an incremental addition of 800 nM (*Synechococcus*) or ~650 nM (picoeukaryotes) H_2O_2 over the course of the daylight portion of a single diel. Within each legend, parentheses represent the addition to a particular strain / treatment. Error bars represent one standard deviation of the geometric mean (n=3).

This incremental H_2O_2 regime was repeated for cocultures of *Prochlorococcus* with picoeukaryotes *M. commoda* strain RCC299 and *O. lucimarinus* strain CCMP2972A. Growth of these picoeukaryotes was unaffected by the H_2O_2 additions. (Fig. 3.5B). Similar to the cocultures with *Synechococcus* under the same H_2O_2 regime, coculturing with picoeukaryotes limited *Prochlorococcus* mortality to one order of magnitude (Fig. 3.6A). This equivalent outcome was surprising, however, as H_2O_2 degradation was slower (Table 1) than cocultures with *Synechococcus* (Fig. 3.4B, 3.6B), ultimately exposing *Prochlorococcus* to higher concentrations of H_2O_2 over the course of the 5-day experiment (Fig 3.4B, 3.6B). And while less degradation may be explained by a lower abundance of picoeukaryotes, this does not explain how similar instances of protection were observed.

To determine how the incremental addition H_2O_2 regime may have shaped the responses of either *Synechococcus* or picoeukaryotes (i.e., the slow addition of H_2O_2 caused lower rates of or a lag in degradation) we repeated the previous coculture experiments with an immediate addition of 800 nM H_2O_2. Compared to the incremental regime, immediate addition increased the mortality of *Prochlorococcus* in monoculture by ten-fold at day two and resulted in no detectable cells by day five (Fig. 3.7). Coculture with *Synechococcus* (Fig. 3.7A) or picoeukaryotes (Fig. 3.7B-C) resulted in *Prochlorococcus* final abundances of about 1,000 and 10,000 cell mL^{-1}, respectively, however H_2O_2 degradation in these treatments was nearly identical to incremental addition of H_2O_2 (Fig. 3.8B-C).

We found that their degradation rates were not dependent upon the concentration of H_2O_2 or its method of addition, but upon the initial abundance of the helper. A 10-fold increase in starting inoculum of either *Synechococcus* (from 10^4 to 10^5 cells mL^{-1}) or picoeukaryotes (from 10^3 to 10^4 cells mL^{-1}) dramatically increased the rate of degradation, particularly for *Synechococcus* (Fig. 3.8B-C). As a result of greater helper abundance, final coculture abundances of *Prochlorococcus* were roughly ten-fold greater (Fig. 3.8A).

Under each H_2O_2 regime both *Synechococcus* and picoeukaryotes have successfully protected *Prochlorococcus*, however *Synechococcus* consistently showed faster degradation rates and lower final H_2O_2 concentrations. Similar coculture abundances of *Prochlorococcus* can be achieved with either organism, but this result is surprising given the differences in H_2O_2 degradation. It is possible cell-mediated interactions between the picoeukaryotes and

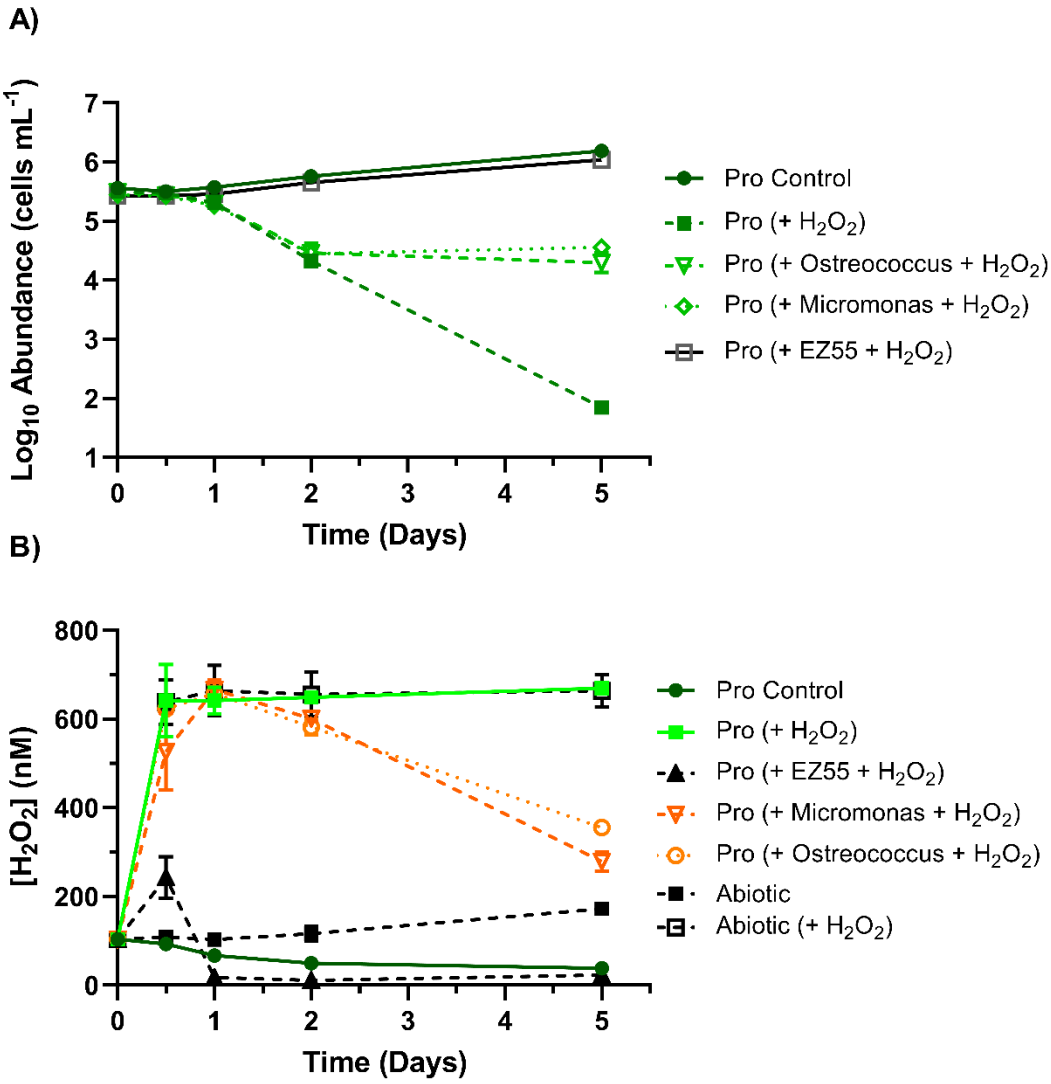

Fig. 3.6. Survival of *Prochlorococcus* with Picoeukaryotes After H_2O_2 Diel: Growth of *Prochlorococcus* strain MIT9215 in mono- and coculture with *Micromonas commoda*, *Ostreococcus lucimarinus*, or *Alteromonas macleodii* strain EZ55 in AMP-A artificial seawater medium exposed to an incremental addition of ~650 nM H_2O_2 over the course of the daylight portion of a single diel (A). Concentrations of H_2O_2 in these treatments were quantified, including abiotic controls, over the course of the experiment (B). Error bars represent one standard deviation of the geometric mean (n=3).

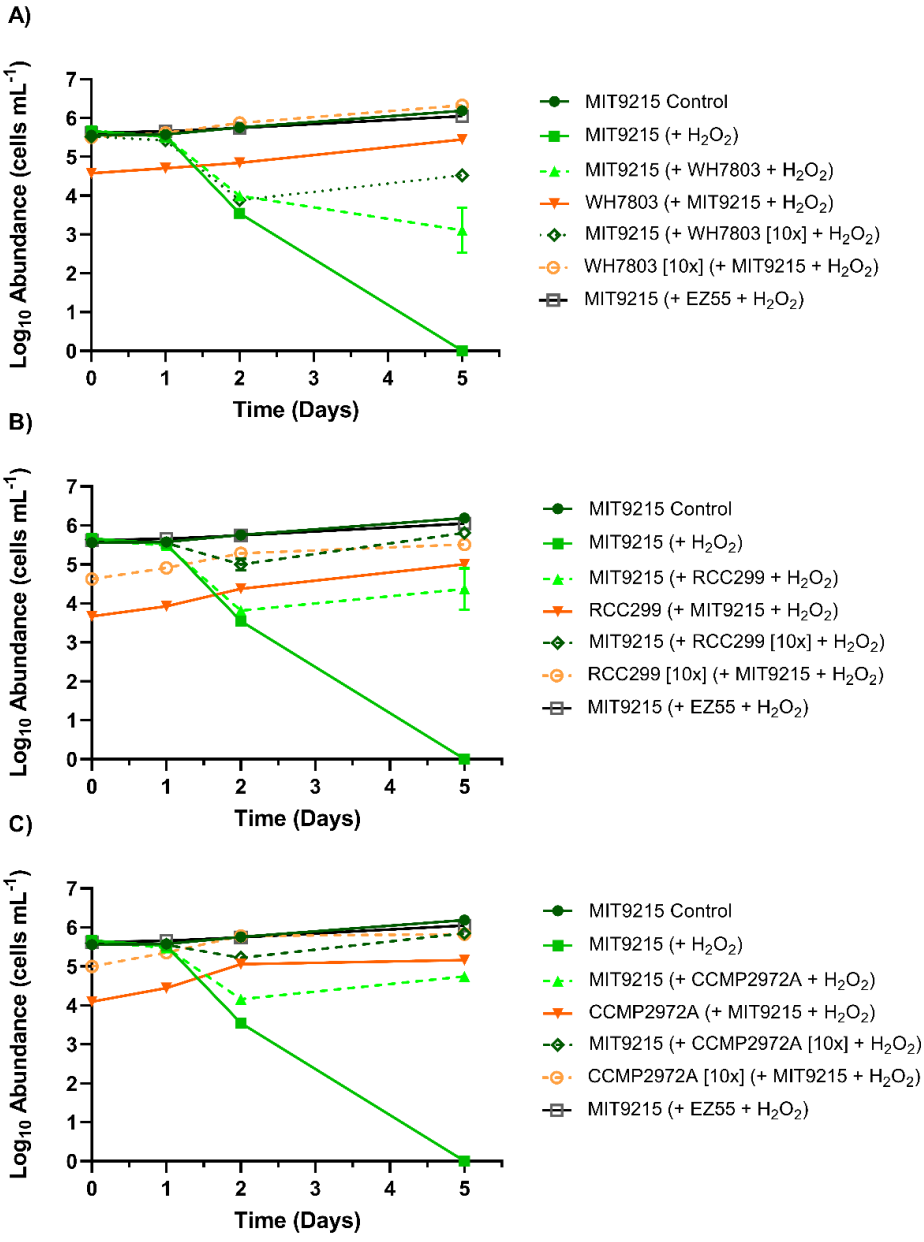

Fig. 3.7. Survival of Prochlorococcus after Instantaneous Addition of 750 nM H_2O_2: Growth of *Prochlorococcus* strain MIT9215 in mono- and coculture with *Synechococcus* strain WH7803 (A), *Micromonas commoda* strain RCC299 (B), *Ostreococcus lucimarinus* strain CCMP2972A (C), or *Alteromonas macleodii* strain EZ55 (all panels) in AMP-A artificial seawater medium exposed to an instantaneous addition of ~750 nM H_2O_2 (A). The initial abundance of photosynthetic helpers was either 1x or 10x ([10x]) their ecologically relevant abundance. Within each legend, parentheses represent the addition to a particular strain / treatment. Error bars represent one standard deviation of the geometric mean (n=3).

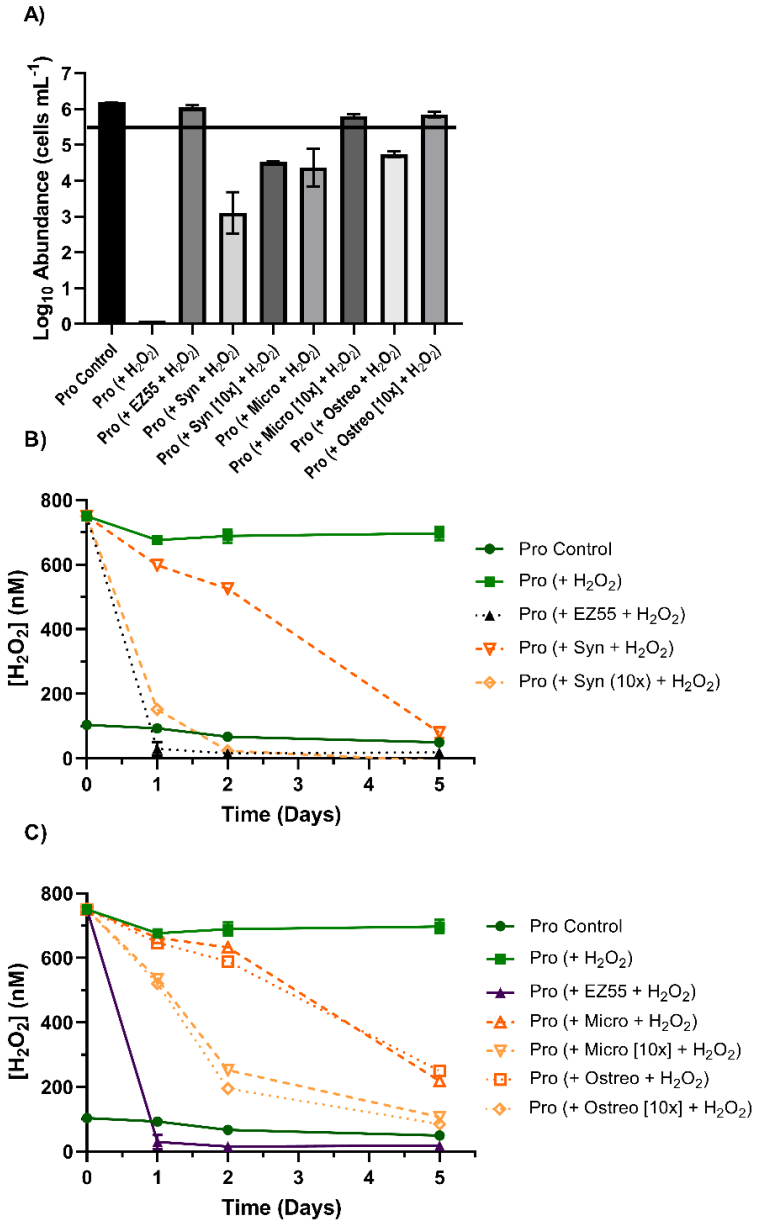

Fig. 3.8. Effect of Instantaneous H_2O_2 Addition and Increased Helper Abundance: Final abundances of *Prochlorococcus* strain MIT9215 in mono- and coculture with *Synechococcus* strain WH7803, *Micromonas commoda*, *Ostreococcus lucimarinus*, or *Alteromonas macleodii* strain EZ55 in AMP-A artificial seawater medium exposed to an instantaneous addition of ~750 nM H_2O_2 (A). The initial abundance of photosynthetic helpers was either 1x or 10x ([10x]) their ecologically relevant abundance. The initial abundance of *Prochlorococcus* (10^5 cells mL^{-1}) is depicted by a horizontal black line. Concentrations of H_2O_2 were quantified over the course of the experiment for cocultures with *Synechococcus* (B) and picoeukaryotic phytoplankton (C). Error bars represent one standard deviation of the geometric mean (n=3).

Prochlorococcus, in addition to or in lieu of H_2O_2 degradation, caused their increased abundance. To address this possibility we exposed monocultures of picoeukaryotes to H_2O_2, then removed these cells by filtration after one, two, three, and six days of growth (Fig. 3.9B). *Prochlorococcus* mortality when cultured in media pre-conditioned for one, two, and three days by picoeukaryotes (Fig. 3.10B) was similar to the *Prochlorococcus* + H_2O_2 controls (Fig. 3.9A and 3.10A). Only culturing in media pre-conditioned by picoeukaryotes for six days significantly reduced *Prochlorococcus* mortality, with a final abundance only ten-fold lower than the starting inoculum (Fig. 3.10B). Importantly, day six of pre-conditioning was the first time H_2O_2 concentrations fell to sublethal levels for *Prochlorococcus* (~200 nM) (Fig. 3.10C). After addition of *Prochlorococcus* to pre-conditioned media H_2O_2 concentrations remained constant and no degradation was observed (Fig. 3.9C-D). When extrapolated to the previous coculture experiments, this data suggests that H_2O_2 concentrations experienced by *Prochlorococcus* during the first three days of coculture with picoeukaryotes are sufficient to later reduce *Prochlorococcus* from 10^5 to $\leq 10^2$ cells mL^{-1}. Final coculture abundances observed at the end of previous experiments were consistently $\geq 10^4$ cells mL^{-1}, presenting the possibility of a cell-mediated interaction between *Prochlorococcus* and picoeukaryotic phytoplankton that reduced mortality in H_2O_2 exposed cocultures.

Discussion

Protection of *Prochlorococcus* by H_2O_2 degrading "helper" heterotrophs has been demonstrated in several previous studies; however, prior to this study contributions of photosynthetic microbes have only been suggested from field observation (Morris et al., 2011, Morris et al., 2016, Morris et al., 2008). Here we showed evidence that cooccurring cyanobacteria (*Synechococcus*) and picoeukaryotic phytoplankton (*Micromonas* and *Ostreococcus*) can protect *Prochlorococcus* when cocultured together at or near environmentally-observed abundances. Importantly, this protection occurred under both instantaneous and incremental H_2O_2 increases that simulated the rainfall and photochemical production sources, respectively, that operate in the open ocean (Morris et al., 2016).

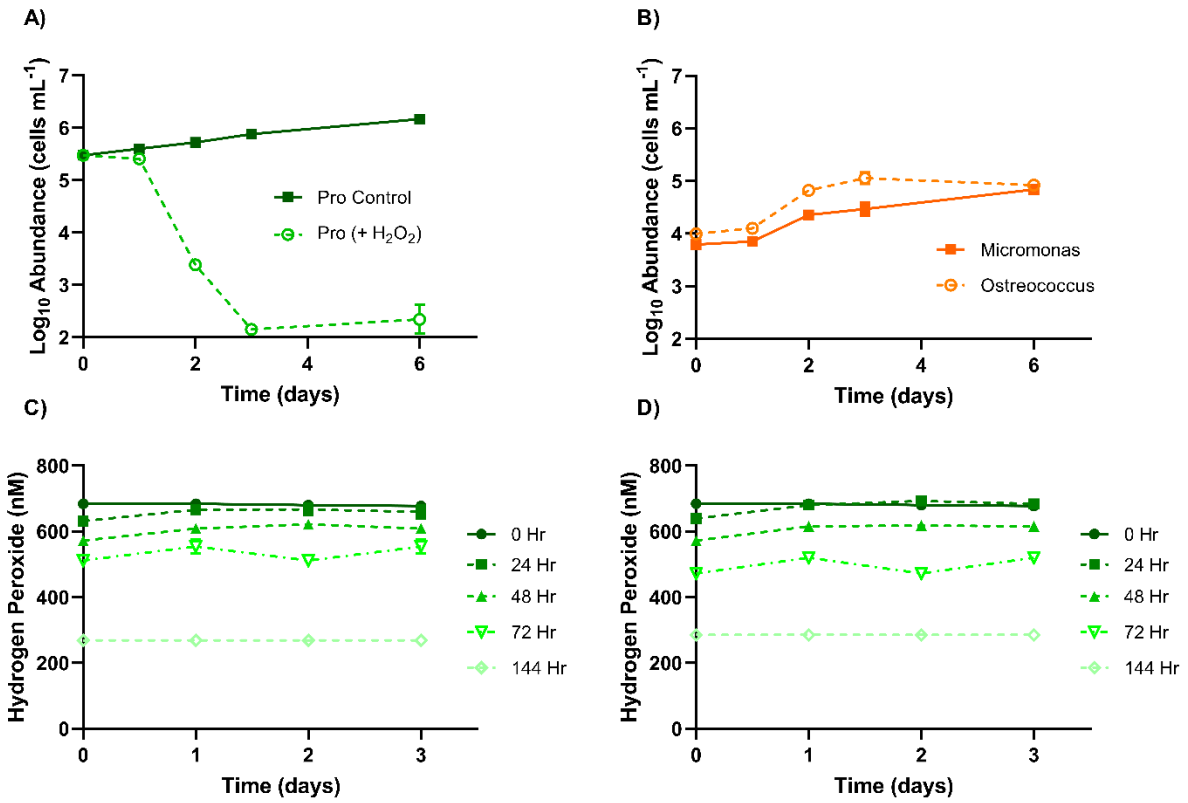

Fig. 3.9 Controls for Prochlorococcus Growth in Picoeukaryote Conditioned Media: Growth of *Prochlorococcus* strain MIT9215 (A), *Micromonas commoda* strain RCC299 (B), *Ostreococcus lucimarinus* strain CCMP2972A (B) in monoculture in AMP-A artificial seawater medium after instantaneous addition of ~700 nM H_2O_2. Concentrations of H_2O_2 quantified for monocultures of *Prochlorococcus* strain MIT9215 in media pre-conditioned by *Micromonas commoda* strain RCC299 (C), *Ostreococcus lucimarinus* strain CCMP2972A (D) for 0, 1, 2, 3, and 6 days. Within each legend, parentheses represent the addition to a particular strain / treatment. Error bars represent one standard deviation of the geometric mean (n=3).

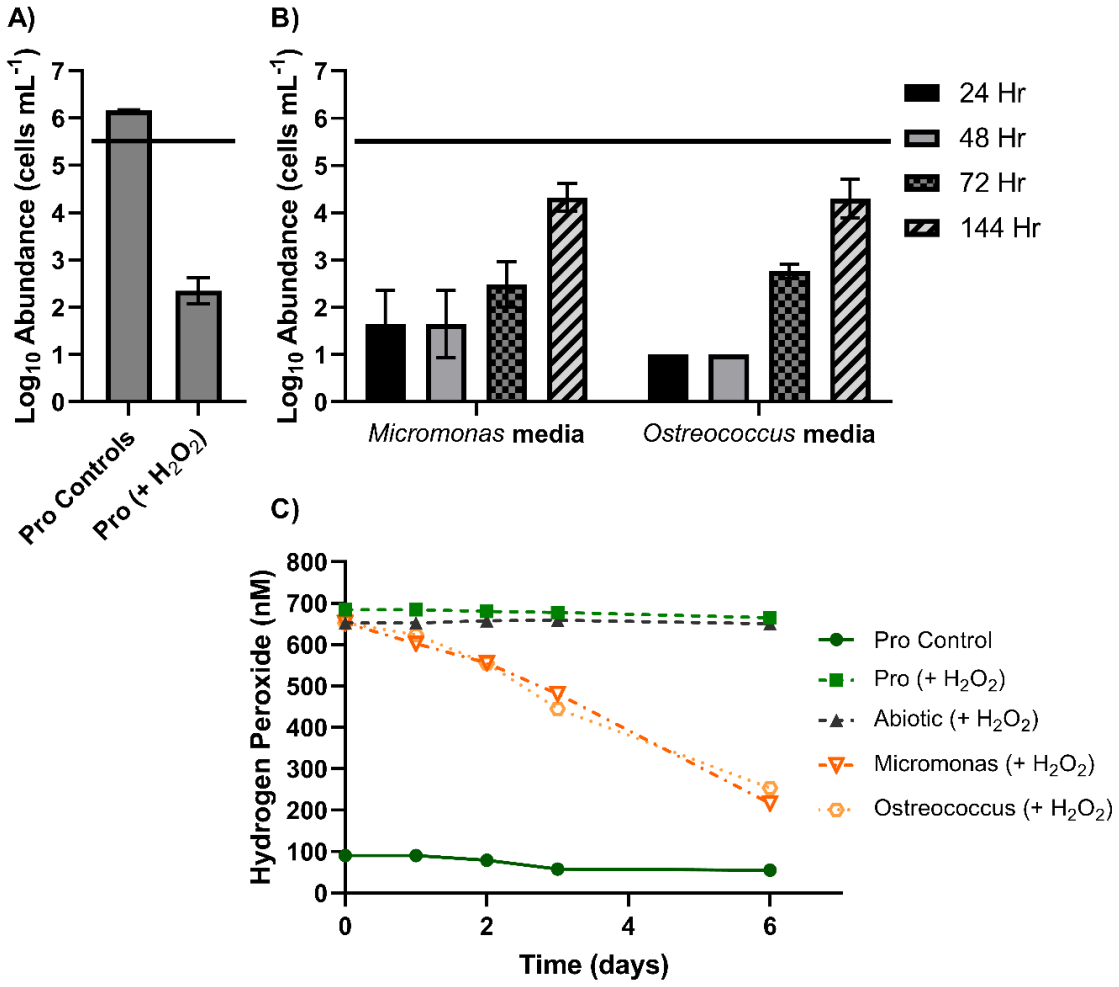

Fig. 3.10. *Prochlorococcus* Survival in Picoeukaryote-Conditioned Media: Final abundances of *Prochlorococcus* strain MIT9215 in monoculture in AMP-A artificial seawater medium ± instantaneous addition of ~700 nM H_2O_2 (A) or pre-conditioned by the growth of *Micromonas commoda* or *Ostreococcus lucimarinus* for 0, 1, 2, 3, or 6 days post instantaneous addition of ~700 nM H_2O_2 (B). The initial abundance of *Prochlorococcus* (10^5 cells mL^{-1}) is depicted by a horizontal black line. Concentrations of H_2O_2 were quantified over the course of the experiment for *Prochlorococcus* and abiotic controls, and during growth of picoeukaryotes (C). Error bars represent one standard deviation of the geometric mean (n=3).

Responses to H_2O_2 exposure

Synechococcus and the picoeukaryotic phytoplankton *Micromonas* and *Ostreococcus* showed marked differences in H_2O_2 degradation when cultured at ecologically relevant abundances. These experiments were designed to test the protective effect when the phytoplankters were set at their natural abundances in the oligotrophic ocean (Flombaum et al., 2013, Visintini et al., 2021); as such, initial *Synechococcus* abundance was 10 times higher than picoeukaryote abundance. Populations of *Synechococcus* consistently showed higher rates of H_2O_2 degradation than either *Micromonas* or *Ostreococcus* during incremental and instantaneous addition (650-800 nM) experiments. This result implied that *Synechococcus* would be responsible for more environmental H_2O_2 degradation in regions where their abundance matches these dynamics. In support of this result, prior studies have shown that at the open ocean station ALOHA a greater number of catalase-peroxidase transcripts (cyanobacteria and other prokaryotes) were detected compared to transcripts of ascorbate peroxidase (eukaryotic microbes) (Morris et al., 2016, Ottesen et al., 2014). However, in this study, *Synechococcus* and picoeukaryotes displayed equivalent rates of degradation when equal abundances were exposed to instantaneous addition of 300 nM but not of 80 nM. This result reinforced that contribution to overall degradation was directly proportional to abundance, but also suggested that *Synechococcus* could detect and reduce lower concentrations of H_2O_2. Past field and laboratory studies reinforce our results through observations that, compared to other green algae (Chlorophyceae), marine *Synechococcus* detected and responded to H_2O_2 concentrations two orders of magnitude lower (Leunert et al., 2013) and showed significantly higher rates of degradation (Wong et al., 2003, Petasne and Zika, 1997). As H_2O_2 concentrations in the open ocean mixed layer are often between 100-200 nM (Cooper et al., 1987, Hanson et al., 2001, Miller and Kester, 1994, Morris et al., 2011, Yuan and Shiller, 2001), the interactions that allow consistent concentrations require further study.

A particularly interesting observation of these experiments was that while less H_2O_2 degradation was observed in cocultures with ecologically relevant abundances of picoeukaryotes than with *Synechococcus*, *Prochlorococcus* achieved similar final abundances in both treatments. However, the full protection from the picoeukaryotes may involve cell-cell interactions, as lower final abundance of *Prochlorococcus* was observed if instead cultured alone in media pre-

conditioned by picoeukaryote growth with identical H_2O_2 addition dynamics. Further studies are required to elucidate this additional mechanism(s).

A limitation of our culturing system should be acknowledged in that while degradation of H_2O_2 and survival of *Prochlorococcus* were tracked for five to eight days, only a single day equivalent of abiotic H_2O_2 production was added on the first day. As it took nearly three to five days for photosynthetic helpers to lower H_2O_2 concentrations below lethality, it is likely protection would not occur if H_2O_2 additions (650-800 nM) had been made daily throughout the experiment. While not solely able to protect *Prochlorococcus* from H_2O_2 abiotically produced on a daily timescale, our data suggests that ecologically relevant populations of cyanobacteria and picoeukaryotic phytoplankton likely provide a significant contribution to community level degradation of H_2O_2 in the open ocean. Using per cell degradation rates calculated in this study and observed environmental abundances of heterotrophic bacteria (10^5) (Church et al., 2002, Johnson, 2013, Li, 1998), *Synechococcus* (10^4), and picoeukaryotes (10^3) (Flombaum et al., 2013, Visintini et al., 2021), the contribution of the two phytoplankton groups was one-third that of heterotrophs (Fig. 3.11A). However when compared with the abundance of the individual heterotroph *Alteromonas macleodii* (conservatively estimated at 10^4) (Beardsley et al., 2003, Eilers et al., 2000, Pedler et al., 2014), the contribution of Synechococcus was double that of the heterotroph, which was now equal to that of the picoeukaryote (Fig. 3.11B). Comparison of per cell degradation rates determined the contribution of individual picoeukaryote cells was significantly greater than the other groups (Fig. 3.11C). Many prior studies have focused on the beneficial interactions between autotrophs and heterotrophs (Beliaev et al., 2014, Biller et al., 2016, Christie-Oleza et al., 2017, Kaur et al., 2018, Sher et al., 2011) but additional focus on mutual or commensal interactions between members of the photosynthetic community, specifically those involving *Prochlorococcus*, will help to elucidate their ecology (Knight and Morris, 2020).

Ecological significance

The Black Queen Hypothesis describes evolutionary outcomes where an increase in fitness or a decrease in physiological costs occurs by utilizing the leaky nature of another's activities, such as nutrient acquisition, polymer degradation, or environmental detoxification (Morris et al., 2012, Morris et al., 2014, Morris, 2015). By challenging these organisms to

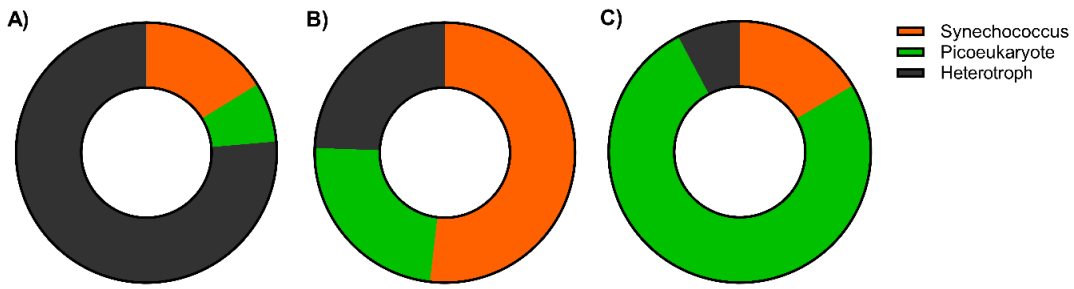

Fig. 3.11. Community H_2O_2 Degradation: Comparison of contributions of H_2O_2 degradation rates (nM day^{-1} $cell^{-1}$) of *Synechococcus*, picoeukaryotic phytoplankton (*Micromonas* and *Ostreococcus*), and heterotrophic bacteria (*Alteromonas*) to total degradation, assuming: environmental abundances of *Synechococcus* at 10^4, picoeukaryotes at 10^3, and *Alteromonas* at 10^5 cells ml^{-1} (proxy for total heterotroph) (A), same abundances of phytoplankton but *Alteromonas* at 10^4 cells ml^{-1} (B), and organisms at a relative abundance of 1:1:1 (C).

protect *Prochlorococcus* from H_2O_2 concentrations that represent the upper limit of abiotic production, we determined the likelihood of these activities occurring in the natural environment. Heterotrophic bacteria can benefit by providing this leaky function (H_2O_2 degradation), as live *Prochlorococcus* cells will exude organic carbon and cycle nutrients necessary for their growth (Sachs and Hollowell, 2012). The long-term coexistence of autotroph-heterotroph pairs has been observed, showing a propensity for mutualistic interactions based upon nutrient transformation and environmental conditioning (Christie-Oleza et al., 2017, Kaur et al., 2018). Yet, presumably devoid of benefits outside self-preservation, populations of *Synechococcus*, *Micromonas*, and *Ostreococcus* all expressed the enzymes for sufficiently leaky H_2O_2 degradation and protected *Prochlorococcus* from H_2O_2-mediated mortality. Recent observations of abundance and prevalence of these phytoplankton alongside *Prochlorococcus* across open ocean environments provide evidence for this interaction's occurrence and importance for survival (Flombaum et al., 2013, Visintini et al., 2021). It is interesting that such different groups of microbes would retain a leaky function also expressed by others, even when with or without a benefit other than H_2O_2 defense. Previously the maintenance of a leaky function in helpers was thought not to be due to selective benefits caused by protection of others but rather by cheaters (ex: *Prochlorococcus*) "race to the bottom", acquiring a fitness benefit due to loss of function (Morris, 2015, Morris et al., 2012). However recent work has proposed that determination of helpers, those that retain a leaky function, is selected by the efficiency with which the function is performed, implying that a helper would be the organism best fit to perform a particular task allowing the loss of function in other organisms (Adkins-Jablonsky et al., 2021). Still, additional work is needed to determine if identical evolutionary forces are acting upon members of different trophic levels.

Influence upon competition outcomes

While our scope was to determine if protection could be observed in laboratory coculture, the data could be extrapolated to better define the interactions of these organisms in natural environments. In Chapter 2 the ability of *Prochlorococcus* to outcompete and achieve higher abundances than other members of the photosynthetic community was highlighted, either through direct competition or by stimulating heterotrophic bacteria nutrient uptake via exudate. However, outside of competition for nutrients, the interactions between photosynthetic microbes that influence their population dynamics have not been well defined. We predict the production

of these nitrogen and trace metal demanding enzymes, catalase-peroxidase or ascorbate peroxidase for *Synechococcus* and picoeukaryotic phytoplankton respectively, represents a net positive interaction for *Prochlorococcus* and a necessary but two-fold detriment for others: production consumes internal nutrient stores, requiring higher cell quotas, and H_2O_2 degradation promotes the survival of their competitor, *Prochlorococcus*.

As predicted for *Prochlorococcus*, fitness increase due to catalase loss has been demonstrated through experimental evolution studies where populations of with or without catalase, but otherwise genetically identical, *Escherichia coli* were competed to show that expression lowered fitness. Coexistence of these populations occurred only when exposed to H_2O_2, emphasizing that costs of expression were offset by shared necessity of catalase function (Morris et al., 2014).

Additionally, many of the most abundant microbes, including heterotrophic bacteria and strains of marine *Synechococcus* (WH8102), in aquatic environments exhibit similar losses of ROS detoxifying or stress response enzymes, hinting that these evolution trajectories have resulted from differing circumstances and trophic levels (Braakman et al., 2017, Gilbert and Fagan, 2011, Giovannoni et al., 2005, Morris et al., 2012, Giovannoni et al., 2014). It is difficult to determine to what extent this interaction influences the numerical dominance of *Prochlorococcus* over these groups or their mutual coexistence, but this represents an interesting direction for future work.

Conclusion

Here we have established a co-culturing system wherein the abundances of and H_2O_2 degradation by multiple groups of cyanobacteria and picoeukaryotic phytoplankton can be observed. We determined that open ocean populations of *Synechococcus*, *Micromonas*, and *Ostreococcus* are all capable of protecting *Prochlorococcus* from exposure to lethal concentrations of H_2O_2, and likely contribute significantly to the degradation activity of the entire microbial community. The ramifications of these results are significant as it is possible the observed interactions determine the environmental abundances of *Prochlorococcus*, *Synechococcus*, and picoeukaryotic phytoplankton by affecting overall fitness or establishing dependencies between populations.

CHAPTER 4

Seasonal distribution of *Prochlorococcus* ecotypes throughout the North Pacific Subtropical Gyre and interactions with surrounding microbial community

Publication Note

This chapter is being prepared for publication. Authors are listed below.

Contributions

Experiments were designed by Benjamin Calfee and Erik Zinser; samples for QPCR were collected by Erik Zinser and processed and analyzed by Benjamin Calfee and samples for 16S rRNA analysis were collected, processed, and libraries were prepared by Jackson Gainer and Steven Wilhelm; data analysis was performed by Benjamin Calfee and T.J. Rogers.; and Benjamin Calfee and Erik Zinser crafted manuscript.

Acknowledgements

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Abstract

Cyanobacterium of the genus *Prochlorococcus* are the most abundant photosynthetic organisms on the planet. This genus is subdivided into phylogenetically distinct clades, termed ecotypes, that each demonstrate varying optima for temperature, light availability, and nutrients. Prior observations of ecotype distributions have determined that the diversity within and adaptations of these ecotypes are directly related to their physical environment, suggesting these differentiations are responsible for their vast biogeographical range and presence throughout the entire euphotic zone. Studies have observed the specific physical parameters that dictate ecotype abundance over latitudinal ranges, but the effect of seasonal variability within an environment and over large spatial regions, such as an oceanic gyre, on *Prochlorococcus* ecotypes is not well characterized. We sought to determine the effect of seasonal variability upon both high and low light adapted *Prochlorococcus* ecotypes spanning the 200m deep euphotic zone from the North Pacific Subtropical Gyre during the winter and summer seasons. We determined that, while temperature was still an important factor, water column stratification contributed significantly to determining the abundance of high light ecotypes between summer and winter, and rival phytoplankton abundance had a negative impact on all ecotypes except eMED4. Additionally unique correlations were observed between high and low light ecotype pairs and between abundant microbial phyla and individual ecotypes, which displayed some aspects of seasonality and suggests possible interactions of ecological importance that will merit further study.

Introduction

Cyanobacteria of the genus *Prochlorococcus* are the most abundant photosynthetic organisms on the planet, are present in almost all open ocean environments from 40 °N to 40 °S (Campbell et al., 1997, Flombaum et al., 2013, Partensky et al., 1999). The genus has been subdivided into phylogenetically distinct groups, termed ecotypes, which all exhibit optima for various environmental parameters such as temperature, light availability, and nutrients (Chandler et al., 2016, Johnson et al., 2006, Rocap et al., 2003, Zinser et al., 2007). While the overall success of this organism has been attributed to genomic and physiological adaptations associated with streamlined, oligotrophic organisms (Bragg, 2011, Dufresne et al., 2005, Ting et al., 2007, Van Mooy and Devol, 2008), it is likely the significant diversity of ecotypes within the genus provide an advantage as well.

Ecotypes of *Prochlorococcus* are present throughout the entire euphotic zone and many studies have helped to resolve their distributions. The ecotypes are typically divided into high-light (HL) and low-light (LL) groups that differ significantly in their preference for light availability, and thus occupy distinct depth regions of the water column (Johnson et al., 2006, Zinser et al., 2007). Major distinction of the HL ecotypes has been resolved over a latitudinal scale, with HLII (eMIT9312; e = ecotype, MIT9312 = type strain) being dominant in warmer, lower latitude waters and HLI (eMED4) dominant in colder, higher latitude waters of the subtropics (Chandler et al., 2016, Larkin et al., 2016). Ecotypes residing low in the euphotic zone and demonstrating sensitivity to sunlight make up the LL ecotypes, of these notably are LLI (eNATL2A) and LLIV (eMIT9313). While eMIT9313 is confined to water below the surface mixed layer, eNATL2A is often present within the surface mixed layer during periods of deep mixing and low stratification (Malmstrom et al., 2010). These LL ecotypes are less sensitive to temperature and have relatively stable abundances over latitudinal regions, but display diversity at the ecotype and sub-ecotype level similar to the surface adapted groups of *Prochlorococcus* (Thompson et al., 2021).

Many studies have focused on the relationship between the HL ecotypes and temperature (Chandler et al., 2016, Larkin et al., 2016), LL ecotype distributions (Thompson et al., 2021), HL and LL distributions across single season transects (Johnson et al., 2006, Zinser et al., 2007), or seasonal variability at stationary timeseries (Malmstrom et al., 2010, Thompson et al., 2018),

however lacking are studies of effects of seasonal variability on the abundance and distribution of both HL and LL ecotypes. To fill this knowledge gap we quantified the abundance of four *Prochlorococcus* ecotypes (HL eMED4 and eMIT9312; LL eNATL2A and MIT9313) in samples collected from 50 stations during two basin-scale transects across the North Pacific Subtropical Gyre (NPSG) during the winter and summer seasons. Seasonal variability during this time showed slight increases in summer temperatures but manifested most dramatically in differences between winter and summer stratification. Increased stratification in the summer reduced surface mixed layer depths, and this had a detrimental effect on depth-integrated abundance and latitudinal range of eMIT9312. We observed novel correlations between the abundance of individual HL and LL ecotype pairs, hinting at environmental factors affecting spatially separated groups. And lastly, we determined significant correlations between abundant microbial phyla and individual ecotypes, suggesting the potential for ecotype specific interactions with the microbial community.

Materials and Methods

QPCR Sample and Oceanographic Data Collection

Samples were collected from 50 stations (26 winter and 24 summer) during two basin-scale oceanographic cruises (Phytoplankton of Warming Ocean Waters – POWOW2: 10 January – 8 February 2013; POWOW3: 1 July – 28 July 2013) in the North Pacific Subtropical Gyre, transects of which have been previous described and depicted (Gainer et al., 2017, Larkin et al., 2016). Sample water (100 mL per replicate filter) was collected using a Niskin rosette system from 5, 15, 25, 50, 75, 100, 125, 150, and 200 meters at the same local time at each station. CTD measurements (conductivity, temperature, density, chlorophyll *a* fluorescence, and photosynthetically active radiation) were obtained for every station. Nutrient concentrations (NH_4, NO_3, NO_2, PO_4, and $SiOH_4$) and flow cytometry counts (total bacteria, *Prochlorococcus*, *Synechococcus*, and picoeukaryotic phytoplankton) were quantified as previously described (Johnson, 2013, Larkin et al., 2016). Isolation, processing, and library preparation of 16S rRNA samples was performed previously (Gainer et al., 2017).

Ecotype Abundance Quantification

Cryopreserved sample filters from each depth and station were processed and ecotype abundances were quantified as previously described (Chandler et al., 2016, Zinser et al., 2006). Briefly, cellular DNA was isolated via physical disruption by bead beating (without beads) samples at 4,000 RPM for 2 minutes after addition of 650 µL Tris buffer (pH 8.0) using a Mini-Bead Beater (Biospec Products, Bartlesville, OK) and heat lysis (15 minutes at 95 °C) after removal of cells from filter. 500 µL of this sample were transferred to a new 1.5 mL Eppendorf tube and stored at -80 °C. QPCR reactions were performed using 10 µL of sample or standard and Quantitect SYBR Green PCR master mixture (QIAGEN, Hilden, Germany), and ecotype abundances were determined by comparing C_T values of samples against those of standards made cell cultures. Primers used to quantify each ecotype were as described previously (Chandler et al., 2016).

Multivariate Analysis

Log transformation and Pearson correlation of data were performed using GraphPad Prism version 9.2.0 for Windows (GraphPad Software, San Diego, California USA), principal components were selected by parallel analysis. Ecotype and 16S rRNA datasets were Hellinger transformed before Canonical Correspondence Analysis and Multiple Linear Regression in R. Code and data frames are accessible at: https://github.com/TJrogers86/distribution_of_Prochlorococcus_ecotypes.git

Results

During the winter and summer of 2013, samples from the North Pacific Subtropical Gyre (NPSG) were collected and analyzed from basin-scale transects that spanned latitudes from 22.75 to 46.69 °N. Winter stations showed surface ocean temperatures from 10.03 to 23.52 °C and mixed layer depths from 69.00 to 139.00 meters. While little difference in surface temperatures were observed at summer stations compared to winter (11.07 to 25.52 °C), higher degrees of stratification compared to the winter caused significantly shallower surface mixed layer depths (3.00 to 42.00 meters).

Maximal *Prochlorococcus* abundance was greater in the summer (Fig. 4.1A), but average mixed layer abundance differed little between winter and summer ($1.41e^5$ cells mL^{-1} and $1.55e^5$ cells mL^{-1}, respectively). For both seasons *Prochlorococcus* abundance was correlated positively with temperature (Pearson r = 0.712, p <0.0001) and negatively with latitude (Pearson r = -0.616, p <0.0001), however these correlations broke down as we examined the abundance of individual ecotype groups. In almost all samples (stations and depths) from both seasons *Prochlorococcus* outnumbered the picocyanobacteria *Synechococcus* 10 to 100-fold (Fig. 4.1B) and picoeukaryotic phytoplankton 10 to 1,000-fold (Fig. 4.1C). These two groups of photosynthetic microbes only achieved greater abundance than *Prochlorococcus* at higher latitude or lower depths where temperatures were not permissive to *Prochlorococcus* growth, evidenced as *Synechococcus* and picoeukaryote abundance was negatively correlated with temperature (Pearson r = -0.553 and -0.768, p <0.0001).

Winter Ordination

We performed canonical correspondence analyses (CCA) to ordinate our samples from each season and assess the variation contributed by specific variables and ecotype abundances. We analyzed the effect of environmental variables on both site (Fig. 4.2A and 4.3A) and ecotype (Fig. 4.2B and 4.3B) ordination. The significance of the winter CCA model was determined by ANOVA (F =52.319, p = 0.0001) and utilized a combination of temperature, light attenuation, depth, and picoeukaryote and *Synechococcus* abundance to account for 45.88% of total variance. Samples at depths within the surface mixed layer clustered but were divided by the differing abundance of eMIT9312 and eMED4 and the environmental variables that influenced them (Fig. 4.2A). Samples skewed toward eMIT9312 were influenced by higher temperatures and lower rival phytoplankton (*Synechococcus* and picoeukaryotes) abundance at lower latitudes. Samples skewed away from the eMIT9312 node clustered equally between the eMED4 and eNATL2A nodes, suggesting potential similarities between these samples, or toward the eMIT9313 node. The latter samples were associated with the depths below the surface mixed layer. CCA plots defining the relationship between ecotype nodes and environmental variables reinforced these trends (Fig. 4.2B). Maximum eMIT9312 abundances co-occurred with smaller populations of rival phytoplankton, suggesting competition or an incompatibility of preferred environment. The proximity of the eMED4 and eNATL2A nodes reinforced the relationship observed in the

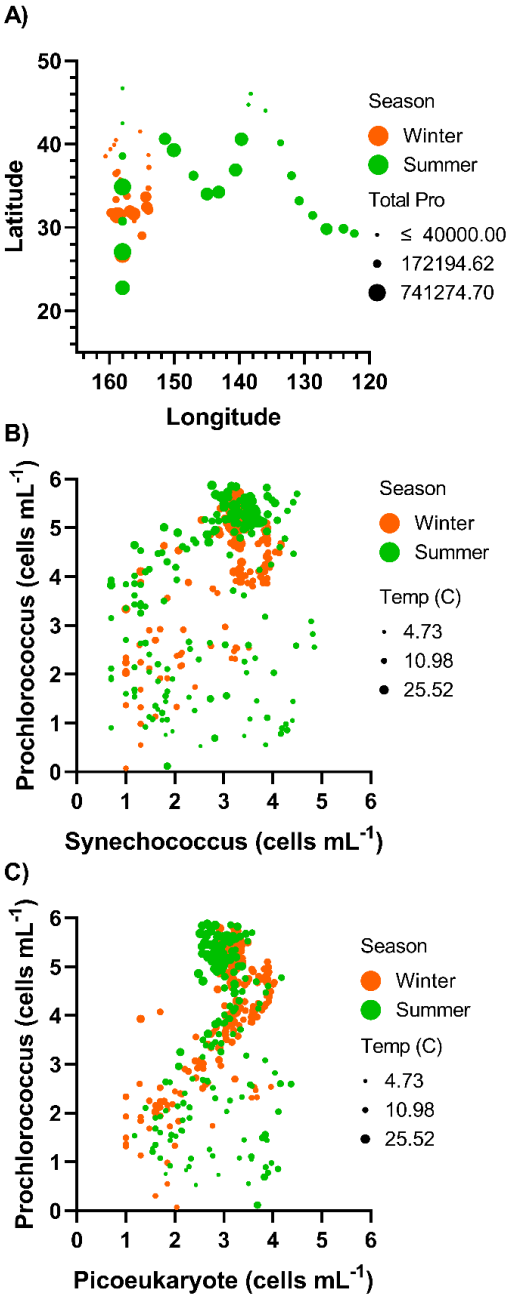

Fig. 4.1. General *Prochlorococcus* Abundance: Comparison of maximum *Prochlorococcus* euphotic zone abundance at each station of winter and summer cruise transects (A), and of *Prochlorococcus* abundance at every measured station and depth to the abundance of *Synechococcus* (B) or picoeukaryotic phytoplankton (C). *Prochlorococcus* abundance was calculated as the sum of four measured ecotypes, while *Synechococcus* and picoeukaryotes were quantified via FCM.

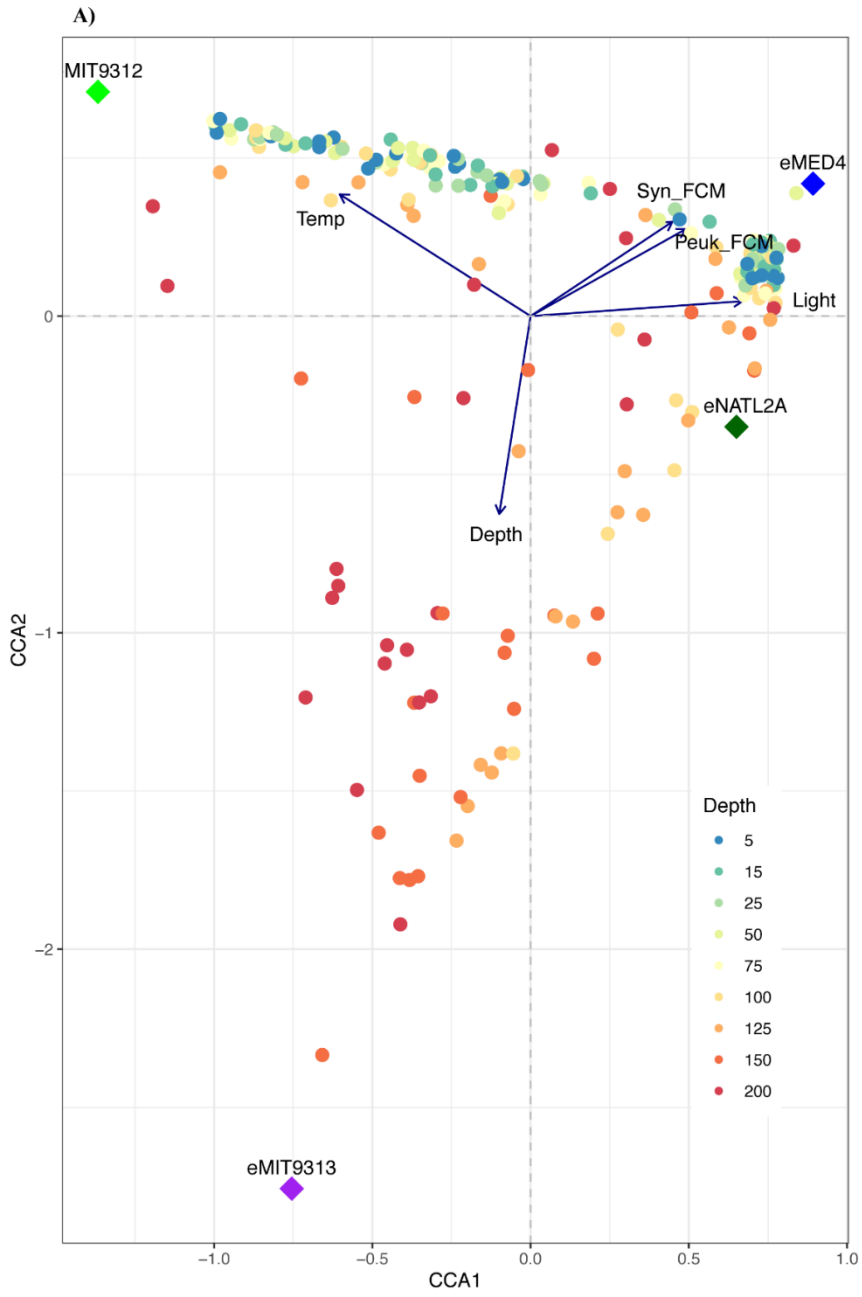

Fig. 4.2. Winter CCA: Canonical correspondence analysis of winter samples (circles), colored by depth. Sample focused - Placement of samples along a variable vector represents the combination and value of factors that best describe that sample. Proximity of samples to ecotype nodes (diamonds) represents the magnitude of ecotype abundance (A). Ecotype focused – Placement of ecotype nodes along a variable vector represents the combination and value of factors that best describe a particular ecotypes distribution / optimal abundance (B).

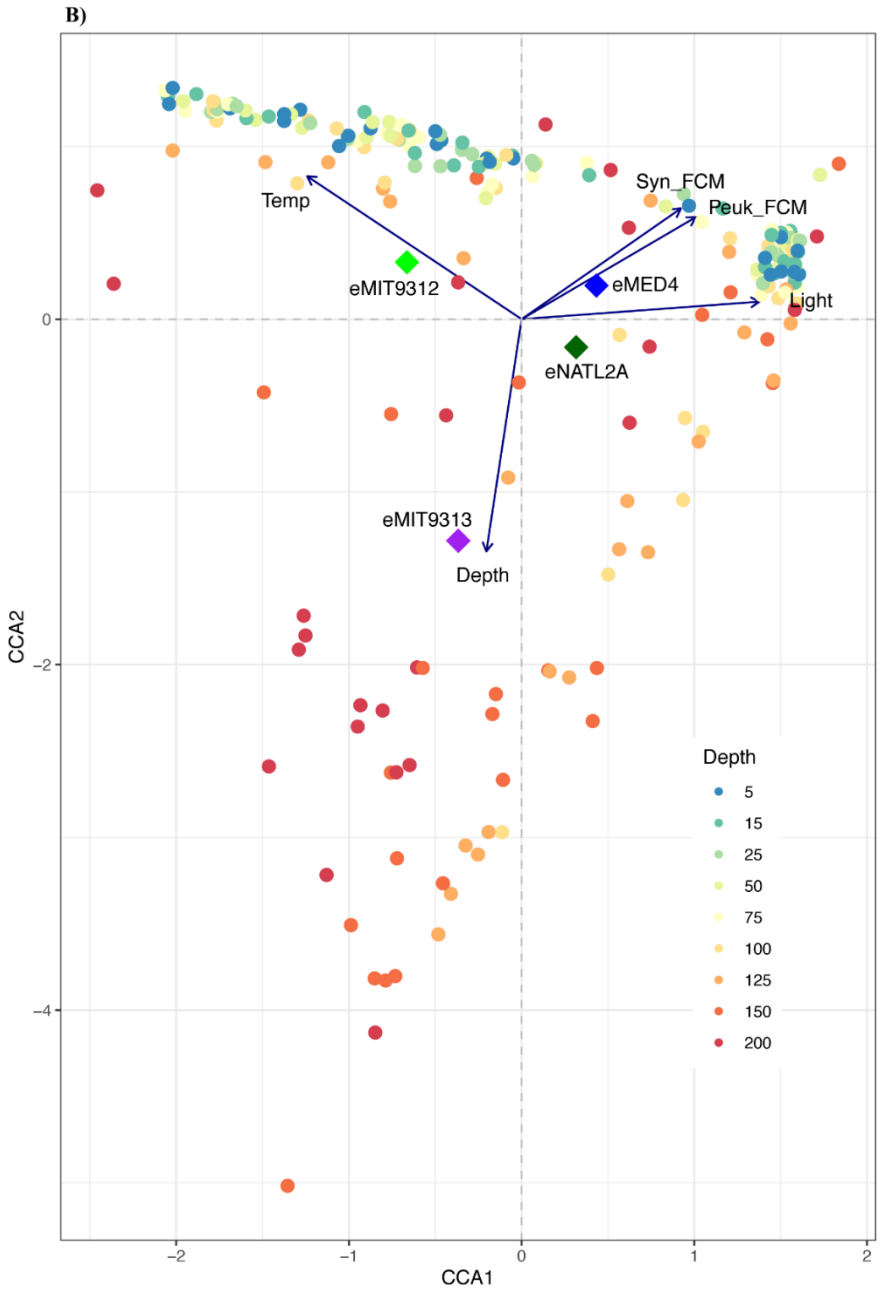

Fig. 4.2. Continued.

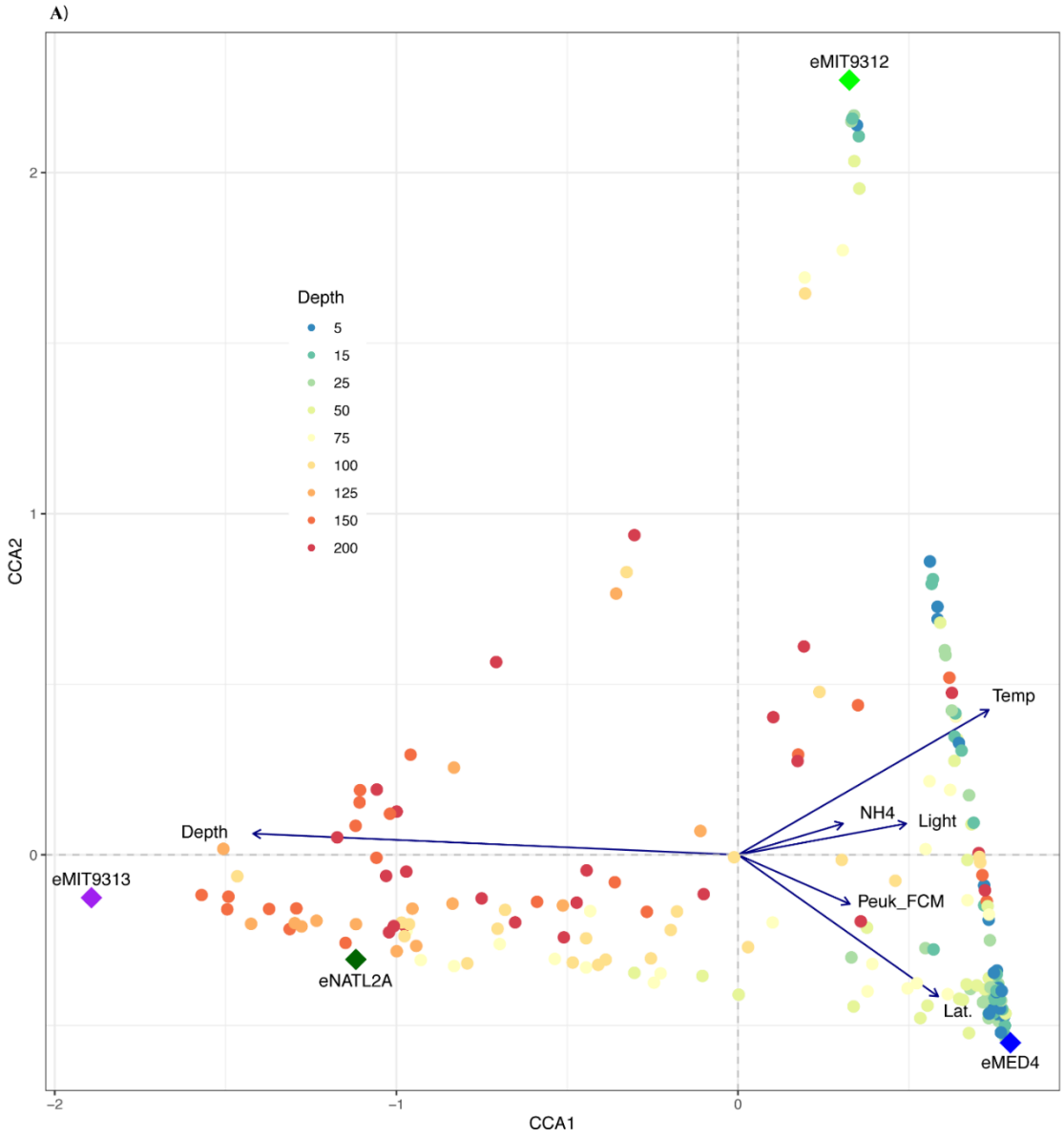

Fig. 4.3. Summer CCA: Canonical correspondence analysis of summer samples (circles), colored by depth (meters). Sample focused - Placement of samples along a variable vector represents the combination and value of factors that best describe that sample. Proximity of samples to ecotype nodes (diamonds) represents the magnitude of ecotype abundance (A). Ecotype focused – Placement of ecotype nodes along a variable vector represents the combination and value of factors that best describe that ecotypes distribution / optimal abundance (B).

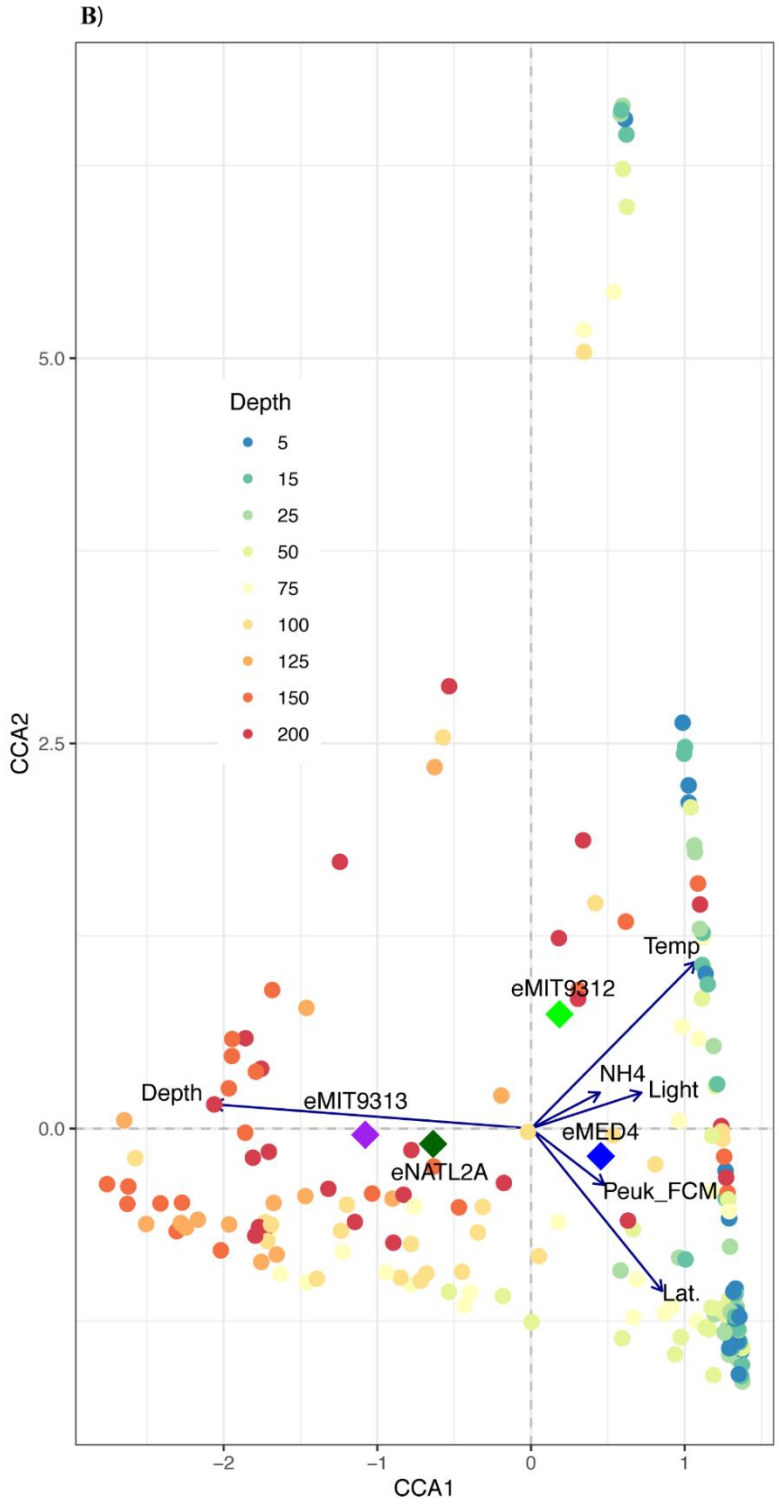

Fig. 4.3. Continued.

site-specific CCA. Interestingly eNATL2A node was further from the other LL ecotype eMIT9313, which was heavily influenced by depth, than would be expected.

Summer Ordination

The significance of the summer CCA model was determined by ANOVA ($F = 22.306$, $p = 0.0001$) and utilized a combination of latitude, temperature, light attenuation, ammonium, depth, and picoeukaryote abundance to account for 44 % of total variance. Sites continued to ordinate by depth, but the clustering of surface samples included fewer sites below 25 meters compared to winter (Fig. 4.3A). Samples at 50, 75, and 100 meters were now more heavily influenced by an increase in depth and abundance of eNATL2A and eMIT9313, as these depths often fell below the surface mixed layer during summer. Surface samples (5-25 meters) clustered strongly to each of the ecotype nodes, but most heavily around eMED4 as it was numerically dominant throughout much of the summer transect. Samples with the greatest eMIT9312 abundance also displayed higher temperature and lower rival phytoplankton abundance at lower latitudes. Abundance of LL ecotype eNATL2A was greater than eMIT9313 in many samples, evidenced by more substantial clustering around the eNATL2A node. Samples with the highest LL ecotype abundance were similar to those with the highest eMIT9312 abundance with respect to latitude and rival phytoplankton abundance but differed due to the effect of depth and temperature (Fig. 4.3A). CCA plots defining the relationship between ecotype nodes and environmental variables showed seasonal differences in the placement of LL ecotype nodes and in the major factors influencing eMIT9312 abundance (Fig. 4.3B).

Integrated Abundance Comparison

To make comparisons and observe trends between HL and LL ecotypes of *Prochlorococcus*, depth integrated abundances of the 200-meter water column were calculated and compared to surface values of physiochemical parameters to describe variations in abundance and diversity over temporal and spatial gradients. The integrated abundances of individual ecotypes showed both intra- and inter-seasonal dynamics (Fig. 4.4A and B). During the winter, eMIT9312 dominated lower latitudes but was eventually overtaken by eMED4 as the transect progressed North (Fig. 4.4A). While the biogeographical niche of these HL ecotypes has been well documented with respect to temperature and latitude, an interesting aspect of this depth integrated data is the relationship of HL ecotypes in the summer. Contrary to expectation,

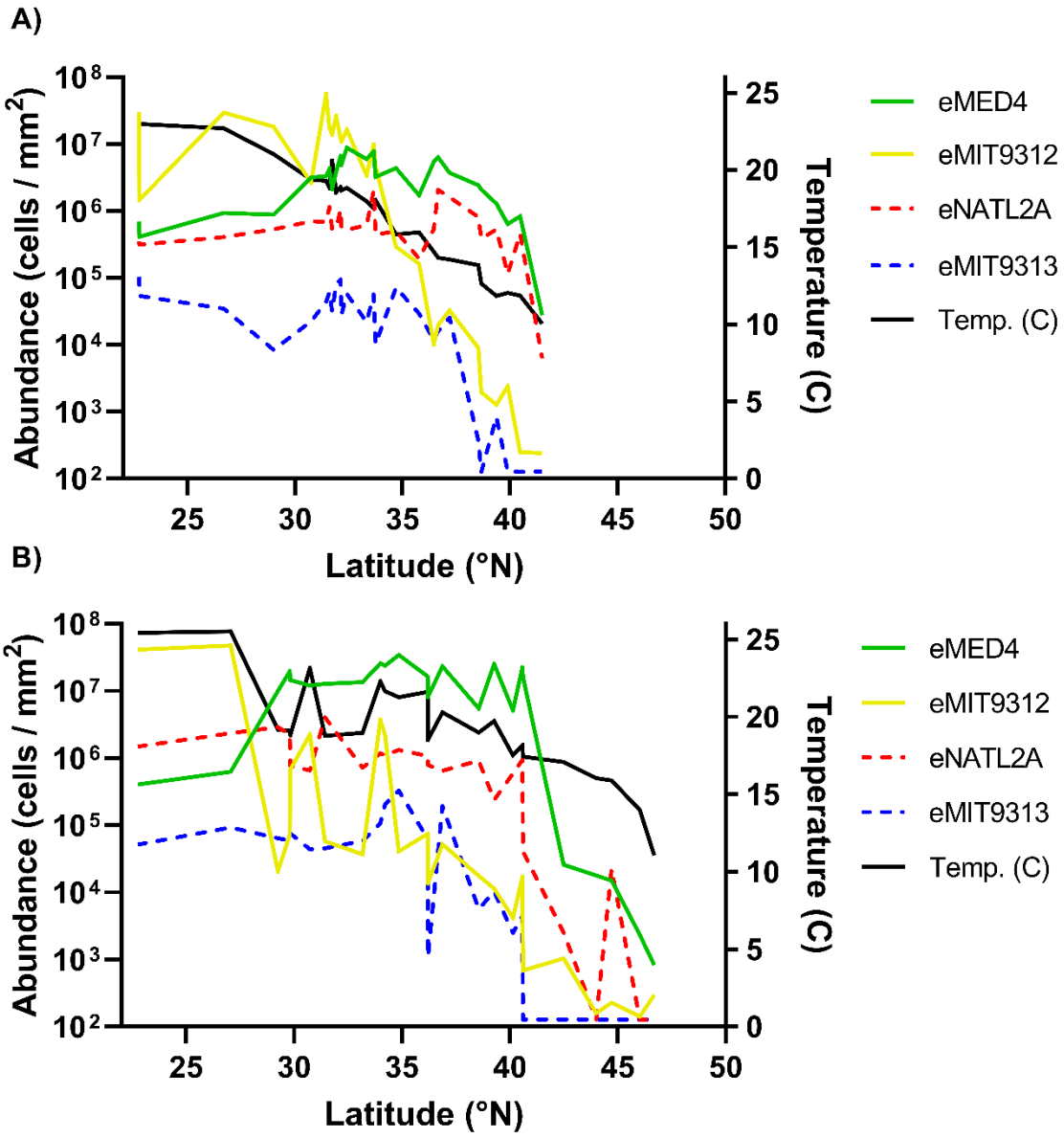

Fig. 4.4. Ecotype Integrated Abundance: Distribution of ecotypes eMED4 (green solid), eMIT9312 (yellow solid), eNATL2A (red dashed), and eMIT9313 (blue dashed) integrated abundance of the 200 meter water column over the latitudinal course of the winter (A) and summer (B) transects.

the geographical range in which eMIT9312 was dominant in the summer shrank compared to winter (Fig. 4.4B) even though summer average surface temperature was two degrees higher. Greater stratification of the water column was observed during the summer resulting in a much shallower surface mixed layer. Integrated abundance of eMIT9312 during this season was negatively correlated with stratification index (Pearson r = -0.581, p = 0.003), suggesting that a shoaling of the mixed layer depth had a far more detrimental effect on the abundance and biogeographical niche of this ecotype. Integrated abundance of eMED4 increased by an order of magnitude in the summer, bolstered by warmer, more stratified waters across the transect (Fig. 4.4B).

Both LL ecotypes (eNATL2A and eMIT9313) displayed very consistent seasonal abundances about an order of magnitude apart throughout the lower latitudes with eNATL2A more abundant, but both began to decline north of 40 °N and 35 °N, respectively (Fig. 4.4A and B). For eMIT9313 this decline at 35 °N could only be related to components of the dataset during winter, with integrated abundances showing direct negative correlation with increases in *Synechococcus* and picoeukaryote abundances, light attenuation, nitrogen, and phosphate (Pearson r = -0.540, -0.530, -0.638, and -0.635, p <0.005). Regardless of similar seasonal latitudinal range, eMIT9313 integrated abundance lacked any significant or meaningful correlations to explain summer population dynamics. Similarly, winter integrated abundance of eNATL2A was enigmatic and showed only a positive correlation with eMED4 (Pearson r = 0.605, p = 0.001), but negative correlation with light diffusion and picoeukaryote abundance in the summer (Pearson r = -0.501 and -0.488, p = 0.013 and 0.018).

An interesting aspect of the dataset is the apparent correlation between HL and LL ecotype pairs in the winter season. As environmental conditions caused a reduction in eMIT9312 integrated abundance at higher latitudes, a simultaneous reduction in eMIT9313 occurred (Pearson r = 0.499, p = 0.009) caused by similar environmental variables (Fig. 4.4A). The integrated abundance of eMED4 and eNATL2A seemed equally linked, overtaking the eMIT9312 population and experiencing decline farther North in a similar manner (Pearson r = 0.605, p = 0.001). The strong correlations between HL-LL ecotype pairs were absent from their summer distributions (Fig. 4.4B) even though the major latitudinal trends did not vary greatly between seasons. This was likely due to seasonal variations in integrated abundance being

observed only in the HL ecotypes whereas LL ecotypes remained consistent over latitudinal scales.

Depth Profile Analysis

To better understand the seasonal differences in environmental forces influencing these ecotypes, we examined their cell densities at all stations and depths. Ecotype depth profiles reinforced previously observed HL trends: eMED4 maintained a tighter distribution of abundances at various depths than eMIT9312 in the winter, due to their more consistent and higher abundance at upper latitudes (Fig. 4.5A and B). While eMIT9313 displayed typical LL distribution (below mixed layer) (Fig. 4.5D), LL eNATL2A had a strong near surface presence (< 25 meters) in the winter. Transition to summer downshifted eNATL2A abundance at this depth range, causing its highest abundances to occur below the mixed layer (Fig. 4.5C). This shift coincided with the marked increase of eMED4 (Fig. 4.5A) and decrease of eMIT9312 (Fig. 4.5B) in the mixed layer during summer, but it is unclear if these shifts are directly related. The abundance of *Synechococcus* (Fig. 4.5E) and picoeukaryotic phytoplankton (Fig. 4.5F) did not vary seasonally by depth.

The QPCR determined abundance of only eMIT9312 seemed directly linked to temperature, with greatest abundances observed at higher temperatures (Fig. 4.6B). Summer abundance of eMED4 seemed to increase with temperature, however winter abundance peaked around 18 °C and reduced at higher temperatures hinting at the influence of additional factors (Fig. 4.6A). All other ecotypes, *Synechococcus*, and picoeukaryotic phytoplankton followed the eMED4 winter trend, peaking in abundance around 15 °C and decreasing at higher temperatures (Fig. 4.6C-F).

Earlier, we noted comparisons of ecotype integrated abundances had suggested eMIT9312 seasonal variability was due to an increase in stratification and a reduction of the surface mixed layer in the summer. We continued this analysis by comparing ecotype abundances at stations with varying mixed layer depths. Instances of high abundance of eMED4 shift upward slightly between winter and summer, as mixed layer depths reduced dramatically (Fig. 4.7A). Instances of high abundance of eMIT9312 displayed a contrasting response and shift downward slightly between winter and summer (Fig. 4.7B). eNATL2A distribution highlighted the concentration of high abundance instances within the surface mixed layer during the winter

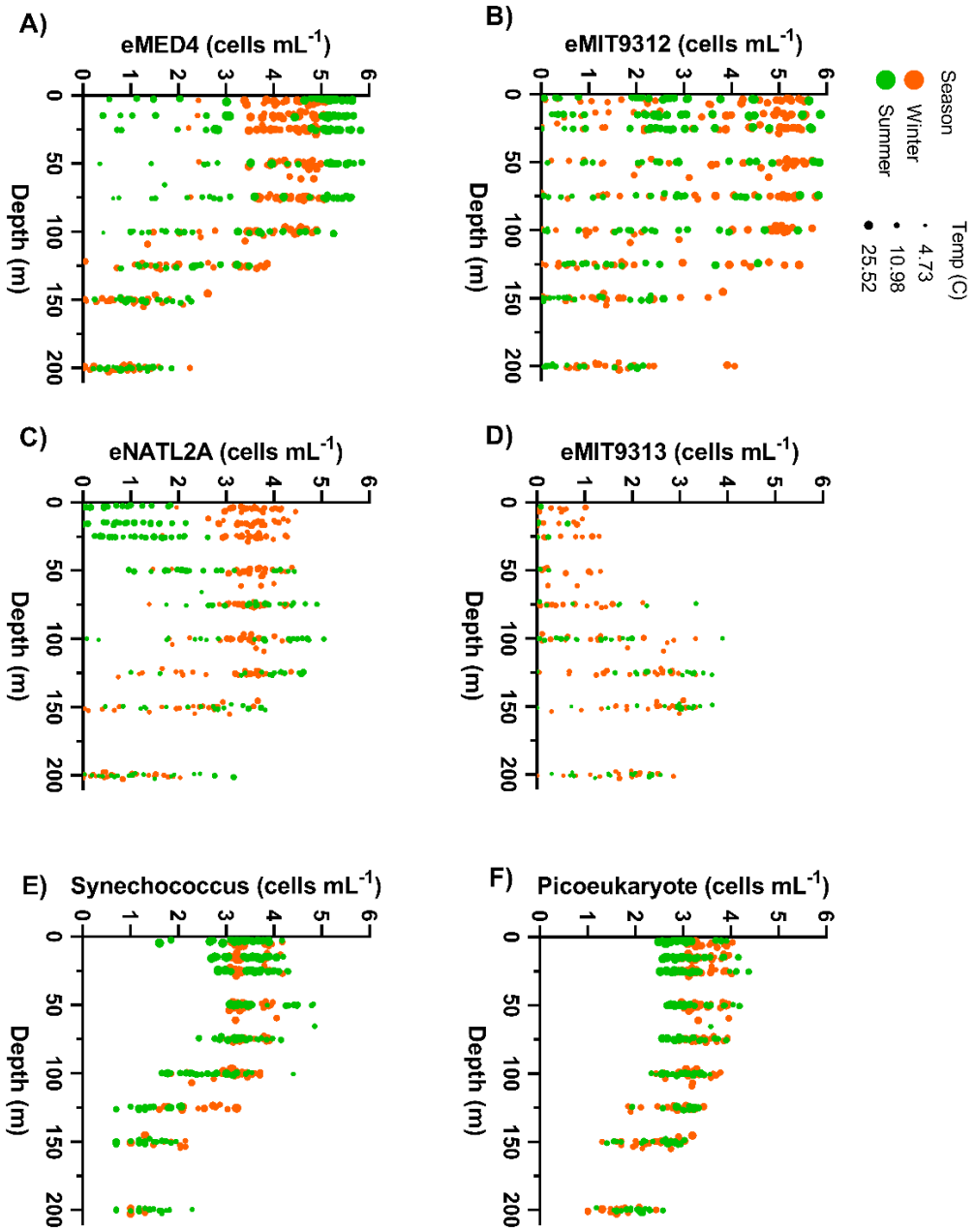

Fig. 4.5. Abundance Depth Profiles: Abundance depth profiles of all ecotypes: eMED4 (A), eMIT9312 (B), eNATL2A (C), and eMIT9313 (D); and quantified rival phytoplankton: *Synechococcus* (E) and picoeukaryotic phytoplankton (F). Ecotype abundances were determined by QPCR while all others were quantified by FCM. Samples shown are from all stations and depths from the winter (orange) and summer (green) transects and are sized based upon temperature.

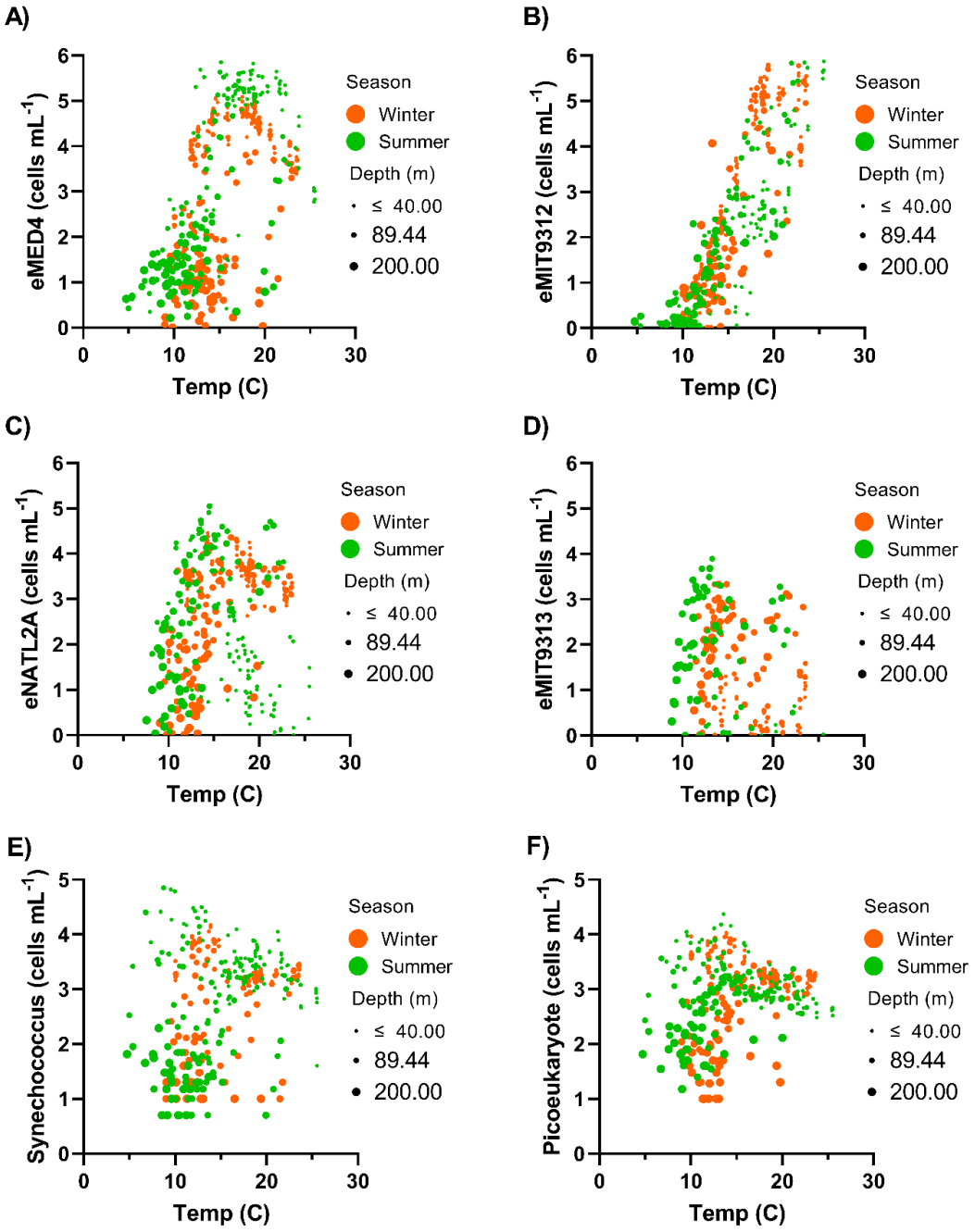

Fig. 4.6. Effect of Temperature on Phytoplankton: Observation of effects of temperature on abundance of all ecotypes: eMED4 (A), eMIT9312 (B), eNATL2A (C), and eMIT9313 (D); and quantified rival phytoplankton: *Synechococcus* (E) and picoeukaryotic phytoplankton (F). Ecotype abundances were determined by QPCR while all others were quantified by FCM. Samples shown are from all stations and depths from the winter (orange) and summer (green) transects and are sized based upon the depth of acquisition.

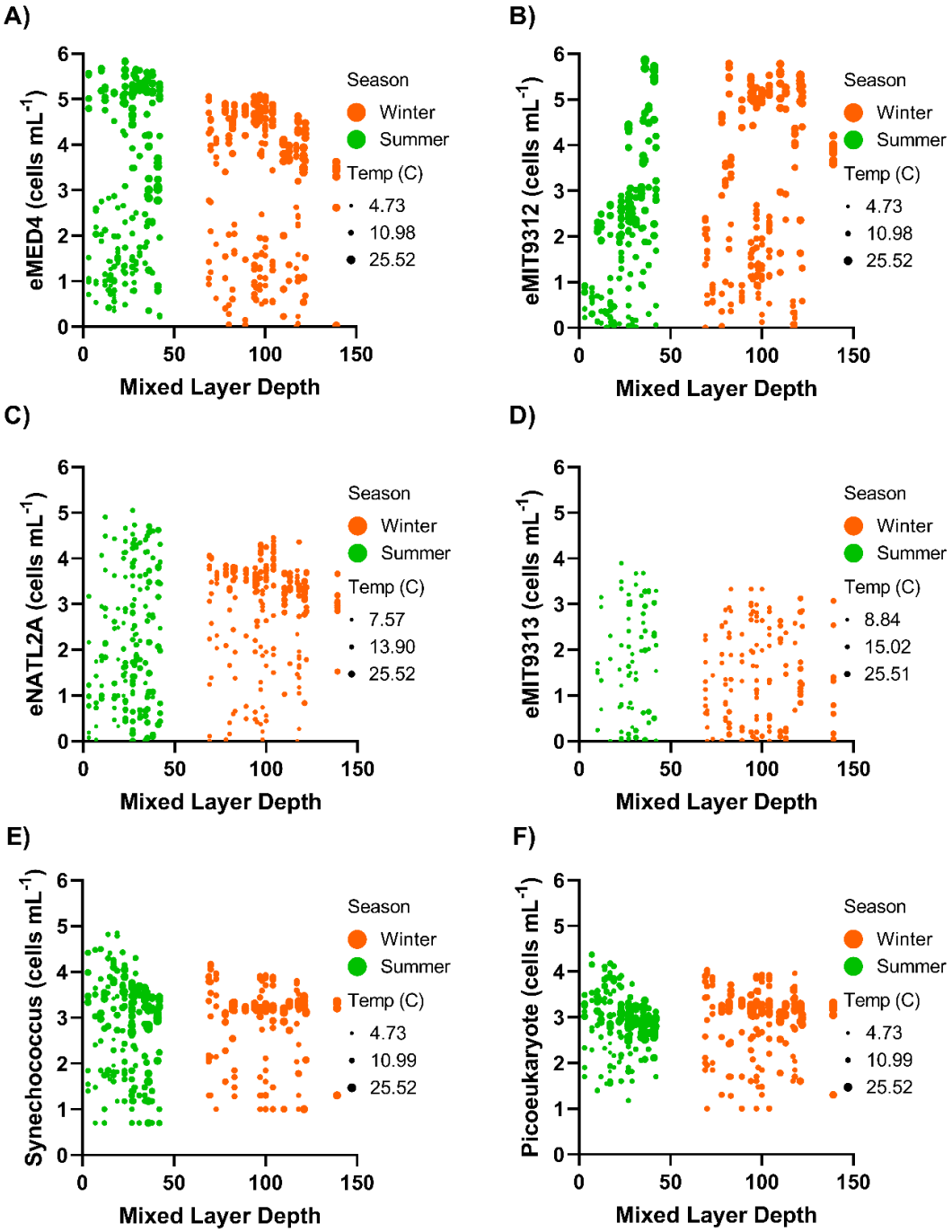

Fig. 4.7. Effect of Mixed Layer Depth on Phytoplankton: Observation of effects of mixed layer depth on abundance of all ecotypes: eMED4 (A), eMIT9312 (B), eNATL2A (C), and eMIT9313 (D); and quantified rival phytoplankton: *Synechococcus* (E) and picoeukaryotic phytoplankton (F). Ecotype abundances were determined by QPCR while all others were quantified by FCM. Samples shown are from all stations and depths from the winter (orange) and summer (green) transects and are sized based upon temperature.

(larger circles), and a shift to more variable abundances at similar temperatures in the summer (Fig. 4.7C). This coincides with the reduction of eNATL2A within the summer mixed layer, presumably because of its reduced depth. eMIT9313, *Synechococcus*, and picoeukaryotic phytoplankton did not vary with changes in mixed layer depth (Fig. 4.7D-F).

As a final constraint we compared ecotype abundances to rival phytoplankton, as our prior analysis associated *Synechococcus* and picoeukaryote abundance with decline of ecotype integrated abundances at higher latitudes. While eMED4 abundance was not negatively affected by rival phytoplankton (Fig. 4.8A and 4.9A), instances of the greatest *Synechococcus* and picoeukaryote winter abundance coincided with reductions in maximum eMIT9312 abundance (Fig. 4.8B and 4.9B). eNATL2A shifted from coexistence in winter to potential competition with *Synechococcus* in summer, and eMIT9313 was negatively correlated with *Synechococcus* in both seasons. Neither LL ecotype showed any negative response to picoeukaryote abundance (Fig. 4.8C-D and 4.9C-D).

Community Analysis

Community 16S rDNA profiling allowed us to identify correlations between *Prochlorococcus* ecotype abundances and cohabiting microbial phyla in the NPSG, by comparing trends within the relative abundance of each. When comparing eMIT9312 against the Phylum in the winter samples, we found this ecotype has significant relationships with Proteobacteria (Fig. 4.10A) ($p < 0.001$) and Chloroflexi (Fig. 4.10B) ($p < 0.001$). Specifically we found a 1% decrease (± 0.2) in eMIT9312 abundance for every 1% increase in Proteobacteria and a 2% decrease (± .8) in eMIT9312 abundance for every 1% increase in Chloroflexi abundance. When comparing eMIT9312 against the Phylum in the summer samples, we found this ecotype has significant relationships with Proteobacteria (Fig. 4.11A) ($p < 0.005$) and Deinococcus-Thermus (Fig. 4.11B) ($p < 0.05$). Specifically we found a 0.4% decrease (± 0.11) in eMIT9312 abundance for every 1% increase in Proteobacteria and a 10% increase (± 4.6) in eMIT9312 abundance for every 1% increase in Deinococcus-Thermus abundance. Though we saw a decrease of .66 in eMIT9312 for every 1% increase in Planctomycetes (Fig. 4.11C), this decrease was not statistically significant ($p > 0.05$).

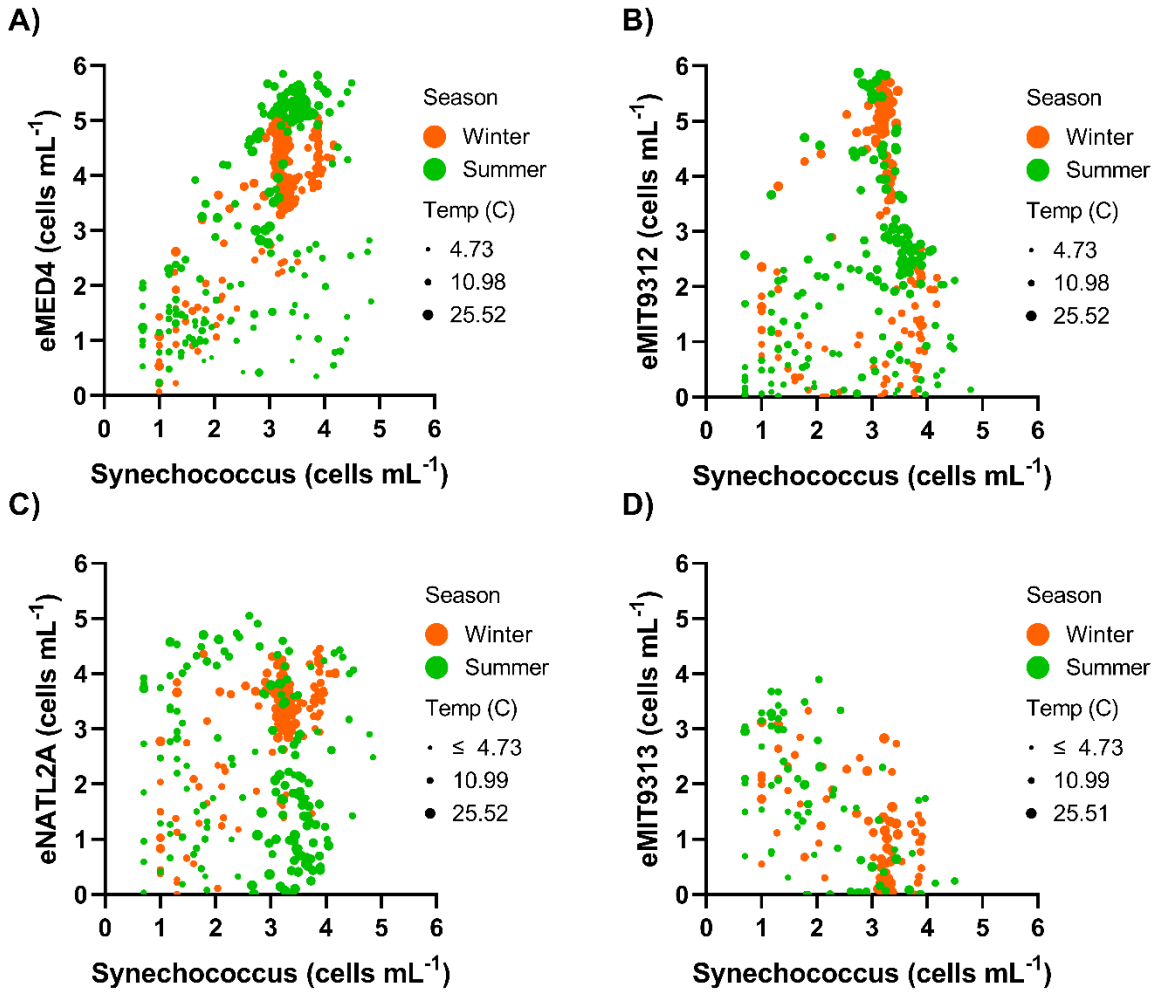

Fig. 4.8. Effect of *Synechococcus* on Ecotypes: Observation of effects of *Synechococcus* abundance on abundance of all ecotypes: eMED4 (A), eMIT9312 (B), eNATL2A (C), and eMIT9313 (D). Ecotype abundances were determined by QPCR while all others were quantified by FCM. Samples shown are from all stations and depths from the winter (orange) and summer (green) transects and are sized based upon temperature.

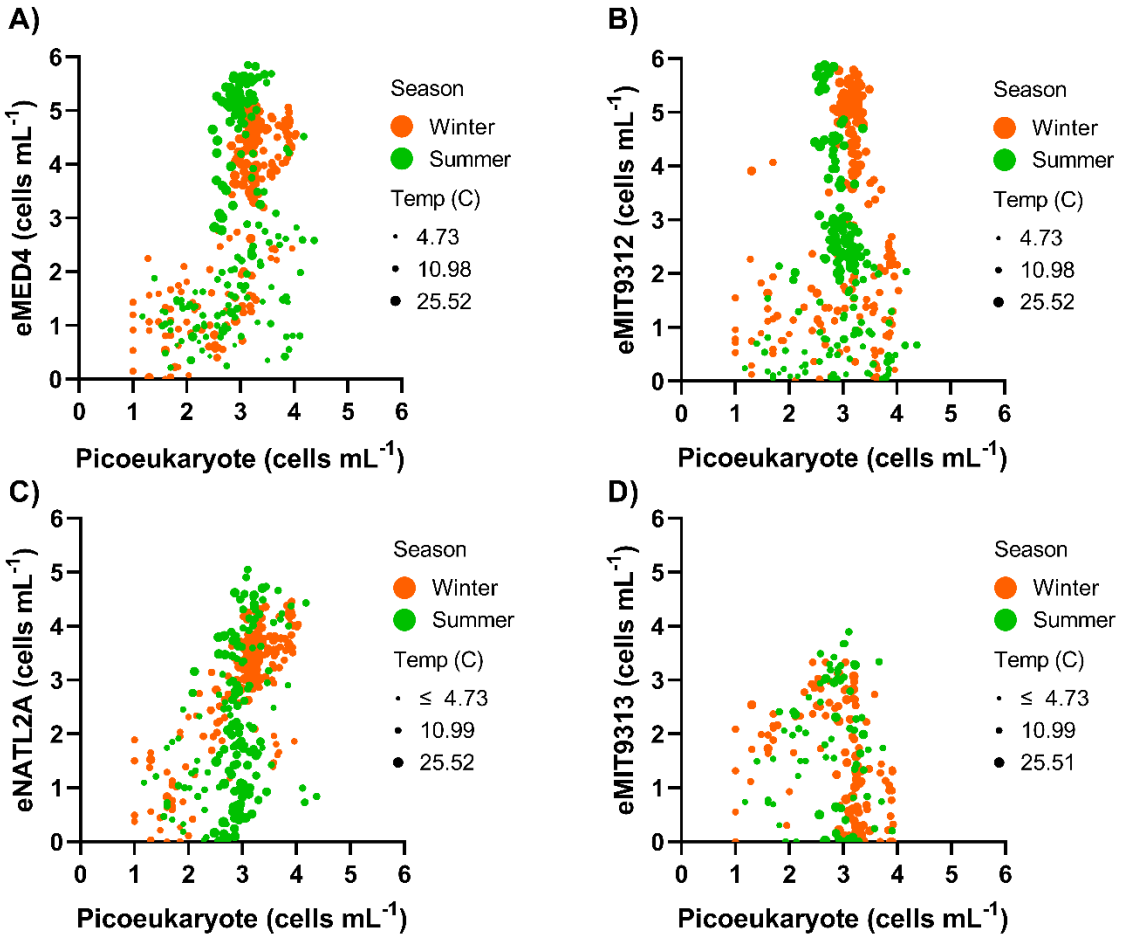

Fig. 4.9. Effect of Picoeukaryotes on Ecotypes: Observation of effects of picoeukaryotic phytoplankton abundance on abundance of all ecotypes: eMED4 (A), eMIT9312 (B), eNATL2A (C), and eMIT9313 (D). Ecotype abundances were determined by QPCR while all others were quantified by FCM. Samples shown are from all stations and depths from the winter (orange) and summer (green) transects and are sized based upon temperature.

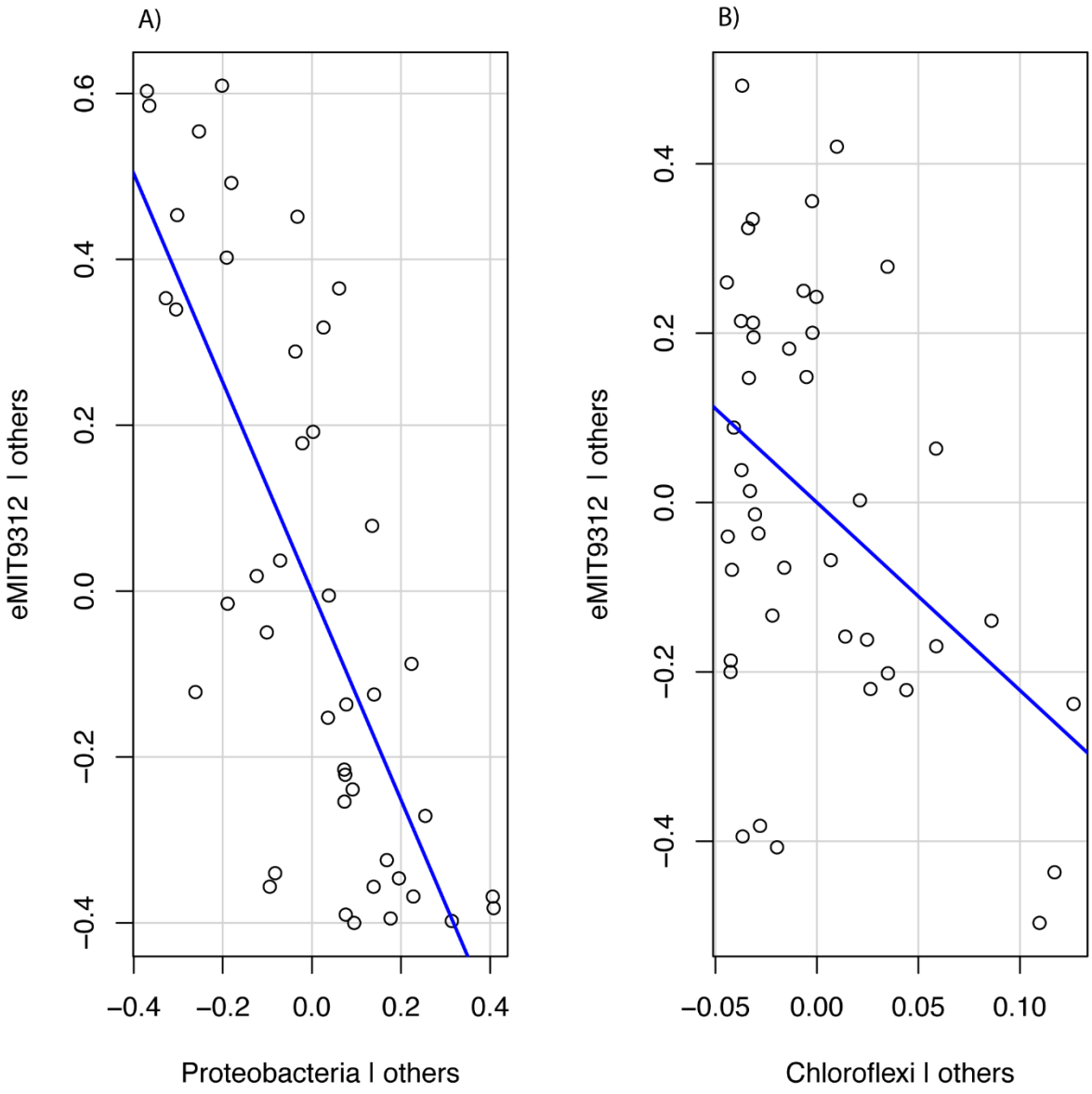

Fig. 4.10. eMIT9312 / Phyla Winter Linear Regression: Multiple linear regression analysis of eMIT9312 and phyla relative abundances from winter season from surface and DCM depths.

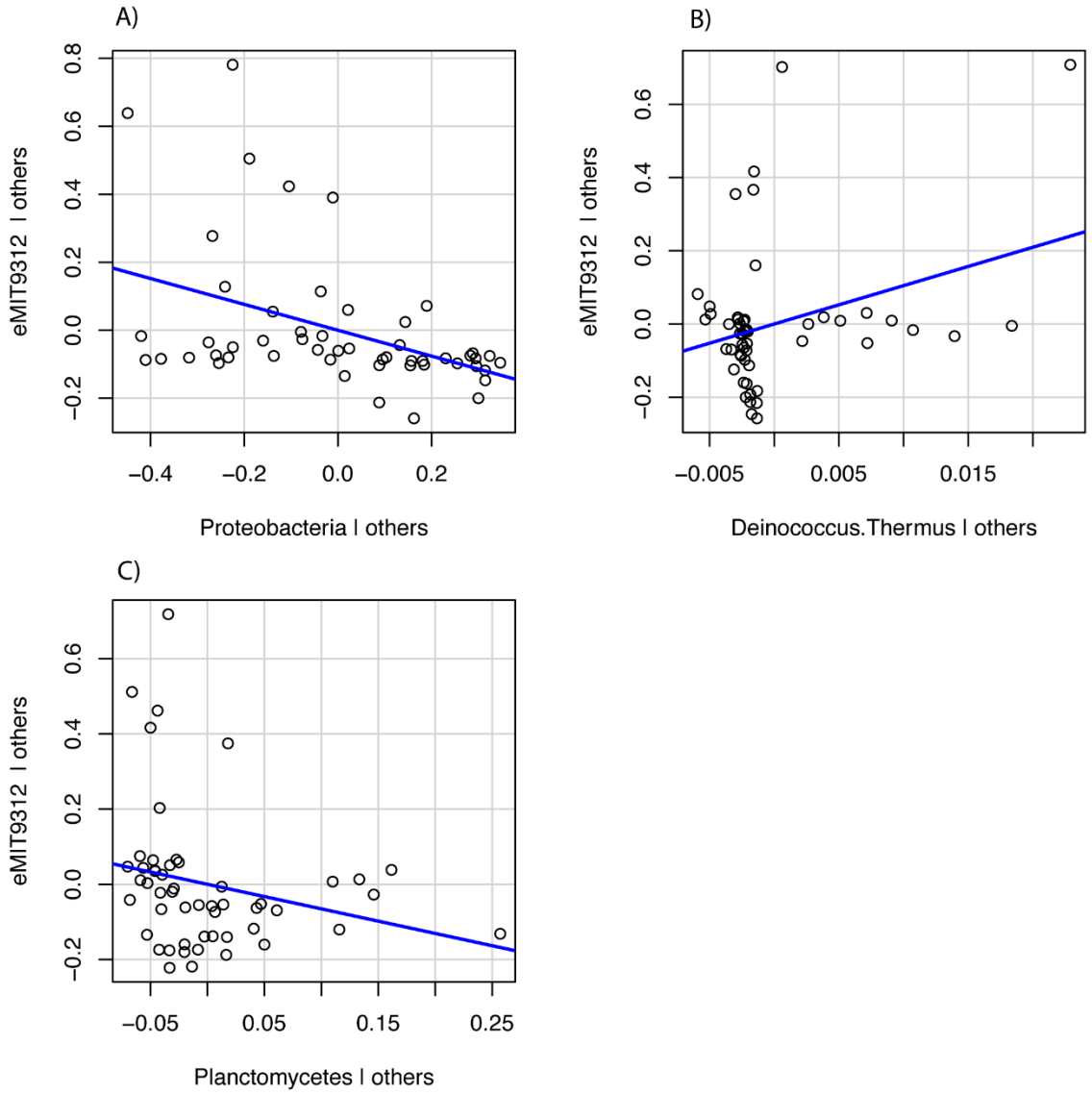

Fig. 4.11. eMIT9312 / Phyla Summer Linear Regression: Multiple linear regression analysis of eMIT9312 and phyla relative abundances from summer season from surface and DCM depths.

When comparing eMED4 against the Phylum in winter samples, we found significant relationships with Proteobacteria (Fig. 4.12A) ($p < 0.05$) Specifically we found a 0.6% decrease (± .1) in eMED4 for every 1% increase in Proteobacteria. While we did find a 0.6% increase in eMED4 for every 1% increase in Planctomycetes (Fig. 4.12B), this was not statistically significant ($p > 0.05$). When comparing eMED4 against the Phylum in summer samples, we found significant relationships with Proteobacteria (Fig. 4.13A) ($p < 0.001$) and Deinococcus-Thermus (Fig. 4.13B) ($p < .05$). Specifically we found a 1.1% decrease (± 0.14) in eMED4 for every 1% increase in Proteobacteria and a 12% decrease (± 5.6) in eMED4 for every 1% increase in Deinococcus-Thermus. Though we saw a decrease of 0.4% in eMED4 for every 1% increase in Euryarchaeota (Fig. 4.13C), this decrease was not statistically significant ($p > 0.05$).

When comparing eNATL2A against the Phylum in winter samples, we found significant relationships with Lentisphaerae (Fig. 4.14A) ($p < 0.05$), the uncultured phylum SHA.109 (Fig. 4.14B) ($p < 0.005$), Gemmatimonadetes (Fig. 4.14E) ($p < 0.05$), and Chloroflexi (Fig. 4.14E) ($p < 0.05$). Specifically we found a 3.4% increase (± 1.5) in eNATL2A for every 1% increase in Lentisphaerae, a 15% decrease (± 5) in eNATL2A for every 1% increase in SHA.109, a 7% (± 3) decrease in eNATL2A for every 1% increase in Gemmatimonadetes, and a 2% increase in eNATL2A for every 1% increase in Chloroflexi. While we found a 0.5% increase in eNATL2A for every 1% increase in Euryarchaeota (Fig. 4.14C), this was not statistically significant ($p > 0.05$). There were no significant relationships in any of the summer samples.

When comparing eMIT9313 against the Phylum in the winter samples, we found significant relationships with Lentisphaerae (Fig. 4.15A) ($p < 0.05$), Chloroflexi (Fig. 4.15B) ($p < 0.0005$), Deinococcus-Thermus (Fig. 4.15C) ($p < 0.01$), Marinimicrobia SAR406 clade (Fig. 4.15D) ($p < 0.005$), and Euryarchaeota (Fig. 4.15E) ($p < 0.05$). Specifically we found a 1.5% increase (± .6) in eMIT9313 for every 1% increase in Lentisphaerae, a 1.1% increase (± .25) in eMIT9313 for every 1% increase in Chloroflexi, a 3% (± 1) decrease in eMIT9313 for every 1% increase in Deinococcus-Thermus, a 1.1% (± .33) decrease in eMIT9313 for every 1% increase in Marinimicrobia_SAR406_clade, and a .3% (± .13) increase in eMIT9313 for every 1% increase in Euryarchaeota. There were no significant relationships in any of the summer samples.

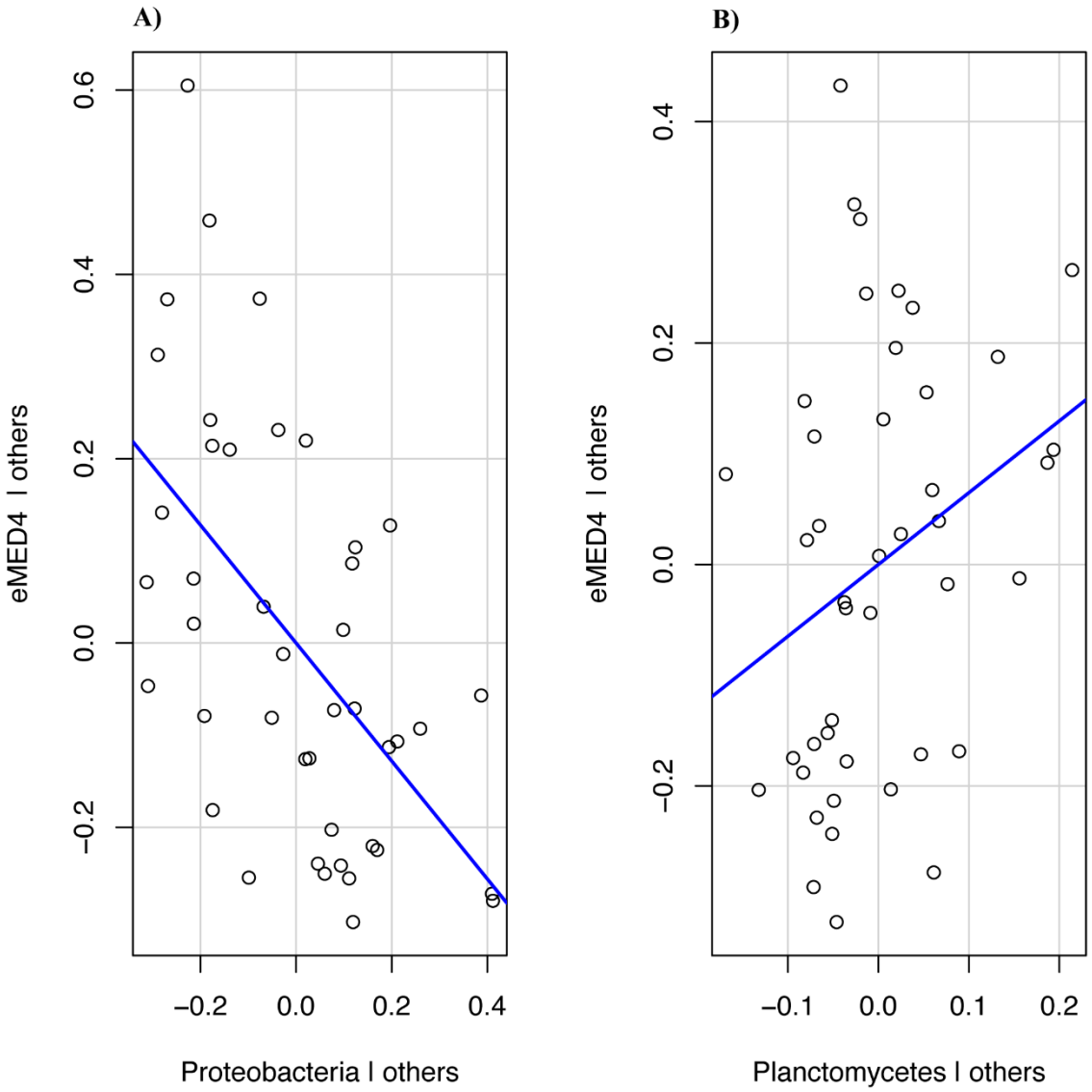

Fig. 4.12. eMED4 / Phyla Winter Linear Regression: Multiple linear regression analysis of eMED4 and phyla relative abundances from winter season from surface and DCM depths.

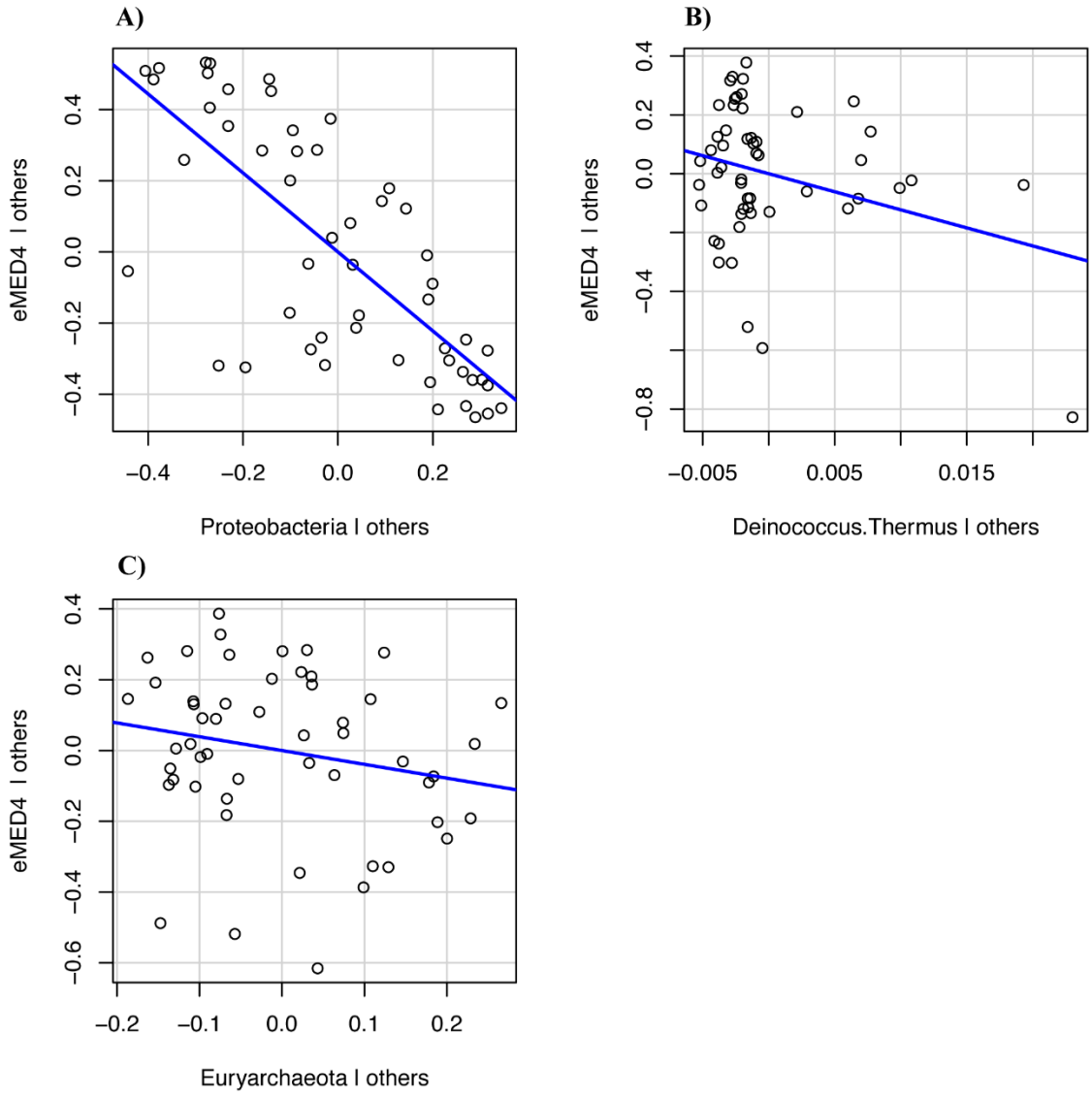

Fig. 4.13. eMED4 / Phyla Winter Linear Regression: Multiple linear regression analysis of eMED4 and phyla relative abundances from summer season from surface and DCM depths.

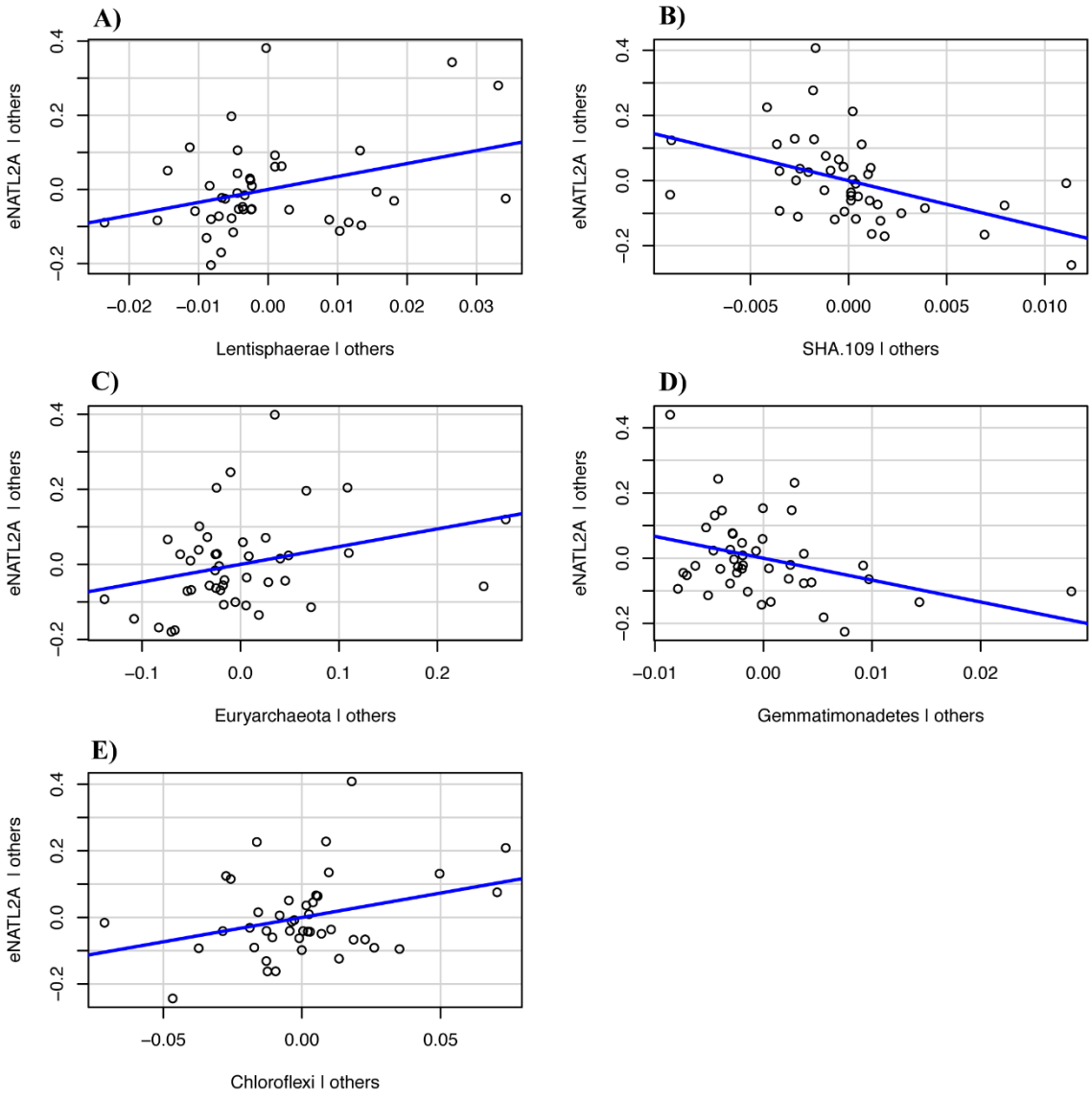

Fig. 4.14. eNATL2A / Phyla Winter Linear Regression: Multiple linear regression analysis of eNATL2A and phyla relative abundances from winter season from surface and DCM depths.

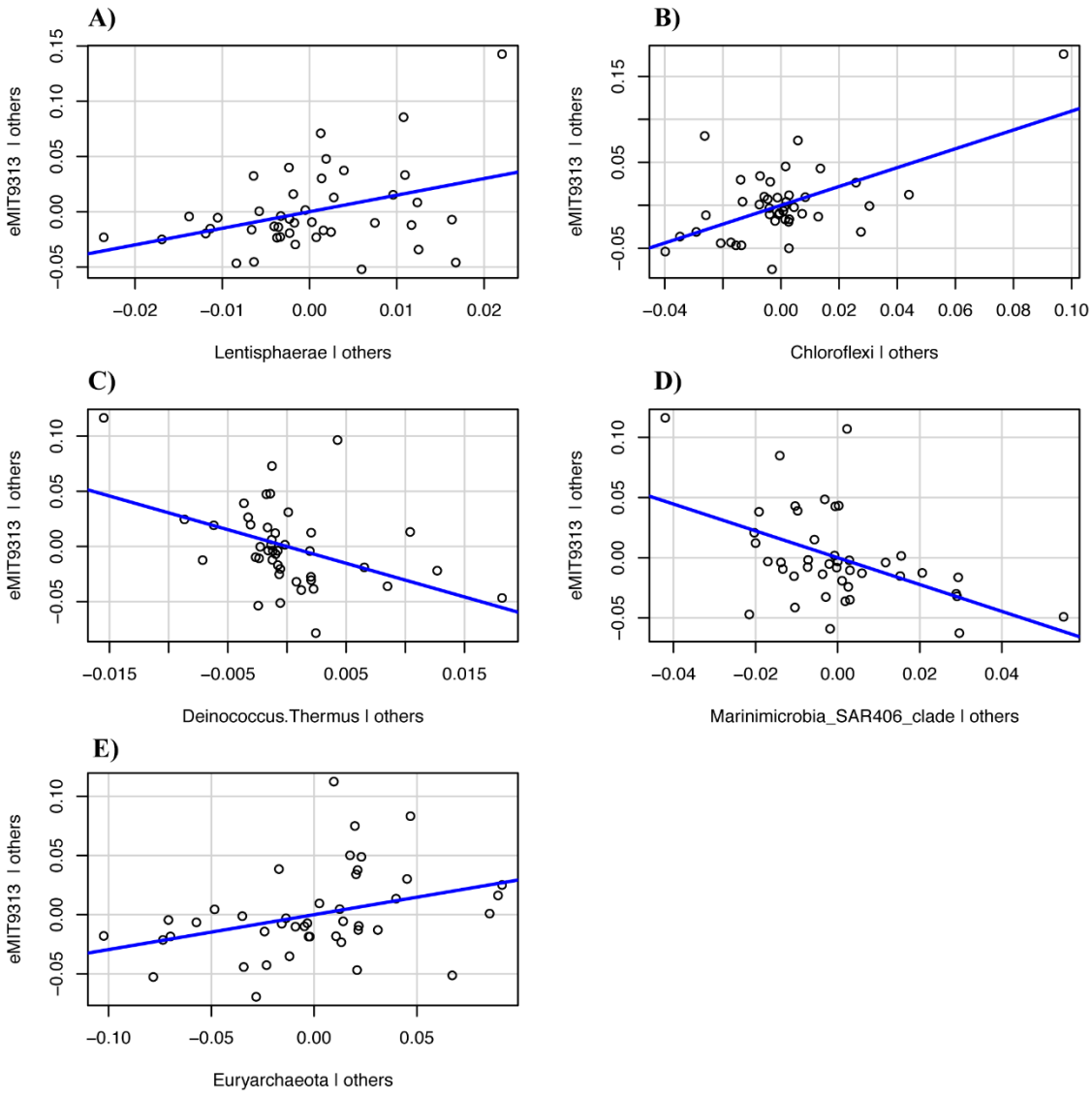

Fig. 4.15. eMIT9313 / Phyla Winter Linear Regression: Multiple linear regression analysis of eMIT9313 and phyla relative abundances from winter season from surface and DCM depths.

Discussion

While the biogeographical distribution of *Prochlorococcus* ecotypes in the NPSG has been previously studied, these focused only upon HL ecotypes, upon samples from surface mixed layers, or upon single stations which limits comprehensive understanding of seasonal ecotype distributions across the region (Chandler et al., 2016, Larkin et al., 2016, Malmstrom et al., 2010). In this study we analyzed euphotic zone depth profiles of HL (eMED4 and eMIT9312) and LL (eNATL2A and eMIT9313) *Prochlorococcus* ecotypes from seasonal, basin-scale transects of the NPSG that covered a similar latitudinal range to assess the seasonal drivers of diversity, ecotype distributions, and potential interactions with associated microbial communities. We determined that seasonal influence by temperature, stratification, and rival phytoplankton abundance influenced the dynamics of all four measured ecotypes with greatest influence on the major HL ecotype relationship, reducing rather than extending the latitudinal range of dominance by eMIT9312. We suggest these findings can be informative not only of current annual trends but also of changes predicted for a future ocean altered by climate change, specifically increases in temperature and stratification.

Major oceanic regions maintain stable annual physiochemical gradients that dictate biological processes. Within annual timescales, seasonal variability can significantly alter the diversity and abundance of microbial organisms. The most well studied ecotype relationship is that of HL ecotypes eMED4 and eMIT9312, for which it has been shown that coexistence or dominance of one ecotype over the other is directly linked to changes in temperature over a latitudinal range (Chandler et al., 2016, Malmstrom et al., 2010, Smith et al., 2021, Zinser et al., 2007). While average seasonal surface temperatures changed little, marked differences in water column stratification were observed between the winter and summer causing a reduction in mixed layer depth throughout the NPSG. Variability in vertical mixing affected both HL and LL ecotypes, regardless of latitude or water temperature. Detection of eNATL2A in surface mixed layer samples had been previously reported, but usually required deep mixing (Malmstrom et al., 2010, Thompson et al., 2021). Winter samples followed this trend, with eNATL2A ($5.60e^3$ cells mL^{-1}) observed at an average abundance an order of magnitude less than eMED4 ($3.57e^4$ cells mL^{-1}) within the mixed layer throughout the transect. Shallow mixed layer depths in summer

nearly excluded eNATL2A (average ML abundance 14.10 cells mL^{-1}) from all near surface depths and are data suggests these seasonal trends are consistent throughout the entire NPSG.

Previous observation describing the effect of vertical mixing comes from stationary timeseries (BATS and HOTS) which are found in the lower latitudes of northern Pacific and Atlantic subtropical gyres. At these locations shoaling of mixed layer depth had only a minor influence on HL ecotype abundances but resulted in the exclusion of LL eNATL2A from near surface depths (0-50 meters) (Malmstrom et al., 2010). In this study we highlighted that shallow mixed layer depths in summer coincided with a reduction in abundance and biogeographical range of eMIT9312. Average surface temperature was 2 °C greater in summer and yet average eMIT9312 mixed layer abundance in summer ($4.51e^4$ cells mL^{-1}) was nearly half the winter average ($9.98e^4$ cells mL^{-1}). Additionally, the latitudinal range over which eMIT9312 dominated was reduced from 34 °N in winter to 28 °N in summer, even though temperatures further north were often above the 19-21 °C transition point of HL ecotypes where eMIT9312 usually dominate above and eMED4 below (Chandler et al., 2016, Larkin et al., 2016). The well-defined relationship of *Prochlorococcus* ecotypes and temperature does not sufficiently describe the differences in seasonal HL abundance in the NPSG. We predict this observation is due to environmental conditions that arose from reduced vertical mixing: closer proximity to low temperature water (below mixed layer), reduced niche space, and increased competition with eMED4 and other phytoplankton. Additional work is needed to sufficiently describe the seasonal differences in eMED4 and eMIT9312 distribution.

Apparent similarities between distributions of HL and LL ecotype pairs persisted across the entire transect, although they were strongest in the winter. It is surprising that environmental factors would act on both HL and LL ecotypes, causing similar trends of abundance and decline, as they are partially or completely spatially separated in the water column. The eMED4 / eNATL2A trend could not be explained by correlation to environmental factors except by a correlation between their abundances, even though they only partially coexist in the water column. The eMIT9312 / eMIT9313 trend could be explained by correlation to rival phytoplankton abundance, latitude, and increases in nutrient concentration. While our dataset provided only limited insight into these HL and LL ecotype relationships, this represents an interesting focus of future research. The need for further research is highlighted in the recent

discovery of nitrate utilization genes, previously found in only eMIT9313, in both HL and LL ecotype populations and subgroups (Berube et al., 2016, Berube et al., 2019). As one of the few aspects of the water column that would affect both HL and LL ecotypes is the presence and abundance of rival phytoplankton (*Synechococcus* and picoeukaryotes), the compounding effects of resource competition and environmental variability must be accounted for when examining the possibility of HL-LL ecotype relationships.

Lastly, we investigated potential relationships and interactions between individual ecotypes and relevant phyla through comparisons of relative abundance. This represents the first time this type of relationship has been examined on a seasonal basis in a major ocean gyre. Examinations of marine microbial community relationships have often found environmental factors of greater influence than microbial and trophic interactions (Chandler et al., 2016, Flombaum et al., 2013, Gilbert et al., 2012). Ecotype distributions are strongly determined by environmental parameters, however specific positive and negative correlations were observed between all four ecotypes and a variety of microbial phyla that varied seasonally for the HL ecotypes. While this analysis was limited, it represents an important avenue of research as interactions between *Prochlorococcus* and other microbial groups significantly impact its ecology.

Here we attempted to contribute understanding of major *Prochlorococcus* population groups and the effects of seasonal variability on their abundance and distribution in one of the largest contiguous environments, the NPSG. This research is of importance as differences in *Prochlorococcus* ecotype abundance due to seasonal variability can be informative of the diversity and prevalence of these organisms and help predict future distributions. Recent studies have determined the possible effects of climate change, namely increased temperature and stratification, on cyanobacterial populations in major oceanic gyres (Flombaum et al., 2013, Knight and Morris, 2020). Increased abundance of *Prochlorococcus* and *Synechococcus* due to warmer ocean waters seems likely but if the consequences of increased stratification observed in this study are predictive of the future ocean, then *Prochlorococcus* population diversity is likely to differ significantly from its current state.

CHAPTER 5:

Conclusions and Future Direction

Conclusions

The overall goal of this dissertation was to identify the ecological and physiological factors that most influence the competitive success of *Prochlorococcus* generally in the oligotrophic ocean and specifically in the NPSG. I approached this objective with a multi-pronged method, combining two ecologically relevant culturing techniques that modeled aspects of nitrogen competition and H_2O_2 dynamics with analysis and observation of environmental samples from the region. The focus of this dissertation has been to highlight ecological and physical factors of influence, as research has shown before, but also to emphasize the significance of microbial interactions on every aspect of *Prochlorococcus*. This was done to be both informative and transformational, as results and conclusions here clash with previous assumptions about the ecology and competitive success of this genus. In the sections that follow, I will draw general conclusions from the findings within each chapter and address the questions put forth in Chapter 1.

What influences the competitive success of Prochlorococcus in the nitrogen limited NPSG?

While a multitude of interactions and activities are occurring simultaneously within nitrogen limited, tripartite cultures, the most important are those causing the dominance of *Prochlorococcus* over *Synechococcus*. For this there are two possible explanations: direct competition where *Prochlorococcus* facilitates the stasis of *Synechococcus,* likely through a combination of competition and interactions, or indirect competition where stasis of *Synechococcus* is the outcome of complex cyanobacteria-heterotroph interactions. The likelihood and implications of each will be discussed.

The more likely of these explanations to account for tripartite outcomes, which I have previously suggested, is that direct competition does occur. The caveat is that competition involves only nitrogen sources *Prochlorococcus* can access (ammonium). The cyanobacteria coexist on separate nitrogen sources, until activity of heterotrophic bacteria depletes that source required by *Synechococcus*. It remains possible that *Prochlorococcus* is unable to drawdown ammonium concentrations to partially or fully exclude *Synechococcus* in competition, i.e., stasis of *Synechococcus* in tripartite is due to competition with the heterotroph. Two aspects of the experimental system prohibited inquiry of these questions: a nitrogen-free base media to access

competition for only ammonium and the lack of a *Synechococcus* mutant incapable of nitrate utilization, both will be addressed in the future directions.

Regardless of the mechanism, the ecological implications of this work are vast. There exists in natural environments exclusive cyanobacteria-heterotroph relationships based upon metabolite transfer and release. It is important to note only the photosynthetic exudate of *Prochlorococcus* elicited a response in marine heterotrophs. Marine *Synechococcus* are known to produce exudate, however carbon limitation of the heterotroph was not alleviated by consuming these compounds, if consumed at all. This suggests that differences in content of cyanobacterial exudate decided competition outcomes or that an evolved relationship exists between sympatric microbes (*Prochlorococcus* and *Alteromonas*) based on the content or timing of exudate release. As stated previously, much work defining the success of *Prochlorococcus* has focused on its competitive ability and streamlined adaptations. Here it is suggested that this success and numerical dominance must be attributed, at least in part, to crucial microbial interactions.

Are rival phytoplankton capable of "helping" Prochlorococcus?

Being unable to quantify the individual protective capacity of rival phytoplankton (*Synechococcus*, *Micromonas*, and *Ostreococcus*) and heterotrophic bacteria in field observation or laboratory coculture, this culturing technique represents the best method for querying this question. I showed unequivocally that rival phytoplankton reduced H_2O_2-mediated mortality in *Prochlorococcus* populations when in coculture. Combined with the data presented in Chapter 2, these findings again show the potential that microbial interactions developed over an evolutionary timescale influence not only the competitive success but everyday survival of *Prochlorococcus* in the NPSG. The application of this data to natural environments to determine the ecology of this interaction or the overall contribution of phytoplankton to H_2O_2 degradation compared to other microbial groups remains difficult, however. Future applications of this culturing technique will be discussed below.

What factors influence total and ecotype abundance of Prochlorococcus in the NPSG?

This analysis of *Prochlorococcus* ecotype dynamics was novel in its inclusion of multiple HL and LL ecotypes, samples from the entire euphotic zone, seasonal comparison of similar latitudinal ranges, and comparison of ecotype and OTU relative abundance, all to better

understand the seasonal differences in ecotype abundance and the physical forces and potential microbial interactions that influence them. I determined that previously described latitudinal trends of temperature explained HL ecotype abundance, but also that changes in stratification shifted dominance toward cold adapted eMED4. While this change in ecotype abundance occurred on a seasonal basis, it is interesting that warmer, more stratified waters, conditions predicted for future oceans, increased the biogeographical range of eMED4. This result has implications for the future ecology of *Prochlorococcus* in warming ocean waters. I also determined specific factors that simultaneously influenced the abundance of HL-LL ecotype pairs (eMIT9312-eMIT9313 and eMED4-eNATL2A); however, the nature of these relationships require further investigation. Specifically, limitations of the dataset restrict the distinction of ecotype relationships from identical reaction to changes in environmental conditions. Lastly, I found significant relationships between ecotypes and individual phyla through multiple linear regression-based models that varied on a seasonal basis. The work in previous chapters highlighted the importance of microbial interactions for the success and survival of *Prochlorococcus,* and this data suggests that some of these relationships could be ecotype specific.

In conclusion, it is my hope that the data presented herein will reinforce the central theme of this dissertation, that microbial interactions greatly influence ecology of the oligotrophic ocean. There is no doubt that *Prochlorococcus* is successful, but to an extent its success and dominance are the result of beneficial relationships, and the sole competitive nature of this organism (one on one) has yet to be demonstrated.

Future Directions

Expansion of tripartite culture system

A total of ten bacterial strains were included in this competition study. With a tool established, the next logical step is expansion of the system. The inclusion of additional *Prochlorococcus* and *Synechococcus* strains is an obvious continuation of this work. However, ecotype field observations in Chapter 4 implicated picoeukaryotic phytoplankton as competitors of certain *Prochlorococcus* ecotypes and their inclusion in resource competition studies could prove significant, especially now that a system for coculturing and quantifying these organisms was established in Chapter 3. The last recommendation for strain inclusion would be

representatives of streamlined heterotrophic bacteria isolated from oligotrophic oceans to begin investigating the role numerically dominant marine heterotrophs play in *Prochlorococcus* ecology. Other than alterations to members of tripartite cultures, changes of limiting nutrients could be made to potentially explore *Prochlorococcus* ecology and microbial actions in regions phosphorus (Atlantic) or trace metal (equatorial and Southern Ocean) limited.

Characterization of Prochlorococcus exudate under nutrient limitation

Given the significance of *Prochlorococcus* exudate in deciding competition outcomes in tripartite culture, attempts should be made to constrain the constituents and concentration of this exudate. Of particular interest would be changes in composition after acclimation to a particular limiting resource. Lastly this content could be compared to *Synechococcus* exudates in hopes of determining the differences that allowing *Prochlorococcus* crossfeeding of heterotrophic bacteria.

Merger of nutrient limitation and hydrogen peroxide culture systems

The resource cost incurred by producing H_2O_2 degrading enzymes was detailed in Chapter 3. It would be insightful to determine if changes in nutrient availability also cause changes in protective capacity of rival phytoplankton or marine heterotrophs, as all estimations of this occurred with replete nutrients. Changes in protective capacity of helper organisms under nutrient limitation could manifest in altered degradation rates, expression levels of degradative enzymes, or of general fitness and growth rate.

Exploration and Expansion of the POWOW Study

Results within Chapter 4 hinted at the possibility of interactions and relationships between HL and LL ecotypes of *Prochlorococcus*, defined by correlating abundance and simultaneous decline at the end of a specific latitudinal range. Limitations of our dataset prohibited attributing this to a reaction to environmental changes or an interaction that tied the fates of spatially separated ecotypes in the water column. Physiological experiments focus primarily on HL ecotypes, but given the current state of flow cytometry it is possible a larger LL ecotype could be distinguished in coculture with a HL. Even if not, side by side comparison of responses to changing conditions, nutrients, or rival phytoplankton abundance would be informative.

Studies detailed in Chapters 1 and 4 described how ecotypes of *Synechococcus* divided into specific regimes that differed based on temperature and nutrients. While ecotypes of these two dominant cyanobacteria have been examined separately, studies determining trends between ecotypes of *Prochlorococcus* and *Synechococcus* are lacking. Quantification of *Synechococcus* ecotypes from field samples (replicate filters) analyzed in Chapter 4 would fill this knowledge gap. And as representatives of many ecotypes of both genera are currently in culture, trends observed in ecotype distribution could be reinforced or quality controlled by physiological experiments.

Lastly, while novel, the attempt to discern relationships between ecotypes and groups of microbial taxa was limited. There obviously are ecotype specific and seasonal trends, but little information can be gleaned from a phyla-level comparison. Future analysis with finer taxonomic levels would be insightful, and again as many representative strains are in culture the determined trends and results could potentially be reinforced by laboratory studies.

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VITA

Benjamin Carter Calfee humbly began his existence on July 18th, 1992, in a Quality Inn hotel room near Falls Church, Virginia. After living in a number of New England states at a young age, his mother found her calling as a registered nurse and the family settled for good in East Tennessee. He graduated from Union County High School of Maynardville, TN in 2010 with a class of about ninety students. Starting at the University of Tennessee later that same year, Benjamin had a passion for cryptozoology, veterinary science, and marine biology, but majored as Undecided his first two years. Soon becoming vastly interested in the study of microorganisms, he was introduced to his future career when taking an introductory microbiology lab course instructed by graduate student Nana Ankrah, now an assistant professor at SUNY. Upon Nana's advice, Benjamin joined the lab of Dr. Erik Zinser as an undergraduate research assistant where he pursued independent research studying the distribution of cyanobacterial ecotypes in the North Pacific Subtropical Gyre, a project which is included in this document. Benjamin graduated with a Bachelor of Science in Microbiology in 2014 and continued onto doctoral studies with Dr. Erik Zinser at the University of Tennessee with an unwavering love of the sea, and all the tiny things within.

Benjamin met his future wife, Michelle Curtis, in 2011 and they have rarely been apart since. They married in 2014 and have two children together: Greyson Carter (2012) and Sadie Gray (2017). In addition, Benjamin is an avid outdoorsmen and scuba diver. He immensely enjoys science fiction literature and nature and is rather fond of reading one whilst in the other.