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Physiological characterization of *Prochlorococcus* under abiotic stressors temperature and hydrogen peroxide

Lanying Ma
University of Tennessee - Knoxville, lma8@vols.utk.edu

To the Graduate Council:

I am submitting herewith a dissertation written by Lanying Ma entitled "Physiological characterization of *Prochlorococcus* under abiotic stressors temperature and hydrogen peroxide." 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:

Steven W. Wilhelm, Elizabeth M. Fozo, Barry D. Bruce, Zackary I. Johnson

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

Physiological characterization of *Prochlorococcus* under abiotic stressors temperature and hydrogen peroxide

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DEDICATION

For my immediate and extended family

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ABSTRACT

Cyanobacteria of the genus *Prochlorococcus* are the smallest and most abundant phytoplankters in the ocean. Temperature is a major influence on *Prochlorococcus* abundance and distribution in the ocean, but the physiological basis for this relationship is not well understood. In other microbes, lipid and fatty acid composition have been shown to be influenced by temperature, and temperature has also been proposed as a relevant factor for setting the elemental allocation in marine phytoplankton. In this study, we found that percentage of fatty acids unsaturation was negatively related with temperature in some *Prochlorococcus* strains, but this was not universal. Temperature had a significant linear positive effect on nitrogen and carbon cell quotas across all strains. As temperature increased 10˚C, nitrogen and carbon quotas rose by 40.0% and 34.6%, respectively. Individual strains displayed negative relationships between growth rate and phosphorus quota.

Studies in other photosynthetic organisms suggested that HOOH and temperature extremes act together as stressors. Importantly, it also has been shown that *Prochlorococcus* is highly susceptible to hydrogen peroxide (HOOH) and co-occurring heterotrophs such as *Alteromonas* sp. facilitate the growth of *Prochlorococcus* at the ocean surface by scavenging HOOH. To address the potential synergistic effects of temperature and HOOH on *Prochlorococcus*, we monitored the growth of environmental-relevant concentrations of cold-adapted (MED4) and warm-adapted (MIT9312) *Prochlorococcus* strains with different initial concentrations of HOOH under a range of temperatures. While not impacting the temperature optima for growth, higher concentrations of HOOH severely diminished the permissive temperature range for growth of both *Prochlorococcus* strains. At the permissive temperatures, the growth rates of both *Prochlorococcus* strains decreased as a function of HOOH, and temperature extremes increased susceptibility of photosystem II to HOOH-mediated damage. While these effects were manifest in both strains, they were more pronounced in the warm-adapted strain. Heterotrophic bacteria, serving as a proxy for the natural community, increased the *Prochlorococcus* growth rate under these temperatures and increased the growth temperature range (MED4), and this was attributed in part to their ability to remove HOOH from the medium.

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CHAPTER I INTRODUCTION

Introduction

An introduction to *Prochlorococcus* ecophysiology

Cyanobacteria of the genus *Prochlorococcus* are the smallest phytoplankton in the oligotrophic open ocean; they are also believed to be the most abundant photosynthetic autotroph on Earth [1, 2]. They are ubiquitous in the open ocean from about 40°N to 40°S latitude including the Pacific, Atlantic, and Indian Oceans, the Mediterranean Sea, and the Arabian Sea [3] [4]. Their vertical distribution typically spans the entire euphotic (sunlit) zone from the surface down to depths of about 200 m [5]. Phylogenetic trees constructed by 16S rDNA and the rRNA internal transcribed spacer (ITS) regions demonstrate that *Prochlorococcus* has distinct evolutionary lineages [6]. These lineages are considered ecotypes [7], as they have distinct distributions and contributions to the open ocean ecosystem [8, 9].

Several high light (HL) adapted (sub-)ecotypes dominate the upper euphotic zone, with distinct zones of dominance that can be associated with key environmental parameters [10]. eMED4 is a low temperature adapted HL ecotype that dominates the colder high latitudes, and eMIT9312 is a high temperature adapted ecotype that dominates the warmer low latitudes [11, 12]. The abundance of *Prochlorococcus* falls sharply at high latitude, which indicates that temperature may play an important role in the establishing the habitat range of *Prochlorococcus* [7]. Other high light lineages are dominant in high nutrient

low chlorophyll regions, and are named as “HNLC” [13]. Another HL clade has been found, and appears thus far to be restricted to South China Sea [14]. Other ecotypes of *Prochlorococcus* - eNATL2A, eMIT9313, eSS120 and eMIT9211 - are low light (LL) adapted ecotypes which are more abundant in deeper waters [11, 15].

Temperature influences *Prochlorococcus* growth rate

Microbes cannot control their cellular temperature, and thus their enzymes, lipids, and other cellular components are subjected to the environmental temperature they live in. Accordingly, microbes have growth rates that vary significantly with temperature, evident from studies of heterotrophic bacteria [16, 17] as well as phytoplankton [18-20] Given that environmental temperature in the biosphere is variable, it is not surprising that microbes evolve distinct lineages that are optimized for one temperature over another. As with thermophilic cyanobacteria [21], *Prochlorococcus* has ecotypes whose growth characteristics match the environment they dominate.

Temperature partitions the high light adapted ecotype into a high temperature adapted ecotype (eMIT9312) that dominates the warm low latitudes (~30 °N-30 °S), and a low temperature adapted ecotype (eMED4) that dominates the colder high latitudes (~30-40 °N or S) [11, 12]. Clear relationships between temperature and growth of these ecotypes in culture were found, with eMED4 outperforming eMIT9312 at low temperature and vice versa at high temperature have different temperature preference and temperature range in terms of growth

rate [11, 12]. The growth rates of *Prochlorococcus* strains as a function of temperature has thus been addressed in prior studies, and are consistent with the distributions of the ecotypes they represent. However, the growth of axenic *Prochlorococcus* has not been investigated. We have no idea how contaminants (possibly other phytoplankton and heterotrophs) influences the growth of these strains, but given their ability to help *Prochlorococcus* deal with an interacting stress, oxidative stress, it could be significant. In this study, we will address these questions.

The influence of temperature on the physiology of microorganisms

It has been shown that low temperature has a negative effect on membrane fluidity, which can be deleterious to the cell, especially as it can inactivate transmembrane proteins [22]. To maintain fluidity at low temperatures, a common strategy of microbes is to increase the extent of unsaturation in the fatty acids [23, 24] which decreases order in the membrane due to the kinks imposed by the C-C double bonds [25]. The degree to which *Prochlorococcus* cells change the degree of unsaturation in their fatty acids in response to temperature is not known. However, they do contain several desaturases in their genome [26], suggesting the potential for this response is there. Additionally, other cyanobacteria are known to change their lipids in response to temperature changes [24], and increasing unsaturation led to a significant increase in protection from photoinhibition at low temperature [27].

Temperature can also impact photosynthesis in cyanobacteria, either directly or via its influence on the membranes in which the photosystems are incorporated. The photosynthesis rate has been shown to be enhanced by increase of temperature in *Synechocystis* [27] to some extent. High temperatures could contribute to a loss in photosynthesis by causing direct damage [28], and/or by inhibiting photosystem II repair, specifically by blocking the processing of protein D1, which contains the reaction center chlorophyll [29, 30]. As for lipid chemistry, the impact of temperature on *Prochlorococcus* is not well characterized, although in one study it was observed that the efficiency of photosystem II was nor significantly impacted by temperature, although this was only for a single strain and a limited range of temperatures tested [31].

Finally, temperature has also been proposed as a relevant factor for setting the elemental allocation, specifically the C:N:P ratio in cells in marine phytoplankton but we currently have limited understanding and data for the quantitative effect [32-34]. Toseland and co-workers showed that phytoplankton produce more ribosomes at lower temperature; putatively to compensate for lower efficiency [32]. Yvon-Durocher and co-workers detected an increase in C/P and N/P (but not C/N) for eukaryotic phytoplankton cells growing a higher temperature [34]. CNP ratios are known for several strains of *Prochlorococcus* [35], but as with lipids and photosynthesis, the extent to which these ratios change as a function of temperature is not known.

The influence of reactive oxygen species on the physiology of microorganisms

There are certainly stresses other than temperature for phytoplankton such as *Prochlorococcus* in the natural world, but one of particular interest is oxidative stress; as discussed below it can have interactive effects with temperature, and can profoundly impact *Prochlorococcus* viability. Hydrogen peroxide (HOOH) is generated in seawater by the photooxidation of dissolved organic carbon (DOC) by sunlight [36]. It is also enriched in rainwater [37-39]. HOOH has been shown to inhibit the growth of diverse marine algae, including cyanobacteria, macro- and microscopic chlorophytes, diatoms, and coral-associated zooxanthellae [40-45], The primary sink of HOOH in natural waters is decomposition by bacteria [46, 47].

HOOH can oxidize and damage multiple components of the cell [48], but two of particular concern for *Prochlorococcus* are likely to be their lipids and photosystems. HOOH can oxidize unsaturated lipids to lipid peroxide (LPO). In eukaryotes, a high LPO concentration in the membrane triggers the cell death program by a stress signaling pathway [48, 49]. *Prochlorococcus* may not have a programmed cell death pathway analogous to eukaryotes, but can nonetheless be damaged via lipid peroxidation, as it is known to have di-unsaturated fatty acids [50], which are uncommon for bacteria but common in photosynthetic organisms [51].

In phytoplankton, reactive oxygen species has also been shown to inhibit the *de novo* synthesis of D1 protein, thus impact the photosynthesis recovery after photoinhibition [52, 53]. In *Synechocystis* sp. PCC6803, increased ROS stimulated photodamage of PSII by inhibiting the repair rather than direct photodamage to PSII. HOOH indirectly inhibits the synthesis of D1 protein by inactivating a translation elongation factor; thus the mRNA of the D1-encoding gene *psbA* gene is not translated into protein [54]. The influence of HOOH on this process is not confirmed for *Prochlorococcus*, but is likely to be similar to its influence in these other cyanobacteria.

Prochlorococcus is especially sensitive to ROS

Unlike most cyanobacteria and many aerobes, *Prochlorococcus* lacks a robust defense against reactive oxygen species [55, 56], and has lost its HOOH-degrading catalase during its evolution [57]. As a result, *Prochlorococcus* is highly sensitive to HOOH, with concentrations as low as 800 nM being lethal for all ecotypes in culture [58]. After 24h of exposure to lethal concentrations of HOOH, axenic *Prochlorococcus* exhibited profound envelope damage [55] perhaps as a result of lipid peroxidation. In nature, however, HOOH concentrations in the open ocean are not found at levels lethal to *Prochlorococcus*, and this is due to the role of the microbial community acting as HOOH sinks [55] . It has been shown in culture studies that heterotrophic "helpers" can eliminate the HOOH and facilitate growth of ecologically-relevant concentrations of *Prochlorococcus* [55, 56]. However, these studies were

performed at a single temperature that was optimal for *Prochlorococcus* growth (24 °C); thus the influence of co-occuring environmental stresses such as temperature on this helping phenomenon, and on the HOOH sensitivity of *Prochlorococcus* as a pure culture, is unknown.

Interactions between temperature and oxidative stresses in microorganisms

Stresses do not always act in isolation in the natural world, and temperature stress is known to act synergistically with oxidative stress. Like oxidative stress, low temperatures decrease the turnover of D1, in this case by slowing the processing of the precursor protein pD1 [59]. Lower temperatures can also increase the production of reactive oxygen species by slowing the rate of the Calvin Cycle “dark reaction” enzymes while not concomitantly slowing down light harvesting in the “light reaction,” resulting in a buildup of NADPH and diminished regeneration of NADP+ as the terminal electron acceptor of the light reaction [60, 61]. In this situation, excess electrons can reduce molecular oxygen to produce the reactive oxygen species (ROS) superoxide and hydrogen peroxide (HOOH). Consistently, a superoxide dismutase deletion mutant of *Synechococcus* PCC7942 defective in eliminating superoxide is more sensitive to chilling stress than wild type [62], and in plants and microbes, the concentration of HOOH was higher at lower temperatures [63] [64]. Higher temperatures also generate excessive ROS [64], leading to oxidative stress [65] and cell mortality [66]. In phytoplankton, high temperatures inactivate Rubisco

activase, leading to a decline in Rubisco and Calvin Cycle activity, that, as for cold temperatures, can generate an imbalance in NADPH/$NADP^+$ which can allow the generation of ROS by the reduction of O_2 by photosystem I [29]. As a result, higher temperatures contribute to a loss in photosynthesis by inhibiting photosystem II repair [29, 30], an in some cases by causing direct photodamage [67].

However, this potential synergistic effect has not been quantitatively studied in cyanobacteria, and the effect of HOOH on the growth of axenic *Prochlorococcus* strains over temperature ranges has not been stressed. We hypothesize that the growth rate of *Prochlorococcus* with higher concentration of HOOH is lower than that with lower concentration of HOOH at permissive temperatures; the high concentration of HOOH decreases the temperature range of *Prochlorococcus* growth; the co-culture with heterotroph may increase the temperature range of *Prochlorococcus*.

Objectives of this study

With the knowledge that temperature is a major influence on the abundance and growth of *Prochlorococcus* in the field, and that *Prochlorococcus* is especially sensitive to HOOH, a major goal of my dissertation is to obtain the information how temperature and HOOH influence the growth and physiology of *Prochlorococcus*. First, we investigated how the lipid profile, CNP ratio in cells, and metabolite pools vary with temperature, using 6 strains of *Prochlorococcus*

as representatives of the high temperature and low temperature adapted ecotypes. Second, the interactive effect of temperature and HOOH on the growth and physiology of *Prochlorococcus* was studied, with the hypothesis that high concentration of HOOH would decrease the temperature range of *Prochlorococcus*, and vice versa. This investigation also addressed the impact of helper heterotrophs on these interactive stressors of *Prochlorococcus*.

This dissertation describes insights into the following features of *Prochlorococcus* physiology as a function of temperatures, with the relevant hypotheses indicated:

A. The lipid profile and fatty acid unsaturation in *Prochlorococcus* as a function of temperature

H1: The percentage of fatty acids unsaturation is higher at lower temperature

H2: The response in fatty acid and lipid composition with respect to temperature is different in the genetically and physiologically distinct high-temperature- and low-temperature-adapted ecotypes

Null: Temperature does not have effect on physiological features of *Prochlorococcus* and different ecotype strains have same profile of the physiological features.

B. The elemental composition of *Prochlorococcus* as a function of temperature

H1: The C:N:P ratio varies as a function of temperature

H2: The ratio changes in an ecotype-specific manner due to the genetic differences in the ecotypes

Null: Temperature does not have effect on elemental composition of *Prochlorococcos* and strains from different ecotypes have same elemental composition.

C. The interactive effects of HOOH and temperature on *Prochlorococcus*

H1: The concentration of HOOH decreases the temperature range of *Prochlorococcus*

H2: the heterotroph would increase the growth rate of *Prochlorococcus* at permissive temperatures with environmental relevantly concentration of *Prochlorococcus*

Null: The growth rate and growth temperature range is not influenced by temperature or HOOH.

H3: The physiology of *Prochlorococcus* cultures including Fv/Fm, red chlorophyll is influenced by temperature and HOOH.

Null: The physiology of *Prochlorococcus* is not affected by temperature and HOOH.

CHAPTER II TEMPERATURE INFLUENCE ON LIPIDS AND ELMENTAL CONTENT OF PROCHLOROCOCCUS

This section is a collaborative project between Campagna laboratory in the Department of Chemistry at UTK and Martiny laboratory at UCI. Components of this chapter are preparation for submission as a manuscript with the tentative title:

Adam C. Martiny, Lanying Ma, Celine Mouginot, and Erik Zinser "Interactions between thermal acclimation, growth rate, and phylogeny influence *Prochlorococcus* elemental stoichiometry"

My contribution to this chapter was the growth of culture, sample collection, lipid extraction, data analysis and literature review and writing.

Abstract

Temperature has been shown to have a major influence on the growth and abundance of the most abundant phytoplankton, *Prochlorococcus*. Responses of *Prochlorococcus* to temperature variation are not well understood as they relate to cell biochemistry, but could involve significant shifts in composition of biomolecules and elemental ratios. The two numerically-dominant ecotypes of *Prochlorococcus* have different temperature adaptations, and these adaptations may involve differences in cell composition as a function of temperature. In this study, we investigated how temperature influences the lipid composition and elemental ratios (C:N:P) among three low temperature adapted strains and three high temperature adapted strains. Lipid composition did not show a clear relationship with temperature or ecotype identity. The main fatty acids were consist of 14 to 18 carbon atoms with mono-, di- and tri-unsaturated double bonds. The unsaturation of fatty acid was negatively related with temperature for some strains but not for others. Temperature had a significant linear positive effect on nitrogen and carbon quotas (QN and QC)

across all strains. As temperature increased 10˚C, QN and QC rose by 40.0% and 34.6%, respectively. Individual strains displayed negative relationships between growth rate and phosphorus quote (QP). In addition, temperature also influenced QP on a per strain basis, but there were no systematic differences between strains nor interactions between factors. Temperature had a significant impact on C/P and N/P but the direction varied between strains. There were thus complex interactions between the strain identity and temperature in controlling the elemental ratios in *Prochlorococcus*.

Introduction

Temperature is a major influence on *Prochlorococcus* abundance and distribution [11, 12], partitioning the high light adapted ecotypes into a high temperature adapted ecotype (eMIT9312) and a low temperature adapted ecotype (eMED4) [68]. Temperature may have many impacts on the physiology of *Prochlorococcus*, but at present we have little understanding of these impacts, and how these impacts may or may not translate into ecotype-specific distributions. One cell component known to be heavily influenced by temperature is the membrane[69]. Cell membranes are lipid bilayers with embedded proteins, and their function as semi-permeable layers is essential for the cell [70, 71]. The early processes of photosynthesis including the absorption of light, photochemical reactions, electron transfer, and the synthesis of ATP, all occur in thylakoid membranes in *Prochlorococcus* cells[72, 73]. The cytoplasmic membrane and outer membrane also protect cells from surrounding

environments, and serve to transport essential nutrients into the cell[74]. Therefore, the characteristics of these membranes are obviously important in photosynthetic organisms.

The thylakoid membranes of cyanobacteria contain three glycolipids, namely, monogalactosyl-diacylglycerol (MGDG), digalactosyl-diacylglycerol (DGDG), sulfoquinovosyl-diacylglycerol (SQDG, and one phospholipid, phosphatidyl-glycerol (PG), as major components [24, 75]. The most abundant lipids in Prochlorococcus are MGDG, SQDG, PG, PE (Phosphatidylethanolamines) and PC (Phosphatidylcholines) [76]. Surprisingly, SQDG dominates membrane lipids in this genus, with levels ranging from 38% to 66% of total lipids, while MGDG and PG account for less than 25% and 10%, respectively, of the total lipids [77]. The dramatic reduction in phospholipids for this species (by replacement with sulfur-containing SQDG) was proposed as an evolutionary adaptation to the low phosphorus-containing open ocean [77]. How the lipid headgroups may change in response to temperature is unknown for *Prochlorococcus*.

It has been shown the desaturation of fatty acids in membrane lipids is related with temperature [69, 78]. When the temperature goes down, the desaturation of fatty acid goes up, which is related with the fluidity of membrane to keep the membrane functional [75, 79]. This unsaturation is determined by the activity of acyl-lipid desaturases. In cyanobacteria, there are several predicted acyl-lipid desaturases: Δ6, Δ9, Δ12 (Δ counted from carboxyl group) and ω3 (ω

counted from methyl group) acyl-lipid desaturases [24, 26, 80]. In a recent study, a *Prochlorococcus* strain (MED4) was found to contain several types of fatty acids, including C14:0; C16:1, C18:1 and C18:2[50, 81]. However, it has not been addressed that how temperature influences the fatty unsaturation of *Prochlorococcus*.

Temperature has also been proposed as a relevant factor for setting the elemental allocation in marine phytoplankton [32, 34, 82]. Toseland and co-workers showed that phytoplankton produce more ribosomes at lower temperature; putatively to compensate for lower efficiency. Hence, temperature was hypothesized to influence the elemental ratios in phytoplankton such that a future warming of the oceans would lead to increasing N/P ratios of marine communities [32]. In support using a meta-analysis of eukaryotic phytoplankton lineages, Yvon-Durocher and co-workers detected an increase in C/P and N/P (but not C/N) for cells growing a higher temperature [34]. However, temperature affects many cellular processes beyond translation with unknown outcomes on cellular elemental composition. In addition, the impact of temperature on growth and elemental composition of phytoplankton is likely modulated by the life history of the organism. Important life history traits include the thermal optimum and more broadly adaptation of cellular processes to various temperature conditions. For example, an increase in temperature may have very different physiological effects depending on whether the rise occurs below or above the thermal growth optimum. Thus, the organismal context should be considered for understanding

the influence of temperature on the elemental composition of phytoplankton.

Here, we hypothesize that the elemental composition and lipid composition with fatty acid unsaturation of *Prochlorococcus* are sensitive to changes in temperature. Such temperature effects will be modulated by changes in growth rate as well as the life history of the organisms. To address this hypothesis, we quantified the effect of temperature on the growth rate, elemental composition and lipid composition of six *Prochlorococcus* strains acclimated to different temperatures. The goal of this study was to provide key information on how temperature influences the elemental composition and lipid composition of this key, abundant lineage and its contribution to global biogeochemical cycles.

Materials and Methods

1 *Prochlorococcus* strains and culture conditions.

Six *Prochlorococcus* strains affiliated with the eMED4 and eMIT9312 clades were analyzed in this study (Table 1). Axenic *Prochlorococcus* strains were cultured in filtered (0.2 μm polycarbonate filter, pressure <10 mm Hg) artificial seawater AMP-J medium [83](per L, 28.1 g NaCl, 6.9 g $MgSO_4$*$7H_2O$, 5.49 g $MgCl_2$*6H2O, 0.67 g KCl. 1.47g $CaCl_2$, 0.504 g $NaHCO_3$ with 2 ml 0.5 M TAPS, pH 8.0, 1 ml 0.4 M $(NH_4)_2SO_4$, 2 ml 0.025 M NaH_2PO_4 pH7.5, 100 μl 10,000 X Pro99 Trace Metal Mix) with 40 μmol quanta m^{-2} s^{-1} light on a 12:12 light:dark cycle using cool white fluorescent bulbs. Axenicity of cultures was monitored by purity test in YTSS and 1/10 ProAC media [56].

2 Quantification of *Prochlorococcus*

Concentration of *Prochlorococcus* was measured by flow cytometry with the Guava EasyCyte 8HT cytometer (Millipore), using an established method [58].

3 Lipid collection

The lipid samples were collected with 0.2 μm Isopore^{TM} membrane filters (Millipore, USA) with high voltage. The filters were flushed with liquid nitrogen right after and stored at -80^{o}C until samples were extracted.

4 Lipid extraction

The filters with lipid samples were unfolded and put sample-side down into eppendorf tubes. 1 ml of fluid mixed with 95% ethanol:water diethyl ethnol: pyridine: 4.2 N NH_4OH with the ratio to 15:!5:5:1:18 were added into the eppendorf tubes and stood for 15minutess. This solvent was moved to a new eppendorf with 100 μl glass beads and vortex and incubated at 60^{o}C water bath for 20 minutes. After then, it was centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed to a new drawn vial. The addition and 1ml fluid was repeated to get more supernatant and this supernatant was added to the same vial. 300μl water saturated butanol and 150μl of water was added to the eppendorf with glass beads, vortexed and centrifuged at 10rpm for 2 minutes. The top butanol phase was added into drawn vials. 300 μl water-saturated butanol was added into the aqueous phase in the eppendorf, vortexed and centrifuged at 10,000 rpm for 2 minutes. The top butanol phase was also added

into the drawn vials. The drawn vials were dried under N_2 and resuspended in 300µl of MeOH with $CHCl_3$ (with V/V ratio as 9:1)

5 Samples analysis

Following resuspension, lipid extracts were placed in a Dionex Ultimate 3000 autosampler cooled to 4 ˚C. A 10 µL aliquot was injected through a Kinetex HILIC column (150 mm × 2.1 mm, 2.6 µM) (Phenomenex, Torrance, CA, USA). Mobile phase A consisted of 10 mM aqueous ammonium formate at pH 3 and mobile phase B consisted of 10 mM ammonium formate at pH 3 in 93% (v/v) acetonitrile. All solvents were HPLC grade (Thermo Scientific). The liquid chromatographic separation proceeded for 35 min with a flow rate of 0.2 mL/min. The gradient began with 100% B from 0 to 1 min, 1 to 15 min B decrease to 81%, 15 to 15.1 min decreased B to 48%, 15.1 to 25 min maintained B at 48%, 25 to 25.1 min increased B to 100% where it was maintained for the duration of the run. The column oven temperature was kept at 25 ˚C for the duration of the separation.

The eluent was introduced into an Exactive Plus orbitrap mass spectrometer (Thermo Scientific) via electrospray ionization set to a spray voltage of 4 kV and a heated capillary temperature of 350 ˚C. The nitrogen sheath gas flow was set at 25 psi with an auxiliary gas pressure of 10 psi. Samples were run in full scan mode with a resolution of 140,000, a scan range of

100 – 1500 m/z, and an AGC target of 3e6. Each sample was run in both positive and negative ionization modes.

Files generated by Thermo Scientific's Xcalibur software during the MS run were converted from their .raw format to the open source mzML format1 via the msconvert software as part of the ProteoWizard package.2 MAVEN3 (Princeton University) was used to automatically correct the total ion chromatograms based on the retention times for each sample. Lipids were manually selected and quantified by mass (± 10 ppm) and retention time for each sample based on known retention times.

Particulate organic matter and data analysis: Particulate organic carbon (POC), nitrogen (PON) and phosphorus (POP) samples were each collected in duplicate from each of three biological replicates (6 total) by filtration of 50 ml of culture onto precombusted (5 h, 500°C) GF/F filters (Whatman, Florham Park, New Jersey) and stored at -20°C. To quantify POC and PON, filter samples were thawed and allowed to dry overnight at 65°C. Filters were then packed into a 30 mm tin capsule (CE Elantech, Lakewood, New Jersey) and analyzed for C and N content on a FlashEA 1112 nitrogen and carbon analyzer (Thermo Scientific, Waltham, Massachusetts) [84]. POC and PON concentrations were calibrated using known quantities of atropine and peach leaves in each run. The amount of POP was determined in each sample using a modified ash-hydrolysis method [85].

All data was plotted using Matlab. Statistical analyses were done using R. To account for non-linear effects of temperature on the elemental content of *Prochlorococcus* strains, temperature was treated as a factor with four levels.

Results

Lipids composition in 6 strains of *Prochlorococcus*

To investigate the impact of temperature on lipid composition in *Prochlorococcus*, we quantified the major lipid percent in cells and fatty acid composition of 6 strains representing the high- and low-temperature-adapted ecotypes (Figure 1, Table 1). We did not observe clear trends for lipid composition as a function of temperature or ecotype identity (Figure 1). However, MGDG and SQDG were generally the most abundant lipids. PC was most abundant in few occasions, such as MIT9515 at 26°C and MIT9215 at 24°C. For low temperature adapted ecotype eMED4, the percentage of MGDG varied from 12% to 59%. Among this ecotype, MED4 had the most MGDG, from 40% to 59%. The percentage of SQDG varied from 6%-59% and Vol29 has the most SQDG from 52%-57%. For high-temperature adapted ecotype eMIT9312, the percentage of MGDG was from 29% to 83% with SQDG from 1% to 35%. UH18301 has the most MGDG from 46% to 83% and MIT9312 had the most SQDG from 10% to 35%.

The fatty acid composition of cyanobacteria membrane lipids has considerable diversity, including saturated fatty acid, mono-and di-

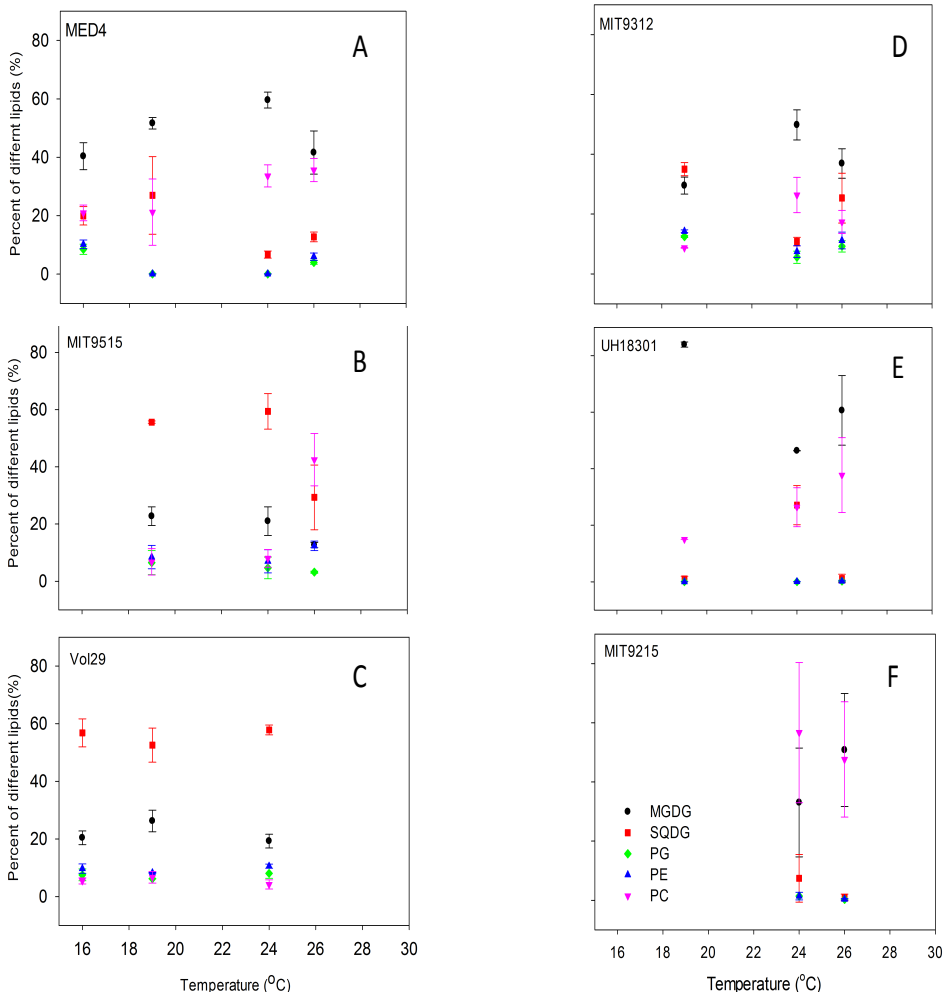

Figure 1 Lipid composition of 6 strains of *Prochlorococous* as the function of temperature.

Percentage of main lipids MGDG(black circle), SQDG (red square), PG (green hexagon), PE (blue up-triangle) and PC (pink down-triangle) were displayed as the function of temperature in different strains. MED4 (A), MIT9515(B) and Vol29 (C) belong to low temperature adapted ecotypes. MIT9312(D), MIT9215 (E) and UH18301(F) belong to high temperature adapted ecotypes

Table 1 Overview of strains and temperature treatments

Strain name	Derived from	Clade	Origin	T_{opt} (˚C)	T treatments (˚C)	References
MED4	PCC	eMED4	Med Sea	24	16, 19, 24, 26	(Moore et al. 1995)
VOL8	MIT9515	eMED4	Eq. Pacific	24	19, 24, 26	(Rocap et al. 2002; Morris et al. 2011)
VOL29	N/A	eMED4	N. Pacific	?	16, 19, 24	NA
MIT9312	N/A	eMIT9312	Gulf Stream	24	19, 24, 26	(Moore et al. 1998)
MIT9215	N/A	eMIT9312	Eq. Pacific	24	24, 26	(Moore and Chisholm 1999)
UH18301	N/A	eMIT9312	N. Pacific	?	19, 24, 26	(Morris et al. 2011)

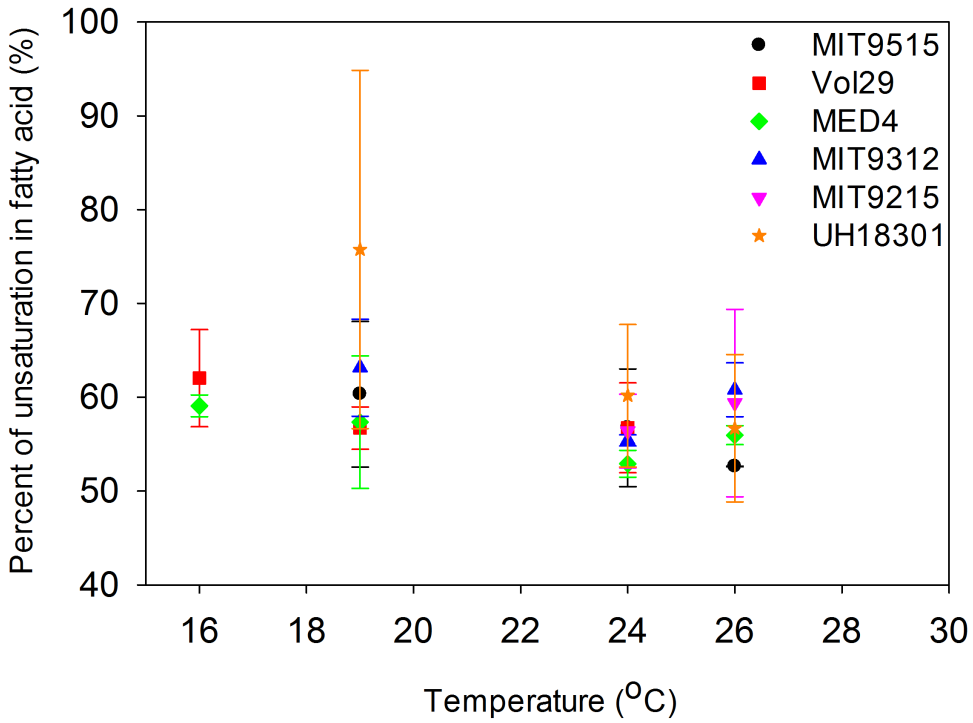

Figure 2 Fatty acid unsaturation in 6 *Prochlorococcus* strains.

eMED4 has three strains: MED4(green diamond), MIT9515(black circle) and Vol29 (red square); eMIT9312 has three strains: MIT9312(blue up-triangle), UH18301(orange star) and MIT9215 (pink down triangle).

unsaturated fatty acid [26, 81].

To investigate the main fatty acid composition, the percentage of different fatty acid in 6 strains along with different temperature was investigated. We found that the main fatty acids in *Prochlorococcus* are C14 to C18, and existed as saturated or mono-, di- or tri-unsaturated fatty acids (see Appendix Table 3).

It has been shown that the unsaturation decreased as temperature went up for UH18301, MIT9515 and Vol29 while it is not the case for MIT9312 and MED4 (Figure 2).

CNP analysis of these 6 strains:

To identify the impact of temperature on the elemental composition of *Prochlorococcus*, we quantified the carbon, nitrogen, and phosphorus cell quota as well as growth rate of six strains (Table 1). The strains covered both the eMED4 and eMIT9312 clades adapted to different temperatures. Median cell quotas across all strains of 0.44 fg P, 6.4 fg N, and 33 fg C were similar to previously measured levels [35, 86]. Temperature had a significant linear positive effect on QN and QC across all strains (Table 2 and Figure 3A-C). As temperature increased 10˚C, QN and QC rose by 40.0% and 34.6%, respectively. We also examined the elemental ratios. C/N showed little variability and was close to Redfield proportions (median C/N = 6.1) (Figure 3D). In contrast, C/P and N/P were above Redfield proportions (median C/P = 174, median N/P = 29) (Figure 3E-F). Both ratios showed some effect of temperature but there were no significant linear trends across all strains (Table 2). We also determined how

changes in growth rate in conjunction with temperature affected the elemental composition of *Prochlorococcus* (Figure 3). We saw a significant negative effect of growth rate on QP, whereas the other cell quotas and ratios did not display any linear trends (Table 2).

We next examined the influence of temperature on the cell quotas in the context of each strain as well as indirectly via changes in growth rate (Table 2 and Figure 4). We observed some similarities as well as difference in the response across the six strains. As seen in the aggregated response for all strains, individual strains displayed negative relationships between growth rate and QP. In addition, temperature also influenced QP on a per strain basis (Figure 4A), but there were no systematic differences between strains nor interactions between factors (Table 2). The Vol8 and Vol 29 strains had higher overall QN and QC and temperature plus growth rate influenced QN and QC across all strains (Figure 4B + C). We also saw some evidence for interactions between strain identity, temperature, and growth rate in setting the overall elemental composition (Table 2).

Temperature and growth rate also affected the elemental ratios of each strain in unique ways (Figure 5). For C/N, we observed differences in the overall level across the strains, whereby strain VOL8 showed the highest and MIT9312 the lowest level (Figure 5A). The strain specific C/N was also marginally affect by growth rate but not temperature (Table 2). The strain specific C/P and N/P also varied considerably between strains (Figure 5B + C). Especially MIT9215 had

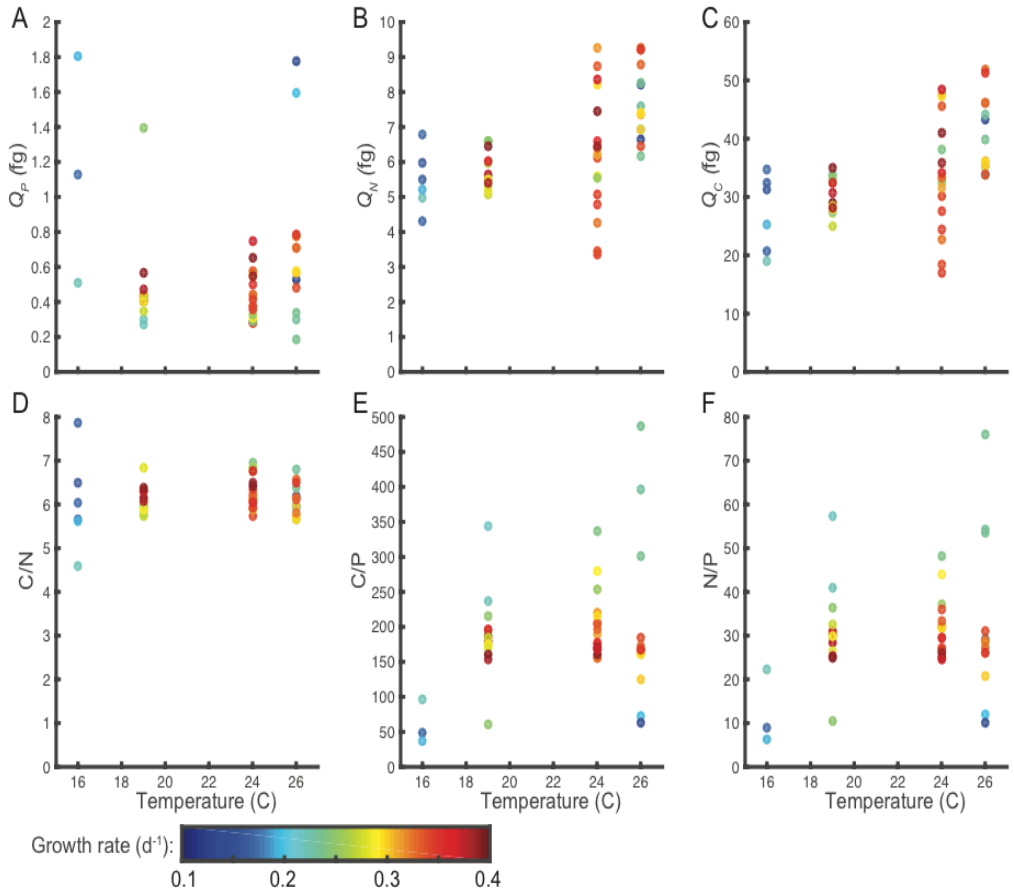

Figure 3 Influence of temperature and growth on the elemental composition and ratios of across *Prochlorococcus* strains.

Factor measured are (A) phosphorus cell quota (QP), (B) nitrogen cell quota (QN), (C) carbon cell quota (QC), (D) C/N, (E) C/P, and (F) N/P. The color of each sample point indicates the observed growth rate. All ratios are molar based.

Table 2 Effects of temperature, growth rate and strain identity on the elemental composition of six *Prochlorococcus* strains.

	Q_P		Q_N		Q_C		C/N		C/P		N/P	
Linear model	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value	Estimate	*p*-value
Intercept	1.2	$4.9x10^{-3}$	2.2	0.08	7.8	0.27	5.8	$7.6x10^{-17}$	53	0.6	14	0.3
Temperature	0.0	1.0	0.2	$2.9x10^{-4}$	1.2	$2.3x10^{-4}$	0.01	0.6	6.8	0.1	0.9	0.1
Growth rate	-2.0	$1.2x10^{-2}$	-1.6	0.6	-3.3	0.8	0.3	0.8	-65	0.7	-14	0.6
ANOVA	SS	*p*-value	SS	*p*-value	SS	*p*-value	SS	*p*-value	SS	*p*-value	SS	*p*-value
Strain	1.1	0.1	22	$1x10^{-4}$	$1x10^{3}$	$6x10^{-6}$	4.3	$7x10^{-3}$	$2x10^{5}$	$2x10^{-5}$	$4x10^{3}$	$1x10^{-4}$
Temperature[1]	1.4	$2.3x10^{-2}$	41	$5x10^{-7}$	$1x10^{3}$	$8x10^{-7}$	0.3	0.6	$3x10^{4}$	$2.5x10^{-2}$	$6.2x10^{2}$	$4.8x10^{-2}$
Growth rate	0.6	$3.0x10^{-2}$	6.5	$1x10^{-3}$	$2.8x10^{2}$	$3x10^{-4}$	0.8	$5.3x10^{-2}$	67	0.9	10	0.7
Strain*T	0.8	0.5	22	$1x10^{-3}$	$5.7x10^{2}$	$3x10^{-3}$	1.8	0.4	$6x10^{4}$	$2.6x10^{-2}$	$1x10^{3}$	$3.9x10^{-2}$
Strain:Gr	0.1	0.9	0.9	0.8	28	0.8	0.3	0.9	$3x10^{3}$	0.9	$1.x10^{2}$	0.9
T:Gr	0.6	0.2	0.8	0.6	6.5	0.9	0.7	0.3	$2x10^{4}$	0.13	$5.8x10^{2}$	$5.7x10^{-2}$
Strain:T:Gr	0.2	0.9	8.6	$7.2x10^{-2}$	$2.4x10^{2}$	$9.6x10^{-2}$	0.8	0.9	$5x10^{3}$	0.9	89	0.9

[1]Temperature was treated as factor in ANOVA

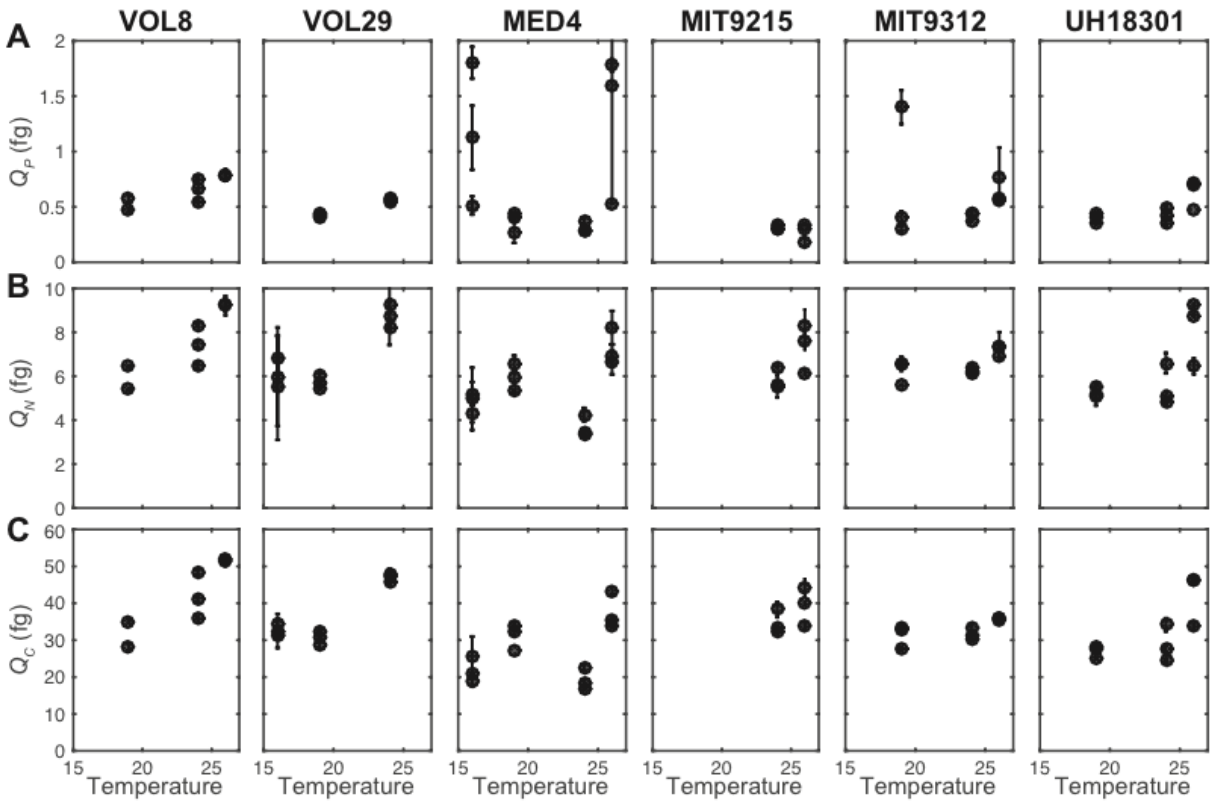

Figure 4 Influence of temperature on cell quotas in six *Prochlorococcus* strains.

Cell quotas include (A) phosphorus (QP), (B) nitrogen (QN), and (C) carbon (QC). The errorbars represent one standard deviation based on technical duplicate sampling of each strain.

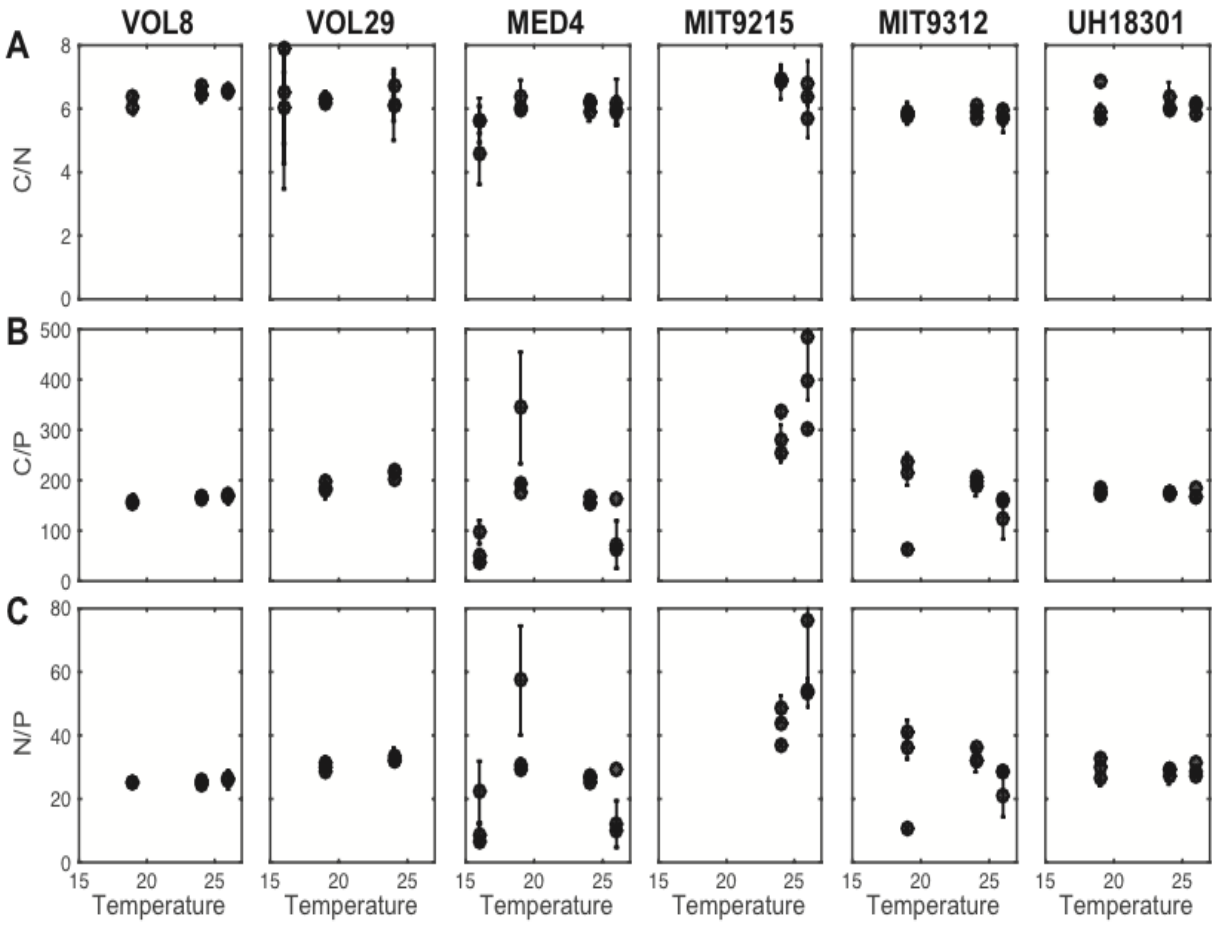

Figure 5 Influence of temperature on elemental ratio in six *Prochlorococcus* strains.

Elemental ratios include (A) C/N, (B) C/P, and (C) N/P. All ratios are molar based. The errorbars represent one standard deviation based on duplicate sampling of each strain.

considerably higher ratios compared to the other strains. Temperature had a significant impact on C/P and N/P but the direction varied between strains. C/P and N/P in strains VOL8, VOL29, and MIT9215 were positively affected, MED4 showed more of a unimodal response, UH81301 showed no response, and a negative response was observed in MIT9312. Thus, there were complex interactions between the strain identity and temperature in controlling the elemental ratios in Prochlorococcus.

Discussion

Temperature and lipid chemistry

The physical properties of a biological membrane depend on the fatty acid composition of its component membrane lipids. Unsaturated fatty acids are essential constituents of polar glycerolipids of biological membranes and the unsaturation level of membrane lipids is important in controlling the fluidity of membranes [87]. Our study showed that SQDG and MGDG are dominant lipids in *Prochlorococcus*, which is consistent with previous studies [76]. In this study we examined how lipid composition and fatty acid composition change in *Prochlorococcus* due to temperature. While lipid composition varied as a function of temperature, there was no clear trend on this relationship. Notably, the composition of lipid in strain MED4 was different from a previous study of the same strain, at the same temperature (22-24 °C) [76]. In our study, the SQDG content was much lower (6%). The value for the MED4 in the prior study (66%), was however more in line with the other eMED4 ecotypes of our study (6-59%),

suggesting that our MED4 24 °C analysis may require additional sampling. MED4 notwithstanding, it was interesting to note that the low-temperature-adapted strains have a higher fraction of SQDG than the high-temperature-adapted strains at 24 °C, suggesting an ecotype-specific difference in lipid chemistry that warrants further investigation. Another difference in our results compared to the prior study is a higher fraction of lipids as PC. This discrepancy likewise warrants further investigation, but it should be noted that our cultures were axenic, while those from the prior study were not, and the impact of heterotrophic bacteria on the lipid concentrations in *Prochlorococcus* and on the mixed community are not known.

The fatty acids in these *Prochlorococcus* strains are mainly from C14 to C18 which is consistent from previous study that cyanobacteria contains fatty acid with 16 and 18 carbon atoms as main fatty acid in their membrane lipids [88]. In a very recent paper, they studied the genomes of several strains of *Prochlorococcus* and found the potential to produce fatty acid with di- and mono-unsaturated 16 and 18 carbon atoms, but not 14 carbon atoms [81]. However, in Biller et al's paper, they did see fatty acid with 14 carbon atoms in *Prochlorococcus* [50], as we did, thus the gene(s) responsible for this fatty acid may be novel for this cyanobacterial lineage.

The unsaturation of fatty acid was negatively related with thermal effect for several, but not all, strains in this study. High temperature adapted strain UH18301 had the most unsaturation content and its content sharply decreased

with temperature (the R^2 of linear trend line between unsaturation and temperature as 98.8%), consistent with what has been observed for other cyanobacteria [89]. The reason of this high content of unsaturation from this strain is not clear yet. The increased unsaturation of fatty acid enhances the protection against low-temperature photoinhibition [27]. It also has been that when the growth temperature is suddenly shifted downward, the fatty acid composition of A. variabilis is rapidly altered [23]. The increase in unsaturation in fatty acid also enhanced the tolerance to photosynthetic machinery to salt stress [89]. It was surprising to find that some strains had no significant change in fatty acid unsaturation as a function of temperature, given the presence of desaturase genes in the genome and the trend in cyanobacteria and other bacteria to do so. This strain-strain difference will warrant further investigation in future studies.

Temperature and elemental composition

Multiple studies have put forward a 'translation-compensation' hypothesis, whereby cells have lower demand for P-rich ribosomes and associated depressed QP when growing at higher temperature [32, 34]. A lower QP will cause elevated C/P and N/P and such an acclimation mechanism should further explain the high elemental ratios observed in cell growing in the oligotrophic gyres [90, 91]. However, we see mixed support for this hypothesis in *Prochlorococcus*. The thermal effect is manifested by increasing QN and QC, whereas QP show little change. This points towards other physiological acclimation mechanisms as the primary drivers of elemental changes in

Prochlorococcus. The observed elemental changes are likely associated with a cell size increase as QN and QC increase in tandem. Thus, our study adds to an emerging concept, whereby changes in cell size due to physiological responses to different environmental conditions are important for regulating the elemental composition and ratios in marine Cyanobacteria.

QP appear linked to changes in growth rate. This would indicate support for the growth rate hypothesis [92]. However, QP is actually decreasing at elevated growth rates in across all strains as well as for most individual strains. The exact biochemical mechanism cannot be elucidated with the current study but as seen in other marine Cyanobacteria. it is clear the growth rate hypothesis cannot explain the elemental composition of *Prochlorococcus* strains.

We observed high C/P and N/P, whereas C/N is close to Redfield proportions. The cells are growing under nutrient replete conditions, which should lead to C/P and N/P at the lower end of the range for an organism [93, 94] . Our observations of elevated ratios in *Prochlorococcus* are consistent with past observations [35, 90, 95] and suggest this lineages have overall high C/P and N/P. As such, the presence of *Prochlorococcus* in low latitude marine communities will contribute to elevated elemental ratios.

We did not observe a strong phylogenetic structuring of the elemental composition and stoichiometry within *Prochlorococcus*. However, we do see extensive strain variability in the elemental content and ratios due to thermal acclimation. Thus, the organismal context appears important for the individual

response. This is consistent with the thermal response in other phytoplankton lineages. In an analysis across nine eukaryotic phytoplankton lineages, Yvon-Durocher and co-workers observed substantial variability in the link between thermal changes and elemental cellular composition [34]. Furthermore, this meta-analysis as well as our study found little thermal effect on C/N, suggesting C/N being fairly invariant to temperature changes.

The broader environmental growth conditions are important to consider when evaluating the elemental outcome in *Prochlorococcus* to thermal changes. In this study, the cells were growing under nutrient replete conditions. Multiple studies have shown the possibility for interactions between factors including interactions between nutrient limitation and temperature [34, 96]. *Scenedesmus* showed stronger thermal responses under nutrient limited vs. replete conditions. Hence, future work studying the interaction between nutrient limitation and thermal conditions would enhance our understanding for how changes in ocean temperature would affect *Prochlorococcus* elemental stoichiometry.

Our study has important implications for understanding both present day and future biogeochemical functioning. The oceans are projected to undergo substantial changes in temperature and nutrient availability due to a rising CO_2 in the atmosphere. Such environmental changes will likely have a large impact on phytoplankton community structure and physiology. This may further affect the elemental composition and the link between the cycles of carbon, nitrogen, and

phosphorus in the ocean. Therefore, we need to understand the biochemical how cycles of key nutrients like nitrogen and understanding current patterns.

Appendix

Table 3 Fatty acid composition in each strain under different growth temperatures

strain	lipid		Fatty acid 14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3
MED4	MGDG	16	0.245	0.070	23.038	0.067	0.000	0.000	0.000	11.231
		19	0.336	0.072	44.455	0.032	0.000	0.000	0.000	1.077
		24	0.101	0.004	31.532	0.007	0.000	0.000	0.000	2.845
		26	0.312	0.227	36.859	0.017	0.000	0.000	0.000	0.997
	SQDG	16	18.900	5.084	0.089	0.000	0.460	0.000	0.345	0.025
		19	0.000	11.947	0.035	0.000	0.056	0.000	0.014	0.007
		24	0.000	3.684	0.019	0.000	0.042	0.000	0.007	0.015
		26	13.647	1.302	0.082	0.000	0.190	0.000	0.109	0.000
	PG	16	0.000	0.011	0.000	0.000	0.023	0.002	0.000	0.000
		19	0.000	0.000	0.000	0.000	0.032	0.000	0.000	0.000
		24	0.000	0.000	0.000	0.000	0.406	0.008	0.000	0.000
		26	0.000	0.026	0.000	0.000	0.038	0.000	0.000	0.000
	PC	16	2.608	0.030	1.073	0.000	0.417	7.421	15.170	0.038
		19	1.679	0.000	12.974	0.000	0.181	2.986	16.464	6.048
		24	1.437	0.000	11.657	0.000	12.159	1.266	23.821	5.136
		26	4.354	0.014	1.982	0.000	0.244	6.562	30.402	0.161
VOL8	MGDG	19	5.036	0.262	5.584	0.039	0.000	0.000	0.000	2.259
		24	5.297	0.187	4.833	0.036	0.000	0.000	0.000	1.557
	SQDG	19	5.391	10.808	29.600	0.000	30.195	0.000	0.518	0.077
		24	4.054	6.294	33.725	0.000	34.185	0.000	0.396	0.064
	PG	19	0.000	0.000	0.000	0.000	0.016	0.013	0.000	0.000
		24	0.000	0.060	0.000	0.000	1.340	0.000	0.000	0.000
	PC	19	1.307	0.040	1.233	0.000	0.742	1.420	2.116	2.116
		24	0.997	0.049	1.002	0.000	0.181	1.461	2.849	2.849
Vol29	MGDG	16	4.999	0.036	3.930	0.022	0.000	0.000	0.000	1.128
		19	5.273	0.059	3.830	0.047	0.000	0.000	0.000	3.440
		24	4.672	0.070	4.115	0.000	0.000	0.000	0.000	0.658
	SQDG	16	9.225	11.914	29.210	0.000	0.000	0.000	0.600	0.066
		19	4.967	4.395	27.185	0.000	0.000	0.000	0.078	0.009
		24	4.687	5.654	29.627	0.000	0.000	0.000	0.148	0.000
	PG	16	0.000	0.072	0.000	0.000	0.000	0.000	0.000	0.000
		19	0.000	0.056	0.000	0.000	0.299	0.000	0.000	0.000
		24	0.000	0.062	0.000	0.000	0.258	0.000	0.000	0.000
	PC	16	0.644	0.006	0.657	0.000	0.086	1.071	2.715	0.000

Table 3 Continued

strain	lipid		Fatty acid 14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3
		19	0.795	0.037	1.100	0.000	5.966	1.039	8.468	0.007
		24	0.661	0.040	1.911	0.000	6.377	0.900	7.966	0.005
	MGDG	19	0.054	0.044	11.349	0.011	0.000	0.000	0.000	14.788
		24	0.060	0.028	16.124	0.000	0.000	0.000	0.000	21.498
		26	0.050	0.018	14.797	0.000	0.000	0.000	0.000	12.807
	SQDG	19	24.829	12.726	0.026	0.000	0.796	0.000	0.630	0.139
		24	7.767	1.776	0.035	0.000	0.191	0.000	0.128	0.028
		26	24.959	6.263	0.013	0.000	0.396	0.000	0.358	0.025
	PG	19	0.000	0.020	0.000	0.000	0.031	0.002	0.000	0.000
		24	0.000	0.000	0.000	0.000	0.019	0.002	0.000	0.000
		26	0.000	0.026	0.000	0.000	0.028	0.000	0.000	0.000
	PC	19	1.628	1.488	0.408	0.000	0.058	1.929	11.318	0.034
		24	1.609	5.682	0.553	0.000	0.198	3.625	17.987	0.053
MIT9312		26	0.868	4.130	0.518	0.000	0.097	1.671	18.077	0.075
	MGDG	24	0.211	0.020	22.490	0.202	0.000	0.000	0.000	0.611
		26	0.072	14.357	19.809	9.884	0.000	0.000	0.000	0.393
	SQDG	24	0.000	5.897	0.034	0.000	0.034	0.000	0.007	0.011
		26	0.000	0.820	0.028	0.000	0.028	0.000	0.003	0.000
	PG	24	0.000	0.000	0.000	0.000	0.847	0.000	0.000	0.000
		26	0.000	0.000	0.000	0.000	0.254	0.000	0.000	0.000
	PC	24	1.185	0.000	6.604	0.000	23.786	2.274	2.274	3.168
VOL1		26	0.267	0.000	4.884	0.000	8.717	1.607	1.607	1.668
	MGDG	19	0.051	25.918	10.117	21.439	0.000	0.000	0.000	0.144
		24	0.079	0.552	37.320	0.216	0.000	0.000	0.000	0.399
		26	0.207	0.063	42.528	0.030	0.000	0.000	0.000	1.669
	SQDG	19	0.006	0.830	0.013	0.000	0.015	0.000	0.001	0.002
		24	0.000	16.559	0.000	0.000	0.004	0.000	0.004	0.000
		26	0.000	11.140	0.017	0.000	0.027	0.000	0.010	0.000
	PG	19	0.000	0.000	0.000	0.000	0.419	0.000	0.000	0.000
		24	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000
		26	0.000	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	PC	19	0.274	0.018	6.289	0.000	0.000	12.381	17.350	1.490
		24	0.759	0.033	8.635	0.000	0.000	0.025	28.448	4.114
VOL6		26	1.690	0.000	12.723	0.000	0.000	0.124	17.963	6.970

CHAPTER III DEGRADATION OF HYDROGEN PEROXIDE BY THE MICROBIAL COMMUNITY FACILITATES THE GROWTH OF *PROCHLOROCOCCUS* AT ITS TEMPERATURE EXTREMES

This Chapter is in preparation as a manuscript to be submitted to ISME J / AEM, with the following authors:

Authors: Lanying Ma[1], Zackary I. Johnson[2], and Erik R. Zinser[1]

Address: [1]Department of Microbiology, University of Tennessee, Knoxville, Tennessee, United States of America, [2] Nicholas School of the Environment, Duke University Marine Laboratory, Beaufort, North Carolina, United States of America

The work I performed for this chapter included the investigation of growth rate, the Fv/Fm measurement, the red fluorescence measurement of MED4 and MIT9312 in presence /absence of heterotroph with different concentration of HOOH to obtain the growth range of *Prochlorococcus* stain under these conditions.

Abstract

Cyanobacteria of the genus *Prochlorococcus* are the smallest and most abundant phytoplankters in the ocean. It has been shown that *Prochlorococcus* is highly susceptible to hydrogen peroxide (HOOH) and co-occurring heterotrophs such as *Alteromonas* sp. facilitate the growth of *Prochlorococcus* at the surface by scavenging HOOH. Temperature is also a major influence on *Prochlorococcus* abundance and distribution in the ocean, and studies in other photosynthetic organisms suggested that HOOH and temperature extremes act together as stressors. To address the potential synergistic effects of temperature and HOOH on *Prochlorococcus*, we monitored the growth of a cold-adapted (MED4) and a warm-adapted (MIT9312) *Prochlorococcus* strain with different initial concentrations of HOOH under a range of temperatures. While not impacting the temperature optima for growth, higher concentrations of HOOH severely diminished the permissive temperature range for growth of both *Prochlorococcus* strains. At the permissive temperatures, the growth rates of both *Prochlorococcus* strains decreased as a function of HOOH, and temperature extremes increased susceptibility of photosystem II to HOOH-mediated damage. While these effects were manifest in both strains, they were more pronounced in the warm-adapted strain. Heterotrophic bacteria, serving as a proxy for the natural community, increased the *Prochlorococcus* growth rate under these temperatures, and this was attributed in part to their ability to remove HOOH from the medium. These studies indicate that in the ocean, the cross-protective function of the microbial community may confer a fitness increase for

Prochlorococcus at its temperature extremes, especially near the ocean surface where oxidative stress is highest, and this interaction may play a significant role in defining *Prochlorococcus'* habitat range with respect to latitude.

Introduction

Stress is a common state for microbes, and it is becoming clear that biological interactions can augment or ameliorate this condition. One stress that has received much attention in microbial ecology is nutrient stress; most microbes spend the majority of their lives in a nutrient limited or nutrient starved state. Relief from this stress comes from nutrient replenishment, and there are many examples where this is facilitated by cross-feeding from other organisms. Microbes and metazoans can serve as benefactors for microbes limited for carbon, nitrogen, iron, and vitamins [97], and this interaction can be general or highly species-specific.

Microbe-microbe interactions can also potentially mediate relief from non-nutritional abiotic stresses, including temperature, pH, desiccation, oxidative, and osmotic stresses. Facilitation is a widely accepted principle in metazoan ecology ([98-100] but see [101]): at the extremes of environments, positive interactions between organisms tend to dominate over negative ones [100, 102]. This is true for both terrestrial and marine systems, with the former tending to involve intra-trophic interactions between plants and the latter inter-trophic interactions often between primary and secondary producers [103]. Examples of facilitation are less extensive for microbes, but reports indicate that microbes can

cross-protect phytoplankton from a number of abiotic stresses, including inorganic carbon limitation and oxygen toxicity [104, 105]. A recent study indicated that by relieving vitamin B_{12} deficiency, heterotrophic bacteria can improve thermotolerance of the green alga *Chlamydomonas reinhardtii* [106]. Microbes can also protect other microbes from oxidative stresses by the removal of the reactive oxygen species from the environment. This has been shown to protect heterotrophic microbes [105], Antarctic diatoms [107] and, as we demonstrated in prior studies [55, 56], marine cyanobacteria as well.

Cyanobacteria of the genus *Prochlorococcus* are the smallest phytoplankton in the oligotrophic open ocean; they are also believed to be the most abundant photosynthetic autotroph on Earth [3] [4]. Prior work by our group has shown that the microbial community can facilitate the growth of the marine cyanobacterium *Prochlorococcus* in the surface mixed layer by the removal of hydrogen peroxide (HOOH) [55]. In absence of the community the HOOH concentrations exceed the lethal limit of *Prochlorococcus* (≥800 nM), but in its presence the HOOH concentrations are maintained within a tolerable range for *Prochlorococcus*, rarely exceeding 200 nM except during rainfall events [55]. Unlike most cyanobacteria and many aerobes, *Prochlorococcus* lacks a robust defense against reactive oxygen species, and has lost its HOOH-degrading catalase during its evolution [57]. Instead, *Prochlorococcus* relies upon the extant microbial community to eliminate the HOOH and maintain the concentrations within the tolerable range.

Stresses do not always act in isolation in the natural world, and temperature stress is known to act synergistically with oxidative stress. Both can inhibit the repair of photosystem II of phytoplankton [59, 108], and temperature extremes can generate excess reactive oxygen species (ROS) [64], leading to oxidative stress [65] and cell mortality [66]. Given the potential for synergistic effects of oxidative and thermal stresses on *Prochlorococcus* growth and photosynthesis, we hypothesized that 1) temperature extremes sensitize *Prochlorococcus* to HOOH-mediated damage, and that 2) by removing the HOOH threat, the extant microbial community facilitates the survival and growth of *Prochlorococcus* at these temperature extremes. As temperature plays a key role in establishing the geographical distributions of the numerically dominant lineages (ecotypes), this interaction between oxidative and thermal stress may have important consequences for the ecology of *Prochlorococcus*.

While multiple ecotypes have been identified, the water column is typically dominated numerically by one of two ecotypes, eMED4 or eMIT9312 (the “e” prefix denotes “ecotype”, named from the type strains MED4 and MIT9312) [68, 109]. These high-light adapted ecotypes partition the surface ocean by latitude, with eMIT9312 dominating the low latitudes, and eMED4 the high latitudes north and south of the equator. Temperature was identified as the key environmental variable correlating with abundance of these two ecotypes [68]. Consistent with the field data, studies with non-axenic cultures of isolated strains confirmed that while both ecotypes share a common temperature optimum (~24 °C), the eMED4

strains grew faster and at a broader range of temperatures below ~19 °C, compared to the eMIT9312 strains, while the opposite held true for cultures grown above the optimum. Collectively, these studies suggested that the different physiological responses to temperature play a significant role in establishing the distribution patterns of these recently-diverged lineages in nature. However, as the culture experiments were performed in the presence of heterotrophic bacterial contaminants [68], the influence of the microbial community on the temperature range of *Prochlorococcus* is unconstrained, but could be significant due to its impact on the concentration of hydrogen peroxide (HOOH).

In this study we used dilute, ecologically relevant concentrations of representatives of the high- and low-temperature-adapted ecotypes of *Prochlorococcus*, grown in the presence or absence a heterotrophic helper, to directly test the hypothesis that HOOH acts synergistically with high or low temperature to restrict growth.

Evidence will be provided that elevated HOOH concentrations are even more deleterious to *Prochlorococcus* cells grown at suboptimal temperature, with impacts on photosynthetic efficiency, and suggest that the activity of the community can facilitate growth of *Prochlorococcus* at its temperature extremes by eliminating a synergistic stress.

Materials and methods

1 *Prochlorococcus* strains and culture conditions.

Axenic *Prochlorococcus* MIT9312 and MED4 were cultured in filtered (0.2 µm polycarbonate filter, pressure <10 mm Hg) artificial seawater AMP-J medium [83](per L, 28.1 g NaCl, 6.9 g $MgSO_4$*7H_2O, 5.49 g $MgCl_2$*6H2O, 0.67 g KCl. 1.47g $CaCl_2$, 0.504 g $NaHCO_3$ with 2 ml 0.5 M TAPS, pH 8.0, 1 ml 0.4 M $(NH_4)_2SO_4$, 2 ml 0.025 M NaH_2PO_4 pH7.5, 100 µl 10,000 X Pro99 Trace Metal Mix) with 40 µmol quanta m^{-2} s^{-1} light on a 12:12 light:dark cycle using cool white fluorescent bulbs. Cultures were acclimated to the test temperature for at least three transfers at high cell concentration (> 10^7cells ml^{-1}), before transferring at low cell concentration (30,000 cells ml^{-1}) for the assessment of temperature and HOOH impacts on growth. HOOH concentration was increased from basal levels in the medium (around 90 nM) where appropriate. Axenicity of *Prochlorococcus* cultures was monitored by purity test in YTSS and 1/10 ProAC heterotrophic growth media [56].

Heterotrophic bacterium *Alteromonas* sp. EZ55 was grown 48 hours in 10 ml YTSS medium [110] (per L, 4.0 g tryptone, 2.5 g yeast extract, 15 g sea salts, autoclaved) on a roller drum at 22°C. Cells were then diluted 1:1000 into 10 ml minimal acetate medium (MAM) (per 200 ml, 150 ml Pro99 [111], 50 ml 18 MΩ H_2O, 0.5% acetate, 100 µl 2000 X VA vitamin mix [112] and incubated for 24 hours on the roller drum. Cells were harvested by centrifugation at 4500 g for 5

min, washed twice with sterile AMP-J medium and resuspended in sterile AMP-J medium. About 10^6 cells ml^{-1} EZ55 were added 24 h before the addition of *Prochlorococcus* cultures, and these cultures were maintained at each corresponding incubators during this pre-treatment step.

2 Quantification of *Prochlorococcus* and *Alteromonas* sp EZ55

Concentration of *Prochlorococcus* was measured by flow cytometry with the Guava EasyCyte 8HT cytometer (Millipore), using an established method [58]. Flow cytometric quantification of EZ55 was performed after staining of the cells with SYBR green [113]. Viable count assays for EZ55 were performed by titration of series dilutions of the culture on YTSS plates. Plates were incubated overnight at 22°C and colonies were counted.

3 HOOH quantification and amendments to media

The HOOH concentration in the media and cultures was measured by Orion L Microplate Luminometer (Titertek Instruments Inc) based on established method using acridinium ester [114]. Concentration of HOOH in the culture media was adjusted as needed to 200, 400, or 800 nM, after the basal concentration of the AMP-J medium was determined.

4 Sodium pyruvate scavenging HOOH

Final concentration of 1 mM sodium pyruvate (Sigma) was used to scavenge HOOH in the medium. Sodium pyruvate was added to medium at the same time

as *Alteromonas* sp. EZ55 was added, which is 24 hours before *Prochlorococcus* was added. 1mM sodium pyruvate has been shown to have the most similar HOOH scavenging ability to EZ55 (Appendix Figure 14); by 24 hours the concentration fell below the limit of detection (10 nM).

5 Measurement of photosynthesis parameters Fv/Fm with lincomycin.

A Fast Induction and Relaxation (FIRE) fluorometer (Satlantic, Halifax, Nova Scotia) was used to measure the photophysiological parameters by the established method [115]. The photosynthetic parameter, Fv/Fm, was obtained by fitting standard models to data in MATLAB [116]. Important values are Fo (initial fluorescence), Fm (maximal fluorescence), Fv (Fm-Fo). Lincomycin (Sigma-Aldrich) was suspended in water and added into tubes with a final concentration of 500 μg ml^{-1}. Higher concentrations had the same impact on Fv/Fm in trial studies (data not shown), so we were confident that maximum inhibition was achieved at this concentration.

Results

Growth of the high temperature-adapted *Prochlorococcus* strain MIT9312 at different temperatures under oxidative stress

Prior work with dense, non-axenic cultures indicated that the high temperature-adapted MIT9312 strain had a growth optimum at 24^{o}C, and growth diminished sharply at higher temperatures [117]. Axenic cultures grown at ecologically-relevant cell concentrations confirmed a 24 ^{o}C growth optimum and a sharp decline at increasing temperatures (see Fig. 6), but also revealed the impact of reactive oxygen species and heterotrophic bacterial activity on this growth response. At 24 ^{o}C, increases in medium HOOH concentrations towards the lethal concentration (800 nM, Morris et al. 2011) led to marginal declines in growth rate (Fig. 6A). However, at 26 ^{o}C, a new, lower lethal HOOH concentration was discovered. While MIT9312 exhibited robust growth in the presence of 90 or 200 nM HOOH, the population could not grow in the presence of 400 nM HOOH, and eventually lost cells detectable by flow cytometry (Fig. 6B). Hence, a shift in temperature of only 2 ^{o}C above optimum resulted in at least twice the sensitivity to HOOH in this strain.

Examination of growth along the entire temperature range for MIT9312 confirmed an enhanced sensitivity to HOOH at the suboptimal temperatures (Fig. 7A). At the unadjusted medium HOOH concentrations (90 nM), the permissive temperature range was 19-28 ^{o}C, similar to the prior study with dense non-axenic cultures [68] (Appendix Figure 17). Increasing the HOOH concentration to 200

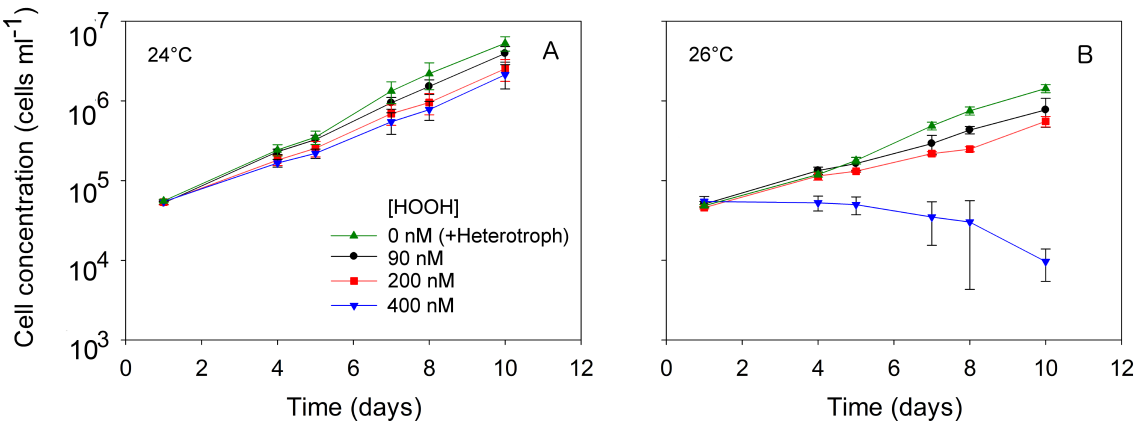

Figure 6 Growth of ecologically-relevant concentrations of MIT9312 at 24°C and 26°C in the presence of different concentrations of HOOH.

Cells from temperature-acclimated dense (>10^7 cells ml^{-1}) mid-log cultures were inoculated into media containing ambient HOOH 90 nM (black circles), 200 nM (red squares), 400 nM (blue down triangles), or in media pre-inoculated with the heterotroph *Alteromonas* sp. EZ55 (around 0nM HOOH, green up triangles).

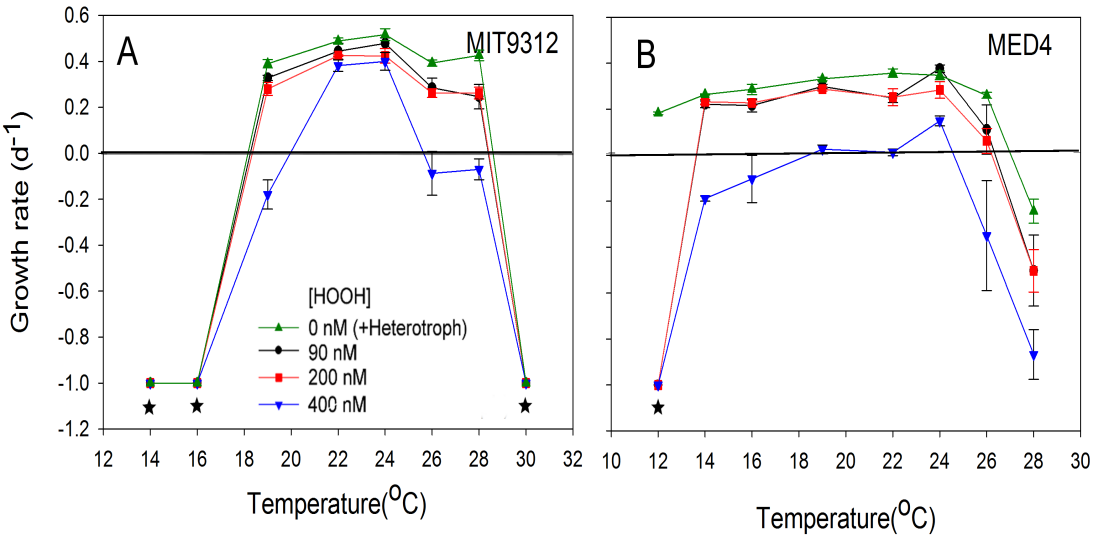

Figure 7 Growth rate of (A) MIT9312 and (B) MED4 at different temperatures and exogenous HOOH concentrations.

Cells in filtered AMP-J medium containing unamended medium HOOH (90 nM; black dots), 200 nM (red squares), 400 nM (blue down triangles), or in media pre-inoculated with the heterotroph *Alteromonas* sp. EZ55 (around 0nM HOOH, green up triangles). Asterisks indicate negative growth rates too large to be detected by 24 hours post-inoculation.

nM had minimal impact on growth over this temperature range, although the overall trend was a slight decrease in rate. However, at 400 nM, the MIT9312 cells could only grow at (24 ^{o}C) or slightly below (22 ^{o}C) their optimum. In fact, with the initial concentration at ~10,000 cells ml^{-1} at 19, 26 and 28 ^{o}C, a slow but steady decline in flow cytometry cell counts was oberved, while at the more extreme temperatures tested, no cells could be detected after only 24 hours post-inoculation, indicating that the cells rapidly lost chlorophyll-based red fluorescence and perhaps cell integrity as well (see Discussion).

Prochlorococcus lacks catalase but can, at slow rates, remove HOOH from the medium[55] . While the degradation rates are too slow to dramatically improve tolerance to HOOH, it was nonetheless notable that the rates of HOOH degradation by MIT9312 varied as a function of temperature and initial concentration of HOOH (Figure 8A). At 400 nM HOOH, degradation was highest at 22 and 24 °C, which is perhaps not surprising since at all other temperatures the population was dying.

However, even at lower HOOH concentrations (90, 200 nM), where cultures grew over a more extended temperature range, removal of HOOH was highest at 22 and 24 °C, coincident with the highest growth rates. This trend is coincident with the enzyme kinetics, that the removal of HOOH was high at optimal temperature and decreased at suboptimal temperatures.

In a prior study it was discovered that heterotrophic bacteria can protect Prochlorococcus from HOOH-mediated oxidative damage by removing HOOH

from the medium [55]. As this earlier work was performed exclusively at 24 °C, we investigated if this helping phenomenon can occur throughout the growth range of Prochlorococcus. At all temperatures assayed, media pre-conditioned by the presence of the heterotrophic bacterium Alteromonas sp. EZ55 had undetectable levels of HOOH (limit of detection = 10 nM), indicating that the HOOH-degrading capacity of this helper is not significantly impacted by temperatures within the growth range of Prochlorococcus. At all temperatures, MIT9312 grew at the fastest rate in this heterotroph-conditioned medium, even when compared to growth at low (90 nM) ambient HOOH concentrations (Figs 6 and 7A). This indicates that even 90 nM may be toxic to *Prochlorococcus*. Alternatively, the growth benefit of the heterotroph could be independent of its HOOH degrading capacity. To address this we used an alternative means of removing HOOH from the medium: pre-conditioning with pyruvate.

Pyruvate has been shown to scavenge HOOH effectively in the medium [118] by a chemical reaction [119]. At 28^{o}C, the growth rate of MIT9312 in pyruvate-treated medium (0 nM HOOH) was significantly higher than in the untreated control (90 nM) (Figure 8B). Thus, by different chemistries the two treatments employed – live bacteria with enzymes and pyruvate – effectively decreased HOOH below detection, and both lead to an enhancement of MIT9312 growth post-treatment (Fig. 8B). Importantly, cultures given pre-treatment with a heterotrophic helper grew faster than those pre-treated with pyruvate. The implications of this difference are explored in detail in the Discussion.

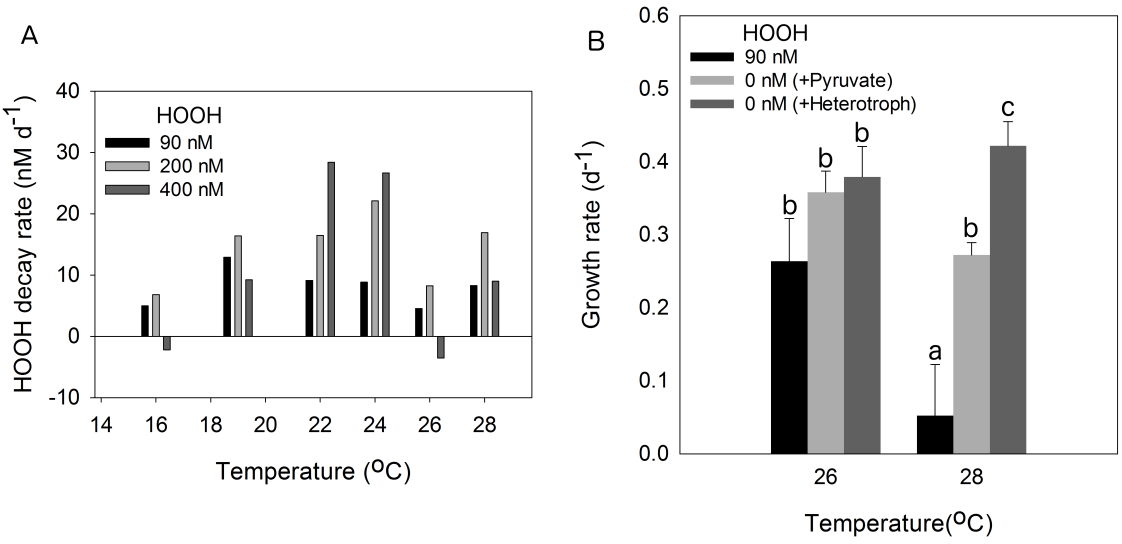

Figure 8 Temperature-dependent HOOH degradation and growth of ecologically-relevant concentrations of MIT9312.

(A) Degradation rates by axenic *Prochlorococcus* MIT9312 relative to uninoculated controls. Black bar: HOOH concentration in MIT9312 with initial concentration of 90nM HOOH. Light gray bar: HOOH concentration in MIT9312 with initial concentration of 200nM HOOH. Dark gray bar: HOOH concentration in MIT9312 with initial concentration of 400nM HOOH. (B) Growth rates of MIT9312 in AMP-J medium (red), and in AMP-J medium pre-treated with pyruvate (orange) or *Alteromonas* sp. EZ55 (green). By two way ANOVA, temperature and HOOH both bring significant different to the growth of MIT9312. The p-value for temperature, HOOH and temperature and HOOH overall are 0.001, 0.000002 and 0.001 respectively.

At a less extreme temperature of 26 ^{o}C the trends are similar but not significant.

Growth of the low temperature-adapted strain MED4 under temperature and oxidative stresses

While some strain differences were observed, in general the low temperature-adapted MED4 strain exhibited similar growth responses to HOOH temperature, and helper treatments. Dilute MED4 cultures grew between 12^{o}C to 26^{o}C, which was cold-shifted relative to MIT9312 (16 to 28 °C) and was similar to its reported range for dense cultures [68] (Appendix Figure 17). Of note, however, MED4 could only grow at 12 °C when in the presence of heterotrophs, suggesting that at this cold extreme, even 90 nM HOOH was lethal. Like MIT9312, growth of pure cultures of MED4 was highly restricted at 400 nM HOOH, in this case only at the growth optimum, 24 °C (Figure 7B). The kinetics of cell loss at the non-permissive temperatures was slow enough that rates of cell loss could be calculated, except at 12 °C, when all cells disappeared by 24 hours. It is interesting to note that the rate of loss increased as a function of distance from optimal temperature, at both temperature extremes. Finally, pre-treatment with heterotroph helpers tended to increase the growth rate or, at lethal temperatures, decrease the death rate of MED4.

Impacts of temperature and HOOH on chlorophyll and photosynthetic efficiency.

Flow cytometry scatterplots used for the quantification of MIT9312 and MED4 cells in the analyses reported above revealed interesting relationships

between red fluorescence (a proxy for chlorophyll content cell^{-1}), temperature, HOOH, and heterotrophic bacteria. Whereas forward scatter (a proxy for cell size) did not change as a function of temperature, a distinct increase in red fluorescence was observed in MIT9312 cells grown above the growth optimum (>24 °C) (Fig 9A). Interestingly, this increase was only observed in axenic cultures (Fig. 9A); co-cultures with *Alteromonas* did not show this temperature-dependent change in chlorophyll fluorescence (Fig. 9B). High HOOH also had an impact on fluorescence: at 19 °C, increasing HOOH concentrations above ambient levels (>90 nM) led to an overall decrease in cellular fluorescence. Depleting the HOOH concentrations from 90 to 0 nM via heterotrophic pre-treatment had no impact on fluorescence (Fig. 9C). Examinations with MED4 cultures revealed the same overall trends (data not shown). Overall, these results indicate that temperature and HOOH can impact chlorophyll-based fluorescence in *Prochlorococcus*, but this effect is eliminated in the presence of heterotrophs.

Temperature and HOOH both impact photosynthesis by blocking the synthesis and/or processing of nascent D1 protein used to replace damaged protein in the PSII reaction center [53, 59, 105, 108]. To assess the contributions of these stresses on *Prochlorococcus*, we quantified Fv/Fm, a measure the quantum efficiency of photosystem II.

During growth in low, ambient concentration of 90 nM HOOH, Fv/Fm for MED4 did not vary from a value of 0.6 throughout the range of 16-26 ^{o}C, which is similar

to the findings of a previous study [31], but did decrease dramatically at 12 °C (Fig 11A). Similarly, MIT9312 also had an Fv/Fm value of 0.6 for the 19-28 °C range, but Fv/Fm was lower at 16 °C (Fig. 11A).

During an 8 hour exposure to elevated HOOH, neither strain experienced a significant decline in Fv/Fm when exposed to 400 nM compared to 90 nM HOOH (data not shown), but did exhibit temperature-dependent responses to 800 nM HOOH (Fig 10, Appendix Figure 15 and 16). This effect was most pronounced for MIT9312, which showed significant temperature-dependent declines in Fv/Fm at 800 compared to 90 nM HOOH (Fig 10A), despite no detectable loss in cell density over the 8 hour period (data not shown). The response was most pronounced at the lower temperatures, but a decline at temperatures above the 24 °C growth optimum was also apparent. This temperature-dependent decline in Fv/Fm was qualitatively shared for MED4 (Fig. 10B), although the trends were less obvious.

Lincomycin, an effective translation inhibitor in *Prochlorococcus* [120] and other cyanobacteria [120, 121], was used to calibrate the drop in Fv/Fm for the different temperature and HOOH exposures. In almost every case, the lincomycin treatment led to a significantly larger drop in Fv/Fm over the 8 hour incubation compared to the HOOH treatment (Fig. 11B and Appendix Figure 15 and 16). If lincomycin treatment leads to complete inhibition of D1 replacement, then this indicates that the HOOH (in combination with any temperature effects) led to only partial blockage of this process which could be demonstrated by

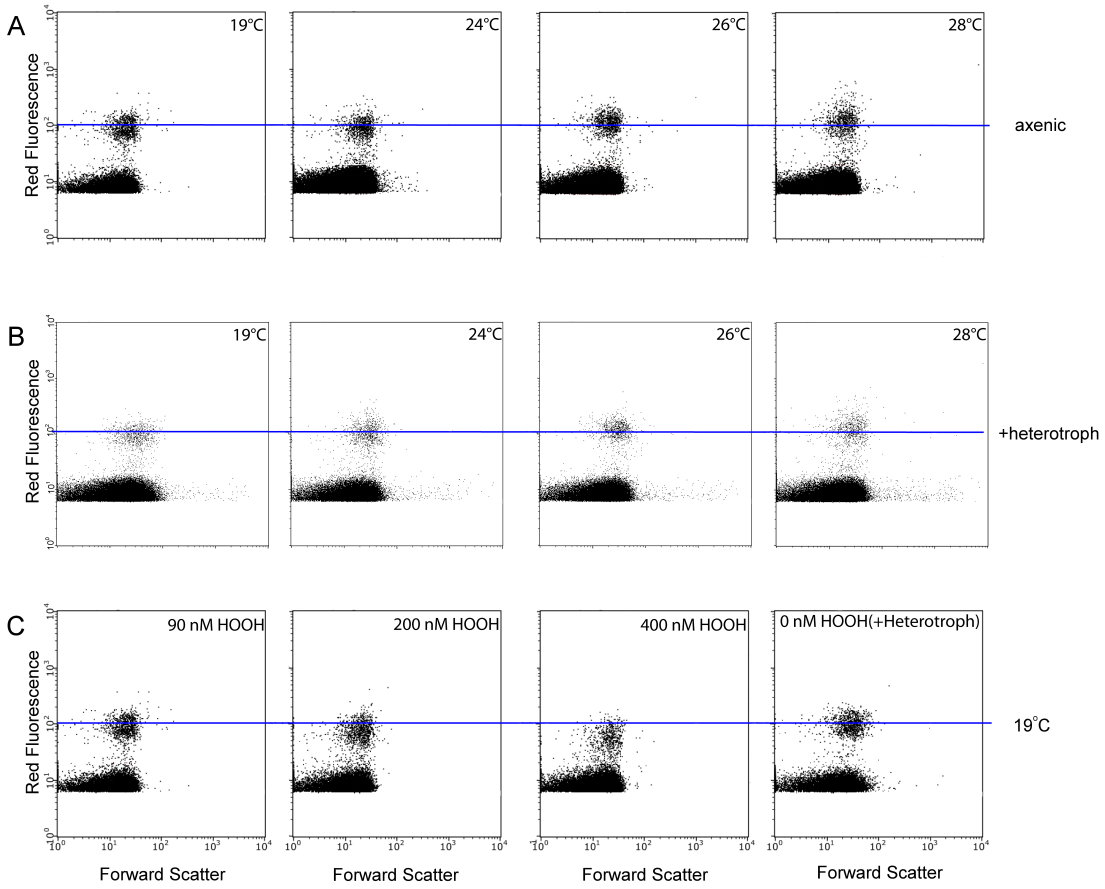

Figure 9 Flow cytometry profiles of MIT9312 at different temperatures and HOOH concentrations.

Forward scatter versus red fluorescence for MIT9312 cells grown axenically (A) or in the presence of *Alteromonas* sp. EZ55 heterotroph (B). Cells grown in the presence of different concentrations of HOOH or in the presence /absence of heterotroph as indicated (C). The blue line is an arbitrary reference to highlight the change of red fluorescence as a function of temperature, HOOH and/or heterotrophic co-cultures.

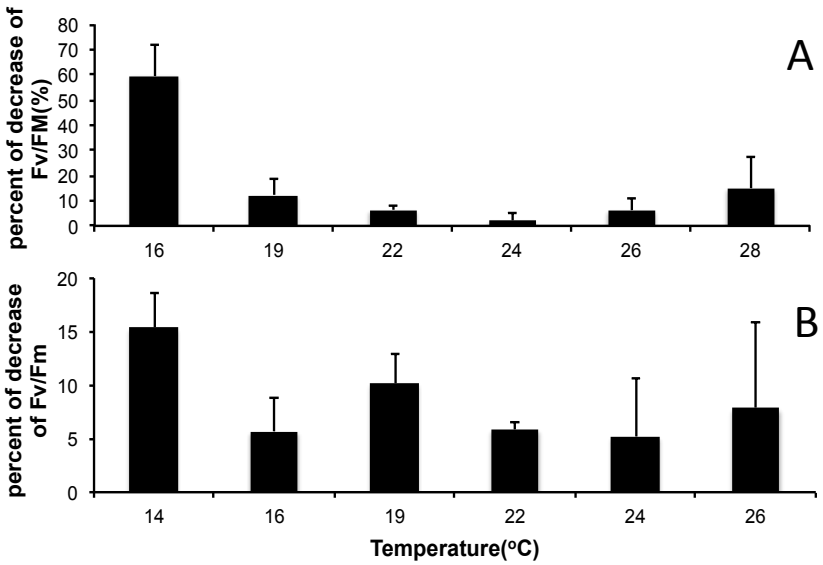

Figure 10 Fv/Fm of *Prochlorococcus* as a function of temperature and HOOH concentration.

Percent decrease of Fv/Fm at 800 nM compared to 90 nM HOOH varied strongly (ANOVA p=0.00007) or weakly (ANOVA p=0.12) as a function of temperature for MIT9312 (A) or MED4 (B), respectively.

Figure 11B: lincomycin treatment (at low, 90 nM HOOH) decreased Fv/Fm to a larger extent than treatment with high (800 nM) HOOH. This difference increased as function of temperature, and was non-significant only at the lowest temperature, 16 °C.

Unexpectedly, these studies indicated that HOOH may be impacting photosynthetic efficiency via mechanisms in addition to blocking D1 replacement. This is evident in comparisons of high and low HOOH treatment in the presence of lincomycin: at most temperatures exposure to 800 nM resulted in greater loss of Fv/Fm compared to exposure to 90 nM HOOH (Fig 11C). Additionally, lincomycin-treated cultures showed enhanced drops in Fv/Fm when also exposed to high HOOH (Fig 11D). In both cases, high HOOH led to decreases in Fv/Fm even when D1 repair was already blocked by lincomycin.

Discussion

This study confirmed our prediction that growth at temperature extremes would heighten the sensitivity of *Prochlorococcus* to hydrogen peroxide, and vice versa. This effect was observed in both strains, chosen as representatives of the two numerically-dominant ecotypes of *Prochlorococcus* in the ocean [7]. While

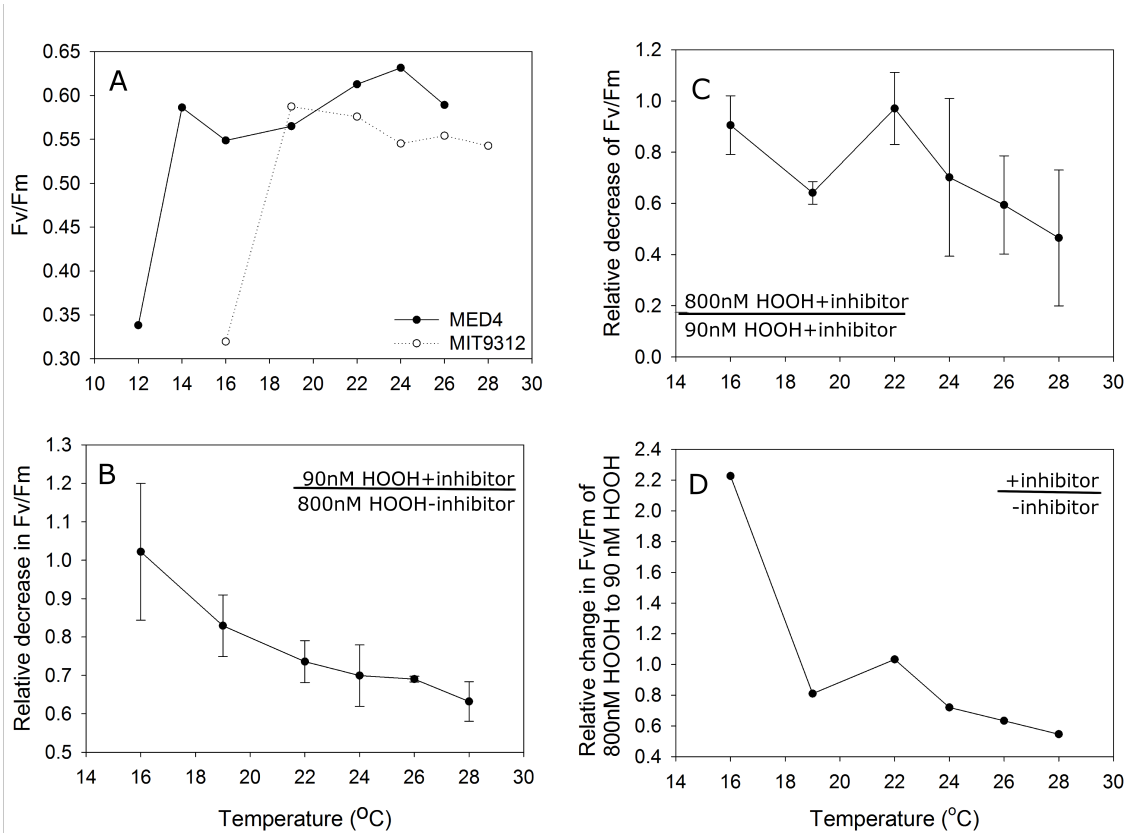

Figure 11 The effects of HOOH on Fv/Fm in MIT9312, in the presence or absence of lincomycin.

Decline in Fv/Fm compared to the ambient 90 nM HOOH, no lincomycin control for the 800 nM HOOH treatment relative to the lincomycin treatment (A). In the presence of lincomycin, the decline in Fv/Fm for the 800 nM HOOH treatment relative to the 90 nM HOOH control (B). The Fv/Fm of MIT9312 at 800nM HOOH with inhibitor relative to that with 90nM HOOH with inhibitor (C). The Fv/Fm of MIT9312 of 800nM HOOH relative to 90nM HOOH in presence of inhibitor over the absence of inhibitor (D)

caution must be used in extending these results into natural populations of these and other ecotypes, it is nonetheless encouraging that the overall patterns were the same for the two strains. Three major interactive effects were observed between temperature and HOOH: 1) within the permissive temperature range, growth rate deceased as a function of HOOH concentration, 2) the range of permissive temperature decreases with increasing HOOH, and 3) for the 400 nM treatment, the death rate (measured as the loss of detectable cells) at the lethal temperatures increases with increasing distance from optimal temperature (24 °C).

Damage mechanisms of oxidative and thermal stress in *Prochlorococcus*

Prior work has demonstrated that oxidative damage from exposure to HOOH can result in diminished photosynthetic efficiency for *Prochlorococcus* [55, 122] and our results are consistent with these earlier reports.. Temperature alone did not significantly impact Fv/Fm over its growth range, similar to prior findings [31], but dropped considerably at cold lethal temperatures (12 and 16 °C for MED4 and MIT9312, respectively). Hence, under low HOOH conditions, *Prochlorococcus* appears to maintain photosynthetic efficiency over its permissive temperature range, suggesting only minimal impacts of temperature on the repair of PSII. However, exposure to temperature extremes, particularly lower temperatures, appeared to sensitize *Prochlorococcus* for a drop in Fv/Fm mediated by oxidative damage. This effect was most pronounced for MIT9312;

the reasons why MED4 did not show a strong temperature-dependence of HOOH sensitivity for photosynthetic efficiency are not known.

The mechanism(s) by which temperature and oxidative stress interact to limit growth in *Prochlorococcus* has not been fully elucidated, but if similar to other cyanobacteria, it includes impeding repair of damaged photosystems. Both temperature and oxidative stress can lower photosynthetic efficiency (Fv/Fm) in cyanobacteria by interfering with the repair of photosystem II via replacement of damaged D1 protein [123, 124]. HOOH inactivates translation elongation factor G, causing a general block in translation [59, 108]. The protein most impacted by this block is D1, which contains the reaction center chlorophyll for photosystem II, and is rapidly turned over during photosynthesis due to continuous photoinactivation [52, 53]. Hence, over short time scales, the primary impact of the HOOH-mediated block in translation is a drop in photosynthetic efficiency. Like oxidative stress, low temperatures decrease the turnover of D1, in this case by slowing the processing of the precursor protein pD1 [59]. Lower temperatures can also increase the production of reactive oxygen species by slowing the rate of the Calvin Cycle “dark reaction” enzymes while not concomitantly slowing down light harvesting in the “light reaction,” resulting in a buildup of NADPH and diminished regeneration of NADP+ as the terminal electron acceptor of the light reaction [60, 61]. In this situation, excess electrons can reduce molecular oxygen to produce the reactive oxygen species (ROS) superoxide and hydrogen peroxide (HOOH). Consistently, a superoxide

dismutase deletion mutant of *Synechococcus* PCC7942 defective in eliminating superoxide is more sensitive to chilling stress than wild type [62], and in plants and microbes, the concentration of HOOH was higher at lower temperatures [63] [64]. Higher temperatures also generate excessive ROS [64], leading to oxidative stress [65] and cell mortality [66]. In phytoplankton, high temperatures inactivate Rubisco activase, leading to a decline in Rubisco and Calvin Cycle activity, that, as for cold temperatures, can generate an imbalance in $NADPH/NADP^+$ which can allow the generation of ROS by the reduction of O_2 by photosystem I [29]. As a result, higher temperatures contribute to a loss in photosynthesis by inhibiting photosystem II repair [29, 30], an in some cases by causing direct photodamage [67].

Interestingly, we observed that both temperature and elevated HOOH stress can change the chlorophyll-based red fluorescence in *Prochlorococcus*. The molecular basis for this phenomenon is entirely unclear, but given that heterotrophs can abolish this relationship, it suggests that there is some link between chlorophyll fluorescence and reactive oxygen species. Since red fluorescence is a common parameter for the quantification of natural populations of *Prochlorococcus* [125-128], these results might a priori cause concern for the gating of the flow cytometers for such applications, especially in transects through varying temperatures or HOOH concentrations. However, in that heterotrophs remove this effect, and that heterotrophs are always co-existent with *Prochlorococcus*, this worry is diminished.

Unexpectedly, we observed evidence of repair-independent damage to PSII upon HOOH exposure. This was observed by the additional decrease in Fv/Fm by elevated HOOH, in cells with lincomycin already blocking the D1 repair pathway. In general, this additional damage occurred at higher, rather than lower temperatures. This might rule out one target of the *Prochlorococcus* cell that could indirectly influence PSII activity. Microbes are unable to control temperature relative to ambient, and so to maintain membrane fluidity at low temperatures, increase the unsaturation of their fatty acids[25]. HOOH can induce membrane damage for cells with di-unsaturated fatty acids via a process known as lipid peroxidation. *Prochlorococcus* has di-unsaturated fatty acids in its membrane [50], and these may indeed increase, but if they follow the rule for other microbes, will increase at colder temperatures. Hence, lipids would be more susceptible to peroxidation at low temperatures, while we see in *Prochlorococcus* an increase in D1-independent PSII efficiency decline at higher temperatures. Hence, the D1 repair-independent target remains elusive.

The role of helper bacteria in protecting *Prochlorococcus* from interactive thermal and oxidative stresses

Consistent with prior studies [55, 56] we observed a growth benefit to *Prochlorococcus* during co-cultivation with the heterotroph *Alteromonas* sp. EZ55. This helping phenomenon has two positive impacts on *Prochlorococcus*, depending on the temperature: at suboptimal permissive temperatures, helpers generally increase growth rate, relative to the unamended (90 nM HOOH)

control; while at the lethal high temperature, helpers slowed the death kinetics of MED4, relative to the unamended control. Using pyruvate as an alternative treatment for HOOH degradation, we observed that the ability of *Alteromonas* cells to help *Prochlorococcus* is due, at least in part, to the removal of HOOH from the medium. This consistent with our prior studies [55, 56], and now suggests that this helping ability is even more important for *Prochlorococcus* cells exposed to temperatures above or below their growth optimum of 24 °C.

The mixed layer of the oligotrophic ocean, especially in the lower latitudes where it is highly stratified, is characterized as a fairly stable environment. In such stable environments, the rate at which the cells die is of minimal consequence to the population at ecological timescales, so long as the rate is greater than zero. However, in the context of HOOH concentration, the mixed layer is not stable: rain is a significant source of HOOH, and rainfall events can provide acute elevations in HOOH concentration. While this additional HOOH is eventually decomposed by the microbial community, it nonetheless can provides a situation in which slower death kinetics at lethal HOOH concentrations would provide a selective advantage for *Prochlorococcus*, which are eventually decreased to the equilibrium concentrations by the action of the microbial community. Therefore, one important role of the microbial community is to minimize the lethal exposure of *Prochlorococcus* to HOOH during such events, by decreasing both the dose and time of exposure. And importantly, this impact

is of increased magnitude at the temperature extremes, where they are especially sensitive to HOOH.

In interpreting the data presented, it is important to recognize what the values of HOOH selected for this study represent in terms of concentrations found in the ocean. In a sterile ocean, the HOOH concentration in the oligotrophic surface mixed layer could reach 800 nM, which is lethal to all ecotypes of *Prochlorococcus* in culture [55]. However, the ocean is not sterile, and 90 and 200 nM HOOH treatments represent the typical range of net HOOH concentrations in the natural ocean: set by the balance between production (primarily though photochemistry) and degradation (primarily through microbial enzymes). The intermediate value of 400 nM can be reached transiently during rainfall events (see below). In this study we have used the marine heterotroph *Alteromonas* as a proxy for the natural mixed community that serves as the microbial sink. However, in the laboratory culture conditions these cells operate without the primary producer of HOOH, the sun, and thus deplete the HOOH below detection, which is more related to waters below the surface mixed layer. As these experiments have demonstrated, the ability of the microbial community to maintain the concentration of HOOH below 800 nM facilitates growth at optimal temperatures, while the ability of the community to maintain HOOH below 400 nM facilitates growth at all other temperatures.

While still in its infancy for microbial interactions, the concept of facilitation is widely accepted in the ecology of macroorganisms. In terrestrial

environments, many examples have been observed whereby plants benefit from the presence of co-existing plants in areas of high environmental severity because the positive effects of environmental modification by the neighbors outweigh the negative effects of competition for resources [98, 99]. These observations have led to the development of the stress-gradient hypothesis (SGH), which predicts that the community-wide prevalence of positive interactions is greater under more severe environmental conditions [102, 129]. Facilitation of growth of one plant species by another have been observed at the extremes of altitudinal, topographic, wind exposure, aridity, and temperature gradients [100, 130-133]. Facilitation of growth at the high temperature extremes occurs via shading or otherwise affecting the microclimate of the sensitive species [103, 130]. Fungi have also been shown to facilitate tolerance and growth at elevated temperatures for plants [134-137], showing that in terrestrial systems inter-trophic interactions can also facilitate range expansion. In some cases, cross-protection against oxidative stress has been proposed as the mechanism of fungal facilitation of plant growth [136].

In marine systems, facilitation between plants has been observed, but appears to be dominated by inter-trophic interactions (reviewed in [103]). For instance, in intertidal regions, canopy-forming macroalgae can facilitate the growth of animal species of undergrowth algae by amelioration of thermal stress [138]. In subtidal regions sessile animals can provide grazing defenses for macroalgae by repelling herbivores, while macroalgae can provide camouflage

for crabs [139, 140]. Hence, severe conditions for metazoans of terrestrial and marine systems, positive interactions between species appear to be quite common in severe environments.

Facilitation of growth via positive interactions under stressful conditions has been observed between microbes, though fewer examples exist. A recent demonstration of algal thermal range expansion mediated by heterotrophic bacteria involves the green alga *Chlamydomonas reinhardtii*. This green alga has two isozymes for methionine synthase, which is required for methionine synthesis. The first isozyme (METE), is temperature-sensitive, while the second (METH) is temperature-resistant, but requires vitamin B_{12}, and is the only enzyme in *C. reinhardtii* with that requirement. B_{12} is not produced by the algae, but growth in the presence of B_{12}-producing heterotrophic bacteria conferred an increased thermal tolerance for the alga by activating the thermal-tolerant METH enzyme. While the molecule in question, B_{12}, is highly specific, the potential donors were phylogenetically diverse, suggesting that the alga could have multiple interacting partners in nature.

Our study represents a second demonstration of increased thermal range of phytoplankton mediated by heterotrophic bacteria. While the mechanism of interaction is different – removal of a toxic molecule, rather than production of an essential one – the overall impact on the phytoplankton (*Prochlorococcus*) is similar, as is the non-specific nature of this facilitation: many species of bacteria can eliminate the HOOH in the environment to facilitate *Prochlorococcus* growth

[55, 56], and it is likely that the removal of HOOH from the surface mixed layer of the ocean is a community-wide activity. In contrast to thermal facilitation by metazoans, which can involve direct cooling of sensitive strain via shading, heterotrophs improve growth at temperature extremes for *Prochlorococcus* by eliminating a synergistic stress: oxidative stress.

The ability of pyruvate to help *Prochlorococcus* grow at higher temperatures confirmed the importance of HOOH decomposition in the helping phenomenon of the heterotrophic bacteria in co-culture. However, as the help from live bacteria exceeded the help from pyruvate, this indicated additional helping mechanisms might be involved, consistent with the observations we have made in prior studies at optimal temperature [55, 56]. This additional mechanism(s) is tangential to the focus of this investigation (i.e. the interaction between temperature and oxidative stress), but is worthy of some additional attention. In our earlier work we were able to rule out the beneficial effects of helpers of other algae that deplete oxygen, replenish inorganic carbon, stabilize pH, and/or provide an essential vitamin or other growth factor [104] [106, 141, 142] [143] [144] [145]. It is formally possible that these mechanisms, while not involved at optimal temperature, might be important for *Prochlorococcus* growth at temperature extremes, and should be addressed in future studies.

The meridional range for *Prochlorococcus* in the oligotrophic ocean is approximately N40° - S40°, survival and growth at higher latitudes are restricted by temperature. Within the permissive range for *Prochlorococcus*, the

concentration of HOOH in the mixed layer varies but rarely exceeds 200 nM. The microbial community maintains this concentration at this low value; in absence of microbes the HOOH concentration exceeds the lethal limit for all known *Prochlorococcus* ecotypes (≥800 nM) [55]. To better appreciate the importance of the impact of the microbial sink on the ecology of *Prochlorococcus*, we used our data to model the growth and distribution of *Prochlorococcus* in the presence of a microbial sink of compromised activity. Applying the growth rate information of both strains, such that the highest growth for either ecotype strain under the conditions tested are used, an upper bound for the growth of the *Prochlorococcus* genus under various temperature and HOOH conditions is produced (Figure 12). As expected, the curves are a composite of the MIT9312 growth rates at higher temperatures, and MED4 growth rates at lower temperatures. And, notably, the *Prochlorococcus* genus as a whole is severely restricted in temperature range when exposed to 400 nM HOOH.

Intermediate concentrations of peroxide, between that permissive for all temperatures for *Prochlorococcus* (200 nM) and restrictive even at optimal temperature (800 nM), if present in the ocean would result in a severe habitat restriction for *Prochlorococcus* in the mixed layer (Figure 13). At a 400 nM standing stock of HOOH, the *Prochlorococcus* habitat range would be only 42% as large as it is currently, and would occur in isolated bands in the northern and southern hemispheres. Survival below the mixed layer, where HOOH concentrations are vey low, would still be possible, but growth and survival would

be subject to light limitation and other environmental restrictions. While certain caution must be made when extrapolating the results of two strains in culture to model the impacts in natural communities, this model provides population-level metrics for the significance of the ability of the community to facilitate the growth of Prochlorococcus, and it emphasizes the heightened importance of protection from oxidative stress at the temperature extremes, which define the major boundaries of the geographical range of this very large contributor to global carbon cycling.

Conclusion

Prochlorococcus is sensitive to HOOH, and this sensitivity is increased when growing at suboptimal temperatures. As is the case at optimal temperature, the presence of a HOOH-degrading heterotroph – serving as a proxy for the natural microbial community – effectively increases the temperature range at which *Prochlorococcus* can grow, and in more extreme scenarios slow the death kinetics. This contributes to the growing body of evidence for facilitation at the microbial scale: while microbes may not be able to directly change the temperature of another microbe, they can improve the response to temperature by eliminating concurrent stresses.

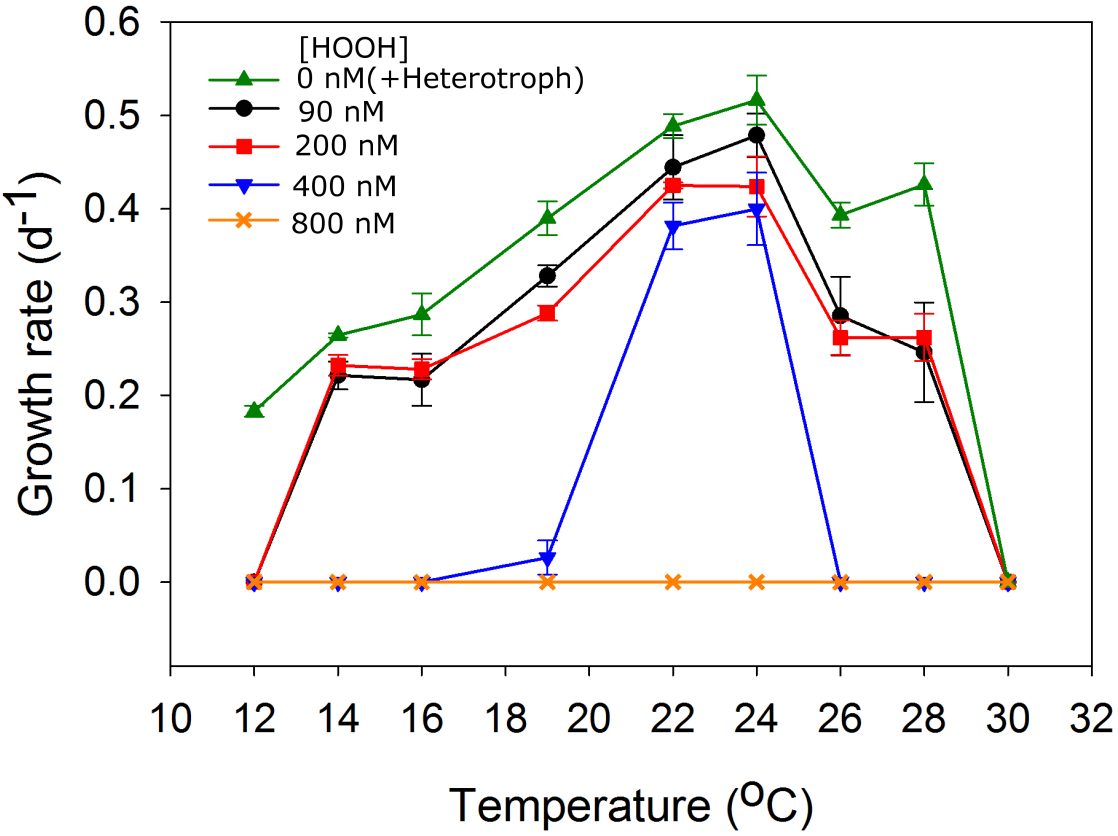

Figure 12 Maximum growth rate of *Prochlorococcus* as a function of temperature and HOOH.

Values were taken as the maximum growth rate for MED4 or MIT9312, which ever was highest under the given condition. Filtered AMP-J media contains ambient HOOH 90 nM (black circles), 200 nM (red squares), 400 nM (blue down triangles), in the presence of EZ55 heterotroph (around 0 nM HOOH, green up triangles), 800nM (cyan cross). The growth rates of *Prochlorococcus* with 800nM HOOH are assumed to be zero or below zero for all temperatures, based on prior studies at the optimal temperature, 24 °C [13].

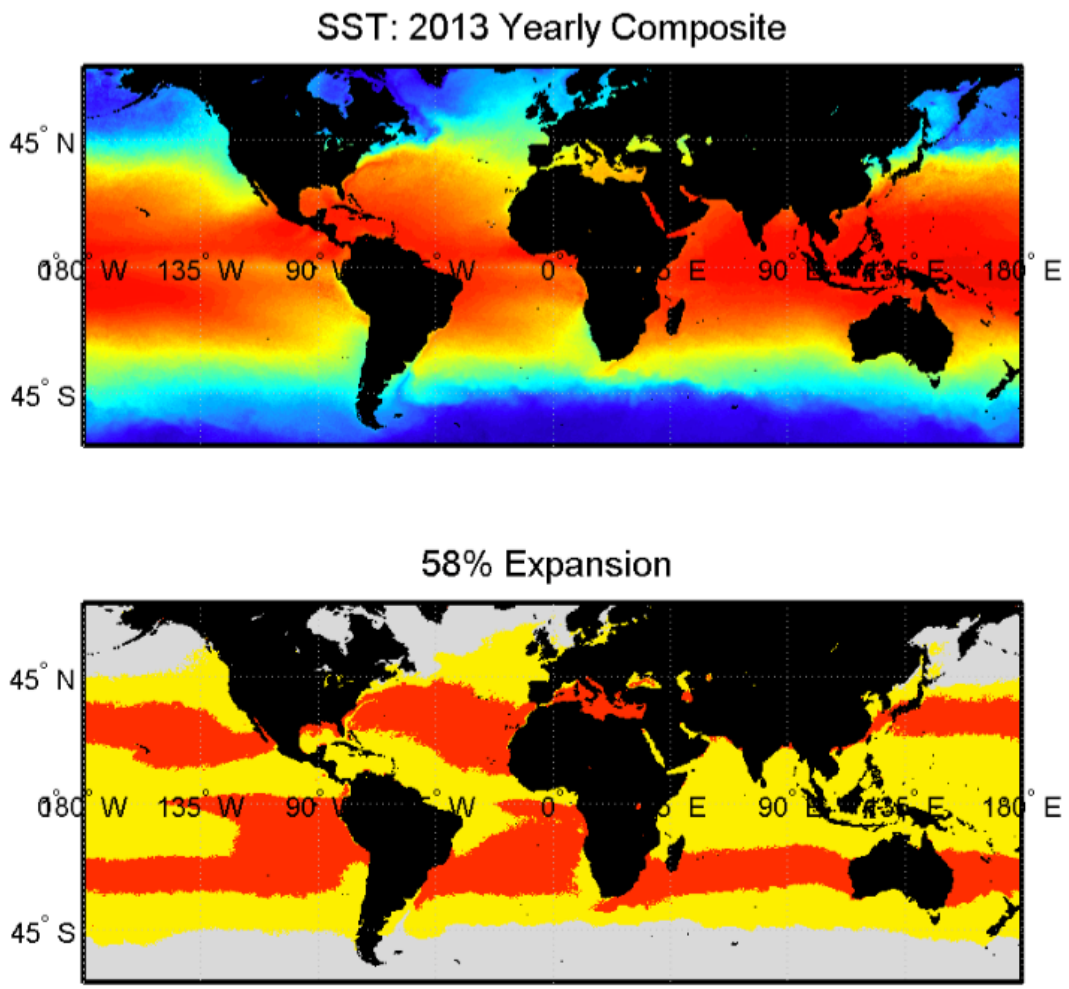

Figure 13 Potential effect of 400 nM HOOH to the distribution of *Prochlorococcus.*

The upper panel is the *Prochlrococcus* distribution in 2013 yearly composite. The lower panel is the potential distribution of *Prochlorococcus* due to 400nM HOOH

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Appendix

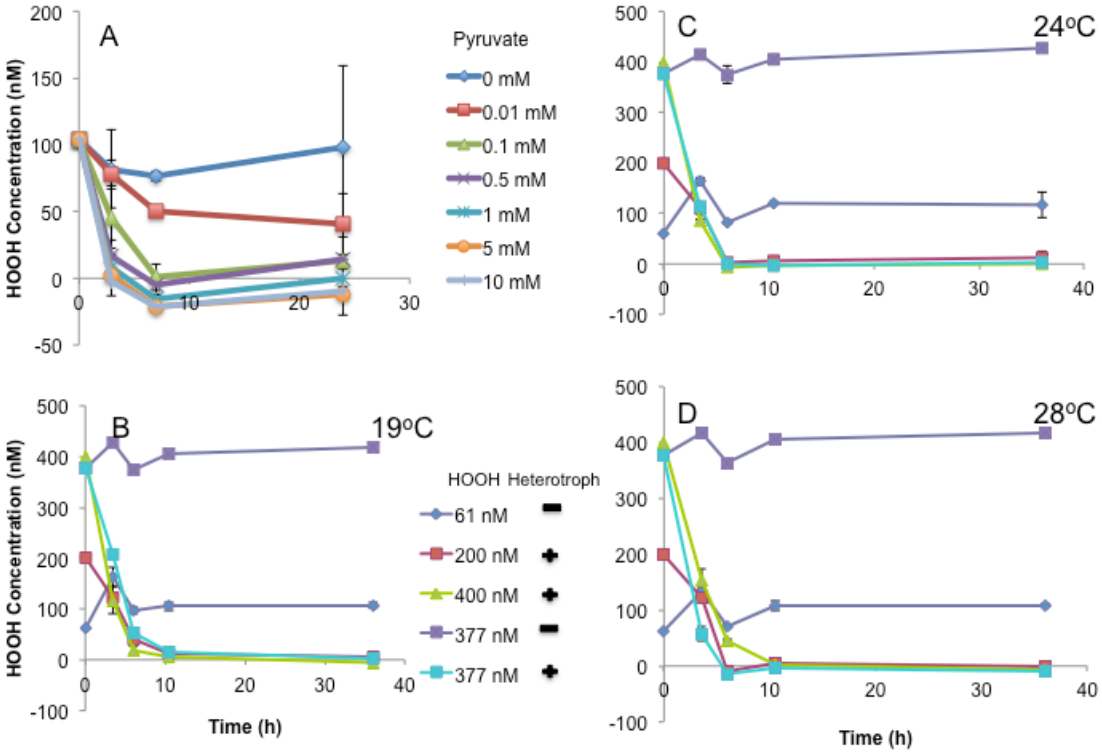

Figure 14 Degradation of HOOH in the medium by pyruvate or the heterotroph *Alteromonas* sp. EZ55.

HOOH in medium degraded by different concentrations of pyruvate as indicated (A). Removal of HOOH from the medium at 19 (B), 24 (C), or 28 °C (D) at various initial concentrations of HOOH; where indicated EZ55 was added at T=0. Panels B,C, and D have the same legend.

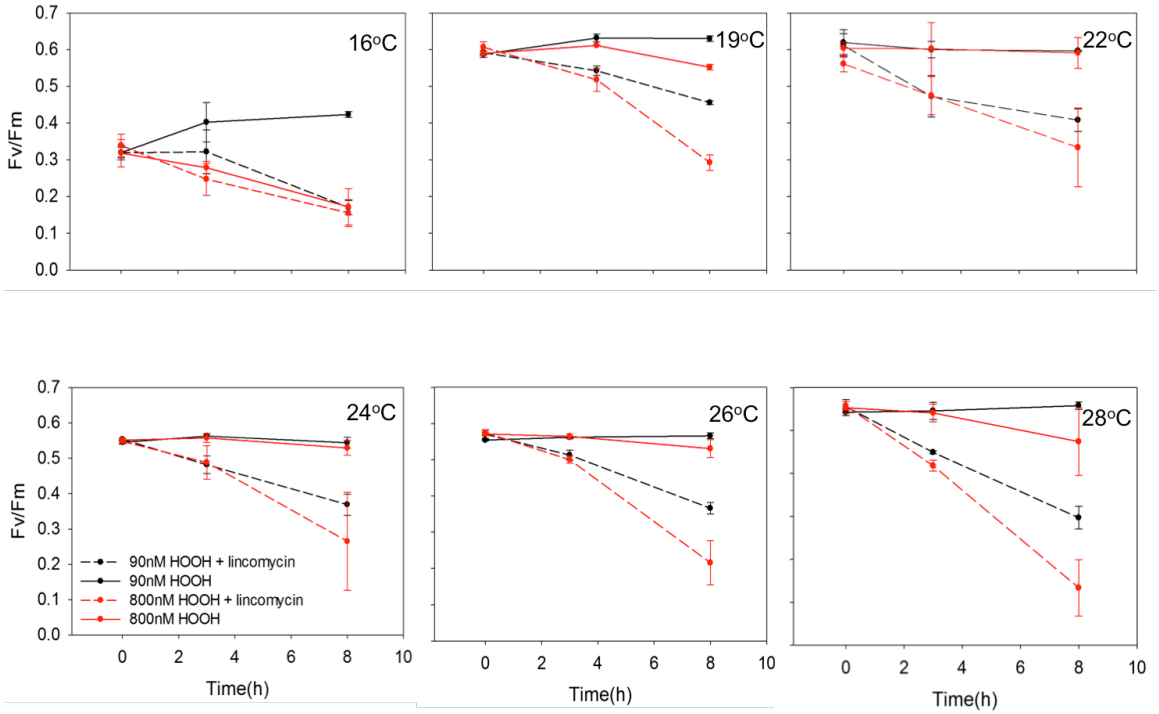

Figure 15 Fv/Fm of MIT9312 as the function of time (h) at different temperatures.

Cells were incubated in the presence (dashed lines) or absence (solid lines) of 500mg ml^{-1} lincomycin, at ambient HOOH 90nM (black circles) or 800nM HOOH (red triangles).

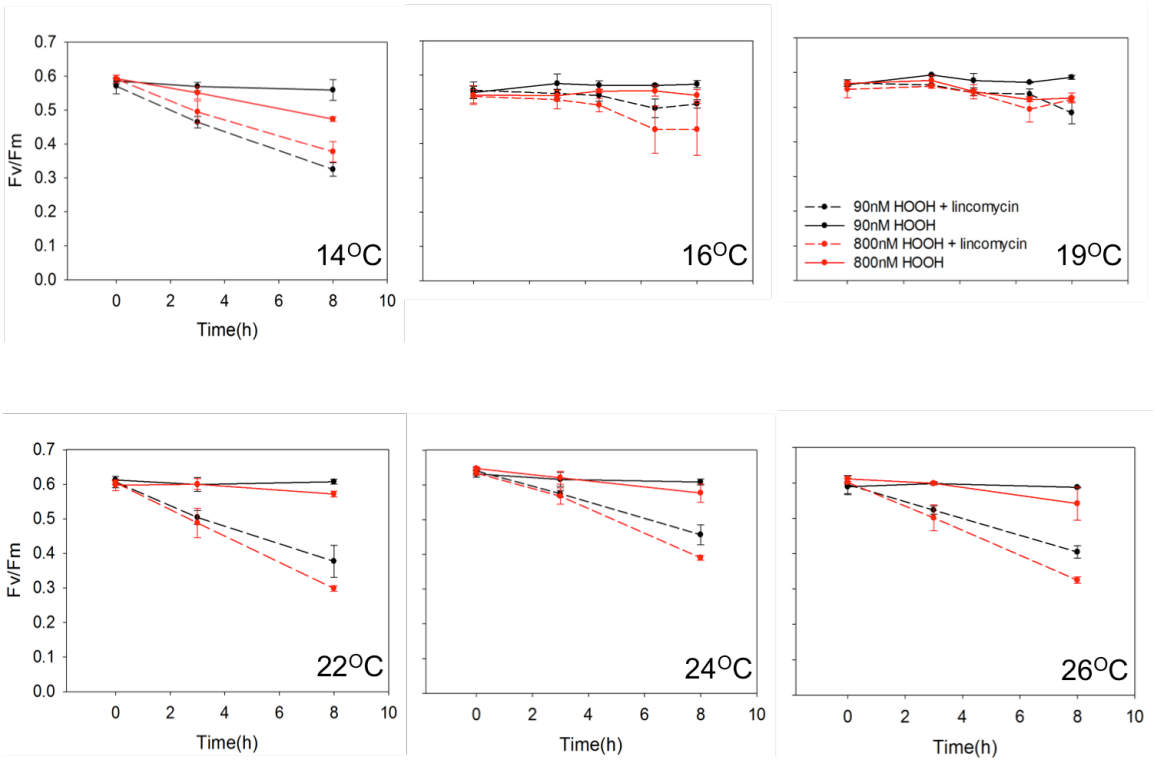

Figure 16 Fv/Fm of MED4 as the function of time (h) at different temperatures.

Cells were incubated in the presence (dashed lines) or absence (solid lines) of 500mg ml^{-1} lincomycin, at ambient HOOH 90nM (black circles) or 800nM HOOH (red triangles).

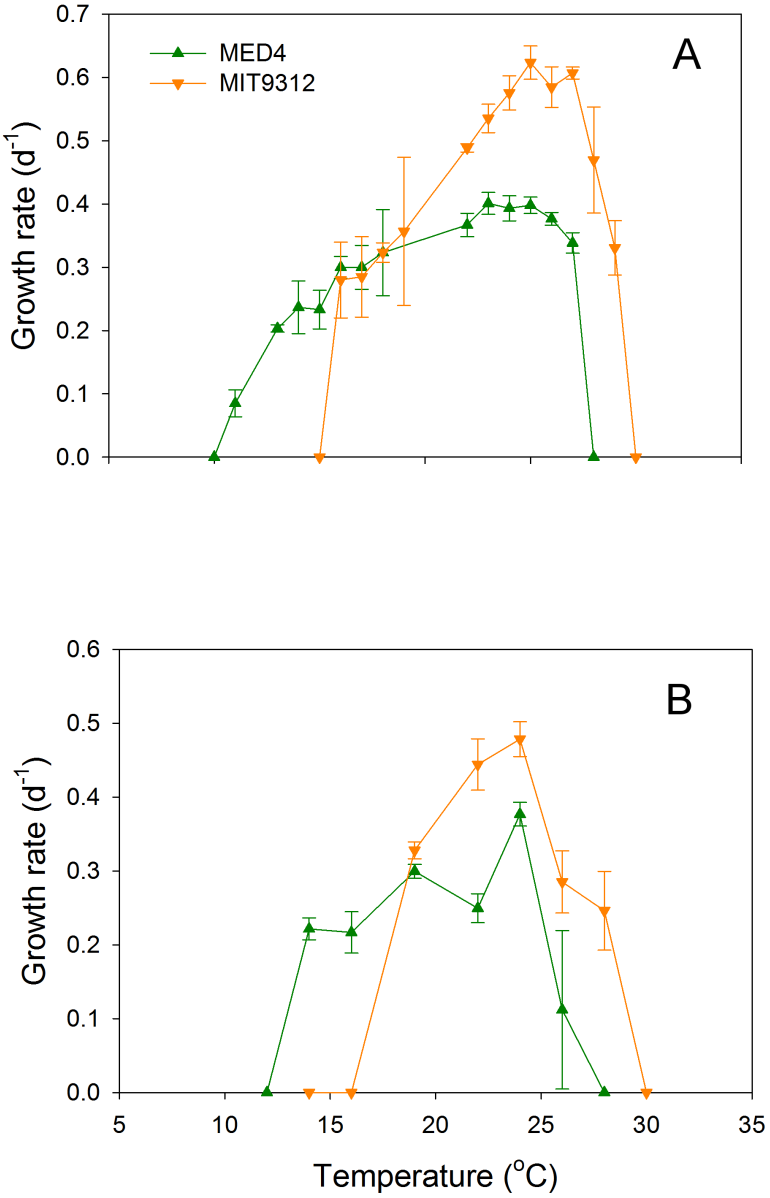

Figure 17 Growth rate of MIT93123 and MED4 as a function of temperature from Johnson *et al* 2006 (A) and this study (B).

Green up triangle:MED4; Orange down triangle:MIT9312

CHAPTER IV CONCLUSIONS AND FUTURE DIRECTIONS

Temperature and cell composition

Conclusions

Temperature was observed to influence lipids, fatty acid composition, and elemental ratios (C:N:P) in *Prochlorococcus*.

1) Although the lipid composition and fatty acid composition were related with temperature, it has not shown a clear negative or positive effect as a function of temperature. Furthermore, this effect was not different between two ecotypes.
2) The main fatty acid were 14 to 18 carbon atoms and mono-, di-, and tri-unsaturated fatty acid were found.
3) The unsaturation of fatty acid was negatively related with temperature for some strains, but not others.
4) Temperature had a significant linear positive effect on QN and QC across all strains. As temperature increased 10˚C, QN and QC rose by 40.0% and 34.6%, respectively.
5) Individual strains displayed negative relationships between growth rate and QP. In addition, temperature also influenced QP on a per strain basis, but there were no systematic differences between strains nor interactions between factors.
6) Temperature had a significant impact on C/P and N/P but the direction varied between strains.

Future Directions

1) As the lipid composition and fatty composition did not have clear relationship with temperature, we have to make sure our samples were properly collected. Thus, we may need to repeat it.

2) As lipids are related with metabolites, we will investigate the metabolites of these 6 strains.

Interactions between temperature and oxidative stress

Conclusions

Our investigation of the potential relationship between temperature and HOOH on growth and physiology of *Prochlorococcus* found:

1) Temperature and HOOH showed synergistic relationship on the growth of temperature on MIT9312.
2) The growth temperature optima were not affect by concentration of HOOH but higher concentration of HOOH diminished the temperature range for growth of *Prochlorococcus*.
3) The co-culturing of heterotroph facilitated the growth of *Prochlorococcus* and it expanded the temperature range. MED4 was able to grow with heterotrophs at 12 °C while axenic MED4 could not.
4) The photosynthesis efficiency, measured as Fv/Fm in this study, under higher concentration of HOOH showed a clear trend with temperature.

Under optimal temperature, its decrease was least and higher under suboptimal temperature.

5) Red fluorescence, a proxy of chlorophyll concentration, was shown to be change with temperature and HOOH. It was positively related with temperature and negatively related with HOOH concentration.

Future Directions

1) As HOOH reacts with di-unsaturated lipids by the process called lipid peroxidation[146, 147], while temperature affects the fatty acids unsaturation, which further influences the membrane fluidity [22, 27, 59, 148], we propose to study the lipid composition, especially the lipid peroxidation, under the interactive effects of temperature and HOOH.

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VITA

Lanying Ma was born in Shandong Province, China, the second daughter of Shouchang Ma and Guifen Dong. She stayed in her home town for primary school to high school. In 2002, she attended Shandong University for Biology department and received her Bachelor's degree in 2006. She received her Master's degree in the same university in 2009. Later in 2009 she came to University of Tennessee for Ph.D study. In 2010, she met her husband Jinlyung Choi in the University of Tennessee and got married in 2012. In 2014, her daughter Arielle Youngeun Choi was born.