Survival of *Prochlorococcus* in extended darkness

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Abstract

Prochlorococcus is the smallest oxygenic phototroph in the ocean, where it can be found in great abundance throughout the euphotic zone in mid-latitude waters. Populations of this picocyanobacterium have been observed below the euphotic zone, but the viability of these cells is unclear. To explore the tolerance of *Prochlorococcus* to extended light-deprivation, we subjected multiple strains of *Prochlorococcus* to varying periods of darkness and examined their ability to recover when placed back in the light. Some strains recovered after 35 h of darkness while others could not; this variability was not related to whether the strains were members of high- or low- light adapted ecotypes. The presence of a marine heterotroph, *Alteromonas macleodii* MIT1002, in the cultures extended their ability to survive prolonged darkness, in the most extreme case by 11 d. This could be attributed at least in part to the reduction of hydrogen peroxide in co-cultures, consistent with known roles of "helper bacteria" in detoxifying hydrogen peroxide, and this effect could be mimicked to some degree by the addition of a known hydrogen peroxide quencher, sodium pyruvate. The addition of glucose alone to the cultures provided marginal enhancement, but when both pyruvate and glucose were added together, all strains were able to survive longer in darkness than they were with only the heterotroph added. Thus, it appears that *Prochlorococcus* dark-survival depends on a multitude of factors. Limited analyses of *Synechococcus* suggest that its dark-survival capacity is longer than that of *Prochlorococcus*, for reasons that are not yet clear.

Found primarily throughout the ocean's euphotic zone between 45°N and 40°S, *Prochlorococcus* is the smallest (< 1 μm) and most abundant oxygenic phototroph (Partensky et al. 1999). The *Prochlorococcus* genus is composed of multiple distinct phylogenetic groups that are physiologically and genetically diverse, known as ecotypes (Moore et al. 1995, 1998; Moore and Chisholm 1999; West and Scanlan 1999; Rocap et al. 2002; Huang et al. 2012). These ecotypes can be broadly classified as either high-light (HL) or low-light (LL)—adapted based on multiple physiological characteristics including the optimal light level for growth in culture (Moore et al. 1995; Moore and Chisholm 1999; Zinser et al. 2007). The distributions of *Prochlorococcus* ecotypes in the euphotic zone show that HL and LL variants have distinct distributions, with HL-adapted cells dominating the surface waters and LL-adapted cells restricted primarily to deeper waters. Cells belonging to one of the LL clades (LLI, also known as ecotype eNATL2A), are relatively better adapted to vertically mixed waters than other LL-adapted clades (Zinser

et al. 2006, 2007; Johnson et al. 2006; Malmstrom et al. 2010). While they are non-motile and neutrally buoyant, *Prochlorococcus* and other cyanobacteria are subject to displacement by vertical mixing or attachment to sinking particles by phytodetritus (Lochte and Turley 1988; Vilibić and Šantić 2008; Jiao et al. 2013). Thus, cyanobacterial cells likely get mixed below the euphotic zone for extended periods of time. Their ability to withstand extended dark exposure could have important consequences for their biogeography, population genetics, long-term evolution, and the biogeochemistry of ocean basins.

Eukaryotic species of phytoplankton have been studied vis-a-vis their ability to survive extended periods of darkness and when these cells can no longer rely on photosynthesis, they must acclimate or die; potential options include resting stage formation (Smetacek 1985), reduction of metabolic rates (Dehning and Tilzer 1989), or using heterotrophic modes of acquiring energy (White 1974). Autotrophic flagellates, ciliates, and cryptophyte species are able to survive by mixotrophy or phototrophy in the summer and solely heterotrophy in winter when the cells experience complete darkness under ice coverage (Roberts and Laybourn-Parry 1999; Laybourn-Parry 2002; Cottrell and Kirchman 2009).

There have been many observations of *Prochlorococcus* and *Synechococcus* cells (collectively known as marine picocyanobacteria)

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in locations and conditions where extended darkness prevails. *Prochlorococcus*, for example, can be found in abundance far below the euphotic zone (DeLong et al. 2006; Shi et al. 2011; Martínez et al. 2012; Jiao et al. 2013; Shibl et al. 2014). Furthermore, the abundance of *Synechococcus* has been shown to change minimally from summer to winter in arctic waters (Cottrell and Kirchman 2009) suggesting they can survive continuous darkness for extended periods under ice coverage. *Synechococcus* has also been found to persist for several months after deep mixing events—down to 600m—in the Adriatic Sea (Vilibić and Šantić 2008), and cells sampled from 300 m in the Suruga Bay (Sohrin et al. 2011) have been observed to maintained their cell density for 30 d in the dark.

The observations of picocyanobacteria deep in the euphotic zone made us wonder whether they, like the eukaryotes described above, have evolved strategies for surviving extended darkness. Picocyanobacterial capacity for mixotrophy—the ability of a phototroph to utilize organic carbon—is now well known (Zubkov et al. 2003; Malmstrom et al. 2005; Zubkov and Tarran 2005; Paoli et al. 2008) and appears to be widespread in the global oceans (Yelton et al in press). More specifically, *Prochlorococcus* genomes contain genes encoding the ability to utilize organic compounds including amino acids (Zubkov et al. 2003, 2004; Rocap et al. 2003; Church et al. 2004; Michelou et al. 2007; Mary et al. 2008), as well as glucose transporters (Rocap et al. 2003; Gómez-Baena et al. 2008; Muñoz-Marín et al. 2013). Recent studies have also shown that *Prochlorococcus* is able to take up glucose and amino acids in both light and dark conditions—in laboratory cultures and in the wild (Zubkov et al. 2003, 2004; Church et al. 2004; Michelou et al. 2007; Gómez-Baena et al. 2008; Mary et al. 2008; Gómez-Pereira et al. 2013; Muñoz-Marín et al. 2013; Evans et al. 2015)—as well as dimethylsulfoniopropionate (Vila-Costa et al. 2006). Amino acids could be used as either a carbon, energy, or nitrogen source, but glucose is likely only utilized for carbon and/or energy.

The goal of the work reported here was to begin to explore the ability of marine picocyanobacteria to survive extended darkness, with a primary focus on *Prochlorococcus*. To this end, we subjected axenic strains grown on a diel light : dark cycle to additional intervals of darkness of varying length, simulating deep mixing events, and then determined if they could recover when placed in the light. We then extended this basic experimental design to examine the effects of co-culture with heterotrophs and the addition of organic compounds on the extent of dark-survival. Finally we carried out a limited number of similar experiments with *Synechococcus* to lay the foundation for further comparative studies.

Methods

Cultures and culture conditions

All *Prochlorococcus* and *Synechococcus* cells were grown in 0.2 μm filtered sterile Sargasso Sea water amended with Pro99 nutrients prepared as previously described (Moore et al. 2007).

Prochlorococcus cells starting at a concentration of 5×10^6 cells mL^{-1} to 1×10^7 cells mL^{-1}, were grown in triplicate in a 13 : 11 light:dark (L : D) cycle with simulated dawn and dusk (Zinser et al. 2009) at 24°C. This simulation creates gradual light transitions at sunrise by ramping light slowly up to midday, remaining at peak light for 4 h, and then decreasing light to sunset over the course of 13 h. This gradual increase was important for reducing light shock on the cultures transitioning from extended darkness back into 13 : 11 conditions. Near optimal peak light levels for maximizing growth rate were used for all *Prochlorococcus* strains involved and included the following combinations: MED4 (78 μmol photons m^{-2} s^{-1}), MIT9312 (75 μmol photons m^{-2} s^{-1}), MIT9301 (54 μmol photons m^{-2} s^{-1}), MIT9202 (87 μmol photons m^{-2} s^{-1}), AS9601 (75 μmol photons m^{-2} s^{-1}), NATL1A (37 μmol photons m^{-2} s^{-1}), NATL2A (37 μmol photons m^{-2} s^{-1}), MIT9211 (33 μmol photons m^{-2} s^{-1}), MIT9313 (29 μmol photons m^{-2} s^{-1}), and MIT9303 (29 μmol photons m^{-2} s^{-1}). Light levels for *Synechococcus* cultures were: WH8102 (29 μmol photons m^{-2} s^{-1}), WH7803 (37 μmol photons m^{-2} s^{-1}). To subject cells to extended darkness, we placed exponentially growing cultures into a 24°C dark incubator at the end of the 13 : 11 L : D cycle for varying durations. Including the 11 h of their last "natural" L : D cycle, these cultures were in the dark for a total of 35, 59, 83, 107, 179, or 275 h, which amounts to an additional 1, 2, 3, 4, 7, or 11 d of darkness. The cultures were then shifted back into the L : D incubator at "sunrise" to reduce light shock effects, and recovery was monitored via bulk chlorophyll fluorescence (10AU model, Turner Designs, Sunnyvale, California) and flow cytometry (see below). All dark sampling and measurements were done in green light using layered neutral density filters #736 and 740 (Lee Filters, Burbank, California) over a white light source, which causes minimal gene expression change in *Prochlorococcus* (Steglich et al. 2006). Cultures used in all experiments were axenic and were tested for purity using three broths ProAC, ProMM, and MPTB (Saito et al. 2002; Morris et al. 2008; Berube et al. 2015), as well as by flow cytometry.

Alteromonas macleodii MIT1002 (Biller et al. 2015) was maintained on ProMM medium (Berube et al. 2015), but was spun down and washed twice with Pro99 prior to addition into *Prochlorococcus* cultures to reduce carryover of organic carbon from ProMM. *Alteromonas macleodii* concentrations ranging between 5×10^5 cells mL^{-1} to 1×10^6 cells mL^{-1} were added at the onset of the experiments and maintained approximately 10-fold lower abundance than *Prochlorococcus* during exponential growth. Five milliMolar stock solutions of glucose and sodium pyruvate (99% ultrapure, Sigma Aldrich, St. Louis, Missouri, U.S.A.) were prepared with Type I ultrapure water and sterilized with a 0.2 μm Supor syringe filter (Pall Corporation, Port Washington, New York, U.S.A.) prior to addition to cultures at the beginning of the experiments. Concentrations used were based on previous work (Morris et al. 2008; Sher et al. 2011; Berube et al. 2015) and

did not influence *Prochlorococcus'* growth rate under normal diel conditions (data not shown).

Flow cytometry and metagenomics

Prochlorococcus cell abundance measurements by flow cytometry were prepared and processed as previously described (Zinser et al. 2006; Malmstrom et al. 2010). Samples were run on an Influx flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.) with 2 μm blue and red excited reference beads. Cells were excited with a blue 488 nm laser analyzed for chlorophyll fluorescence (692/40 nm), SYBR Green I stained DNA fluorescence content (530/40 nm), and size (forward scatter). Calculations for relative cell size and chlorophyll per cell were performed by normalizing forward scatter and red chlorophyll fluorescence per cell to 2 μm diameter Fluoresbrite beads (Polysciences Inc., Warrington, Pennsylvania, U.S.A.) as previously described (Olson et al. 1990). Co-culture samples were also stained with 1x SYBR Green I (Invitrogen, Grand Island, New York, U.S.A.) and incubated for 10 min in the dark prior to running. All flow cytometry files were analyzed using FlowJo version 7.6.5.

Total *Prochlorococcus* abundance depth profiles were characterized from samples taken from a Hawaii Ocean Time-series (HOT152) cruise in October 2003 using flow cytometry (methods described above) and metagenomics. Metagenomic DNA samples from 50, 600, and 1000 m depths were extracted from frozen filters (100 mL raw seawater filtered onto a 0.2 μm polycarbonate filter) using a phenol:chloroform based method (Urakawa et al. 2010), and 1 ng of DNA was used to generate sequencing libraries using the Nextera XT DNA library prep kit (Illumina, San Diego, California, U.S.A.). Libraries were sequenced on the Illumina NextSeq platform at the MIT BioMicro center, yielding $\sim$ 20–30 million 150 + 150 nt paired-end reads per library. Illumina adapters were removed from the raw sequence data using cutadapt (Martin 2011), overlapped using clc_overlap_reads (CLC bio, Aarhus, Denmark) and remaining low-quality regions were removed by clc_quality_trim (CLC bio, Aarhus, Denmark) using default settings. To determine the *Prochlorococcus* and *Synechococcus* content of each library, all overlapped and trimmed reads were first searched against a custom database of all sequenced *Prochlorococcus* and *Synechococcus* genomes (Biller et al. 2014a) using UBLAST (Edgar, R.C., unpublished, http://drive5.com/usearch). Any reads that had a significant hit (e value <= 1e-4) against this database were then searched against the NCBI nr database (downloaded April 25, 2015) with UBLAST, using the following options: "-evalue 1e-4 -maxhits 1 -accel 0.5". The number of reads whose best hit in NR was to either *Prochlorococcus* or *Synechococcus* (according to the NCBI taxonomy ID for that best hit) were counted using a custom Python script. The metagenomic datasets were deposited in the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under accession SRP065727.

Viability

Viability was tested by adding 1 μM SYTOX Green (Invitrogen, Carlsbad, CA) to the cells for 10 min prior to flow cytometry (as per manufacturer's instructions) on a Guava easyCyte HT flow cytometer (EMD Millipore, Billerica, MA). Glutaraldehyde-killed negative controls were created by adding glutaraldehyde (final conc. 0.125% v/v) to samples and incubating these in the dark at room temperature for 1 h. Cells were excited with a blue 488 nm laser and analyzed for chlorophyll fluorescence (692/40 nm) and size (forward scatter). Using events within the *Prochlorococcus* defined gate, dead cells were determined by examining SYTOX Green content (580/30 nm) and size (forward scatter) and the events that had values similar to a glutaraldehyde-killed control were defined as dead cells.

Extracellular hydrogen peroxide

Hydrogen peroxide measurements were obtained by using an Amplex Red kit (A22188, Life Technologies, Grand Island, NY). Directions from the manufacturer were followed with the exception of using Pro99 medium (instead of buffer) and reducing the Amplex concentration to 50 μM (instead of 100 μM as suggested). All *Prochlorococcus* cultures were grown in a medium buffered with 3.75 mM TAPS pH 8 to maintain enzymatic functionality of the Amplex Red. Samples were filtered through a 96 well multiscreen HTS vacuum manifold (MSVMHTS00, EMD Millipore, Billerica, MA) using a 0.22 μm Multiscreen HTS GV sterile filter plate (MSGVS2210, EMD Millipore, Billerica, Massachusetts, U.S.A.). The filtrate was collected and transferred to black walled plates with H_2O_2 standard curves (0–2.5 μm), which were made by adding known peroxide concentrations to a seawater background to obtain the slope of the linear peroxide-fluorescence relationship. We made a no-H_2O_2 control by adding 1 mM sodium pyruvate (a H_2O_2 scavenger) to the seawater background 30–60 min prior to the assay; this consumed all of the peroxide the seawater sample and thus allowed us to determine the intercept of the peroxide-fluorescence relationship. As pyruvate quenches peroxide at a 1 : 1 molar ratio (Holleman 1904; Bunton 1949), this concentration of pyruvate is vastly in excess of what is needed to remove previously measured seawater media peroxide levels. Peroxide treatment yields results similar to reactions done with catalase, a light sensitive chemical used in previous studies for creating no-H_2O_2 control in seawater (Rose et al. 2010). These data and results from another report that used Amplex Red and sodium pyruvate (Zhu et al. 2010), confirm that sodium pyruvate is not inhibitory to Amplex Red. Plates were read on a fluorometer (BioTek Snergy2, Winooski, VT), using 530/25 nm excitation and 590/20 nm emission filters.

Results and discussion

Prochlorococcus below the euphotic zone

Adding to the growing body of evidence that *Prochlorococcus* is found well below the euphotic zone (DeLong et al. 2006; Shi et al. 2011; Martínez et al. 2012; Jiao et al. 2013; Shibl et al. 2014), we found *Prochlorococcus* and *Synechococcus* across a gradient of depths up to 1000 m at station ALOHA in the Pacific, as determined by both flow cytometric and metagenomic

approaches. *Prochlorococcus* and *Synechococcus* concentrations were relatively consistent between 600 m and 1000 m depth, at $3–4 \times 10^3$ cells mL^{-1} and $2–6 \times 10^2$ cells mL^{-1}, respectively (Supporting Information Table S1). These deep populations were dominated by HL adapted *Prochlorococcus* ecotypes (Supporting Information Table S1) as was observed for deep water samples from the Luzon Straight off China (Jiao et al. 2013) and the Red Sea (Shibl et al. 2014). Subduction of surface waters in which HL adapted ecotypes are dominant is the likely explanation for their abundance in deep water but it is not clear how long they, or *Synechococcus*, might maintain a recognizable flow cytometric signature in the perpetual darkness at these depths.

Limits to *Prochlorococcus* culture survival in extended darkness

We wondered how "old" cells detected at 1000 m might be and to address this indirectly, we subjected a *Prochlorococcus* MED4 culture to complete darkness for 200 d, and measured its flow cytometry signature over this period (Fig. 1). Cell abundance remained roughly the same during the entire experiment (Fig. 1a), but the relative chlorophyll per cell and cell size (as inferred from forward scatter) decreased gradually (Fig. 1b,c). Flow cytometry dot plots after 197 d in the dark revealed a population that remained well above the noise level and recognizable as an intact population (Fig. 1d). We made attempts to revive cultures by returning them to the light conditions they were originally grown in, but none resumed growth (data not shown).

To determine the limit of "photon deprivation" beyond which the cells cannot recover in the absence of alternative sources of energy and whether LL-adapted ecotypes are better at surviving extended darkness, we took exponentially growing cultures of strains representative of the cultured high-light and low-light adapted *Prochlorococcus* clades and subjected them to 35, 59, and 83 h (1, 2, and 3 extra days of darkness beyond their 11 h night time dark cycle) of dark stress and then re-exposed to light to see if they could recover (Table 1). All but 3 of the 10 strains were able to recover after 35 h of darkness, but none were able to after 59 or 83 h in the dark. The three strains unable to tolerate even 35 h in the dark were members of the HLI (MED4), HLII (MIT9202), and LLII/III (MIT9211) clades. Thus there was no relationship between dark survival capacity and whether or not a strain was high or low-light adapted. Two *Synechococcus* strains (WH8102 and WH7803) were also examined for their dark survival times (Table 1) and both were able to recover even after 83 h of darkness, two days longer than other *Prochlorococcus* strains (further discussed below).

The role of co-cultured heterotrophs in dark survival

Evidence is mounting that the fitness of *Prochlorococcus* can be positively affected by the presence of heterotrophic bacteria when growing under continuous light or a diel light/dark cycle, due at least in part to the reduction of oxi-
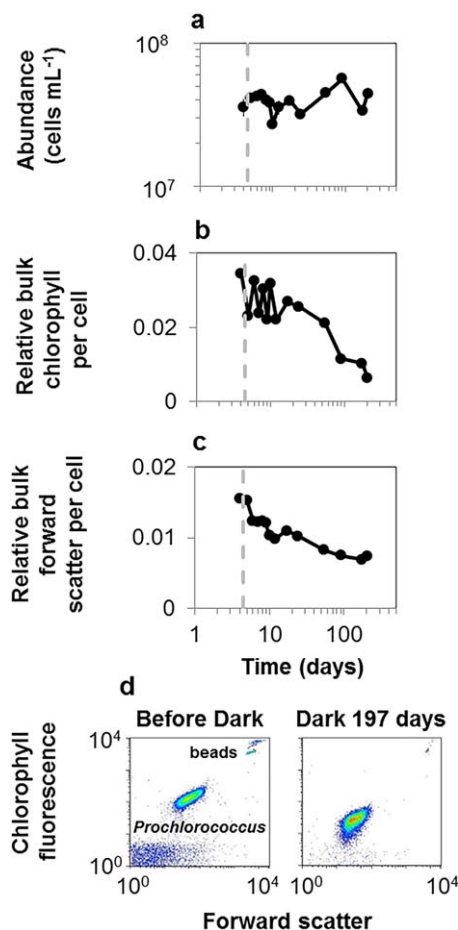

Fig. 1. Changes in cell abundance (a), bulk chlorophyll per cell (b), bulk forward light scatter per cell (c), and chlorophyll fluorescence (d), in axenic *Prochlorococcus* MED4 cultures as a function of time in continuous darkness (data to the right of gray dotted vertical line). Flow cytometric signatures (d) of the cells at the beginning (before dark) and end (197 d of darkness) reveals that the population maintained its distinct flow cytometric signature, slightly shifted, even after 197 d in darkness. Populations in the upper right corner (d) are 2 μm diameter internal standard beads. Error bars represent the standard deviation from triplicate samples and are smaller than the data points when not visible.

dative stress in the cultures (Morris et al. 2008, 2011; Sher et al. 2011). To examine if the presence of heterotrophs might also affect the ability of *Prochlorococcus* to survive the stress of extended darkness, we subjected cultures of MED4, NATL2A, and MIT9313 in co-culture with *Alteromonas macleodii* MIT1002 (hereafter referred to as *Alteromonas*), a heterotroph which was isolated from the original *Prochlorococcus* NATL2A culture, to periods of extended darkness and monitored their ability to regrow when re-introduced to the light. While the presence of *Alteromonas* did not influence the growth rate of these three strains in exponentially growing cultures grown on a diel cycle (Supporting Information Fig. S1), survival of MED4 (a HL—adapted strain) and NATL2A (a LL—adapted strain) in the dark was significantly enhanced

Table 1. Regrowth of axenic high-light (HL)- and low-light (LL)-adapted *Prochlorococcus* and *Synechococcus* cultures following extended darkness conditions. Cultures were placed into the dark for an additional 24, 48, or 72 h (for a total of 35, 59, or 83 h) before re-exposure to light. Cultures were monitored for regrowth based on increases in bulk chlorophyll fluorescence. Plus symbols (+) indicate that the cultures grew when placed back into the light, minus symbols (−) a lack of growth following dark stress.

	Strain	Clade	Growth following time in dark		
			35 h	59 h	83 h
HL *Prochlorococcus*	MED4	HLI	−	−	−
	MIT9202	HLII	−	−	−
	MIT9301	HLII	+	−	−
	AS9601	HLII	+	−	−
	MIT9312	HLII	+	−	−
LL *Prochlorococcus*	NATL2A	LLI	+	−	−
	NATL1A	LLI	+	−	−
	MIT9211	LLII/III	−	−	−
	MIT9303	LLIV	+	−	−
	MIT9313	LLIV	+	−	−
Synechococcus	WH8102	III, 5.1A	+	+	+
	WH7803	V, 5.1B	+	+	+

by its presence (Fig. 2a,d and 2b,e respectively). When alone, *Prochlorococcus* MED4 was unable to survive even 35 h of continuous darkness (Fig. 2a), but in co-culture it could survive an additional 6 d (Fig. 2d). Similarly, *Prochlorococcus* NATL2A was able to survive an additional 10 d of darkness when with *Alteromonas* (Fig. 2e).

For MIT9313 (LLIV clade), the presence of *Alteromonas* extended the dark survival time from 35 h to 83 h, but could not mediate recovery for longer dark periods (Fig. 2c,f). Furthermore, its recovery was much slower relative to that of MED4 and NATL2A for the same period of darkness (Fig. 2d–f), consistent with observations of extended recovery lag periods in co-cultures of *Alteromonas* and *Prochlorococcus* relative to pairings with other heterotrophs (Sher et al. 2011). It is not clear what aspect of MIT9313 cell physiology would influence the "helping" function of the heterotroph vis a vis dark survival. One possibility is that it could be relatively more stressed upon reintroduction into the light as previous work has shown that MIT9313 is more sensitive to light shock than MED4 or NATL2A (Berta-Thompson 2015).

Effect of oxidative stress on the survival of *Prochlorococcus* in the dark

Heterotrophs play a key role in reducing oxidative stress of *Prochlorococcus* via the production of extracellular catalase-peroxidase (Morris et al. 2008). *Prochlorococcus* lacks the gene (*katG*) that encodes this enzyme, and co-culture with catalase-

peroxidase producing heterotrophs has been shown to increase the fitness of *Prochlorococcus* both in culture and in the field (Morris et al. 2008, 2011). These observations have even led to a new theory about the evolution of dependencies and genome streamlining (Morris et al. 2012). While the production of hydrogen peroxide is typically thought to be due to photochemical reactions—either biologically or abiotically mediated—in sunlit ocean waters (Cooper and Zika 1983; Zepp et al. 1987; Johnson et al. 1989), biological production of H_2O_2 can also be significant in the dark (Palenik and Morel 1988; Yuan and Shiller 2004; Vermilyea et al. 2010). Thus we designed experiments to see whether detoxification of H_2O_2 could explain the ability of *Alteromonas* to partially mitigate the stress of prolonged darkness for *Prochlorococcus*.

We measured hydrogen peroxide levels in MED4 (Fig. 3a–d) and NATL2A (Fig. 3e–h) cultures during extended darkness and upon re-exposure to light, with and without additions of *Alteromonas* (Fig. 3a–d, blue and red lines). As before (Table 1 and Fig. 2), in the absence of other treatments MED4 cells were unable to survive even 35 h in the dark (Fig. 3a,b); NATL2A cells were able to tolerate 35 h of darkness but no longer (Fig. 3e,f). In all the untreated cultures that were unable to recover, hydrogen peroxide concentrations peaked 3–4 d after the cells were placed in the dark (Fig. 3e,f,h). In the untreated NATL2A culture subjected to 35 h of darkness (Fig. 3g) that did recover, hydrogen peroxide levels remained near media background levels throughout. In the 83 h dark treatment of both strains, hydrogen peroxide concentration increased before the cells were re-introduced into the light (Fig. 3f,h) indicating that its production was not due to light shock. Thus, for the three conditions tested in which the cells were unable to recover from dark stress, hydrogen peroxide concentrations reached levels 2–4 times those of media alone (Fig 3e–h, black dashed line).

When *Prochlorococcus* was co-cultured with *Alteromonas*, hydrogen peroxide concentrations stayed close to that of the media background, and all cultures recovered (Fig. 3a–h, red lines). From these data, we infer that the presence of *Alteromonas* reduced hydrogen peroxide concentrations in the cultures and promoted dark survival of *Prochlorococcus*. To try to explore this causality more directly we added sodium pyruvate, a reactive oxygen species (ROS) scavenger and organic carbon source, to cultures in the absence of *Alteromonas* (Fig. 3a–d green lines). Sodium pyruvate is commonly included in media used for *Prochlorococcus* isolations or purifications (Berube et al. 2015) and has no influence on its growth under standard conditions. We were unable to use exogenous catalase as it is highly sensitive to light and degrades quickly, so it would not be effective in these long-term experiments. Sodium pyruvate, on the other hand, has a very quick and effective 1 : 1 molecule reaction with hydrogen peroxide (Holleman 1904; Bunton 1949)—reducing the concentrations of H_2O_2 in all the cultures to zero (Fig. 3a–d, green lines).

The presence of pyruvate in the *Prochlorococcus* NATL2A cultures extended their dark survival times from 35 h to
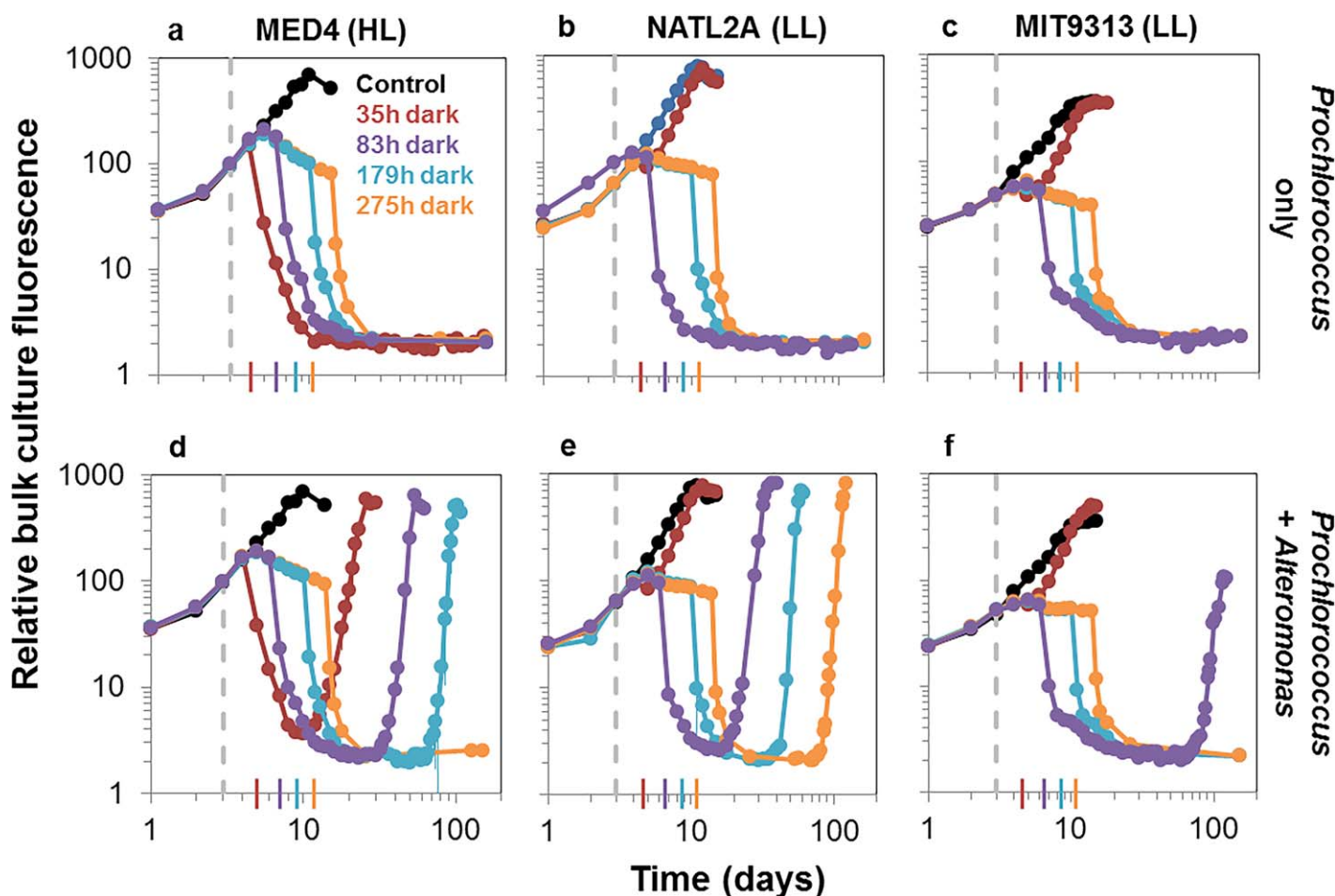
Fig. 2. Response of axenic *Prochlorococcus* high-light (HL)-adapted MED4 (a) and low-light (LL)-adapted NATL2A (b) and MIT9313 (c) to extended darkness compared with the same strains cocultured with the heterotrophic bacterium *Alteromonas macleodii* (d, e, f) under otherwise identical conditions. Cultures were inoculated with *A.macleodii* at the beginning of the experiment and placed into the dark (gray dotted vertical line) for an additional 24, 48, 72, 168, or 264 h beyond the normal "night" (for a total of 35, 83, 179, or 275 h of darkness) and then re-exposed to light (represented by colored ticks on axis). Experimental cultures were monitored using bulk chlorophyll fluorescence for over 100 d to detect whether they could be revived when placed back in the light; cultures which did not regrow during this timeframe were monitored visually for an additional 2 months, but no growth was observed. Note that both axes are on a log scale.

83 h (Fig. 3c,d), mimicking the effect of *Alteromonas*. While the addition of pyruvate allowed MED4 to survive 35 h of darkness—which it was unable to do in the no treatment control—the time MED4 took to recover was delayed relative to that in the presence of *Alteromonas* (compare red and green Fig. 3a). Furthermore, *Alteromonas* could facilitate MED4's survival of 83 h of darkness, while pyruvate could not (Fig. 3b). This suggests that its role in promoting dark survival does not lie solely in H_2O_2 detoxification. One possibility is that organic exudates from *Alteromonas* can be used as sources of organic carbon by *Prochlorococcus*.

Use of alternative carbon and energy sources as a means to survive darkness

As discussed previously, *Prochlorococcus* has the genomic capacity for mixotrophy, and is known to take up glucose

and other organic compounds. To test whether non-ROS scavenging organic compounds could enhance dark survival, we grew *Prochlorococcus* MED4 with 5 mM glucose and subjected cultures to 59, 83, or 107 h of darkness (Fig. 4a–d). While this is a very high concentration of glucose relative to that found in the wild, so is the concentration of cells in our experiments; thus proportionally it is not that high. Since we know that this amount of glucose does not inhibit or enhance growth rates under standard growth conditions in the light (data not shown), we opted to use this concentration to insure that there would be a sufficient supply available to the cells to determine whether it might have an effect in the dark. That is, our intention was simply to see whether saturating levels of glucose might extend dark-survival. In the absence of any treatment, MED4 cells are unable to recover after 59 or more hours of darkness (Fig.
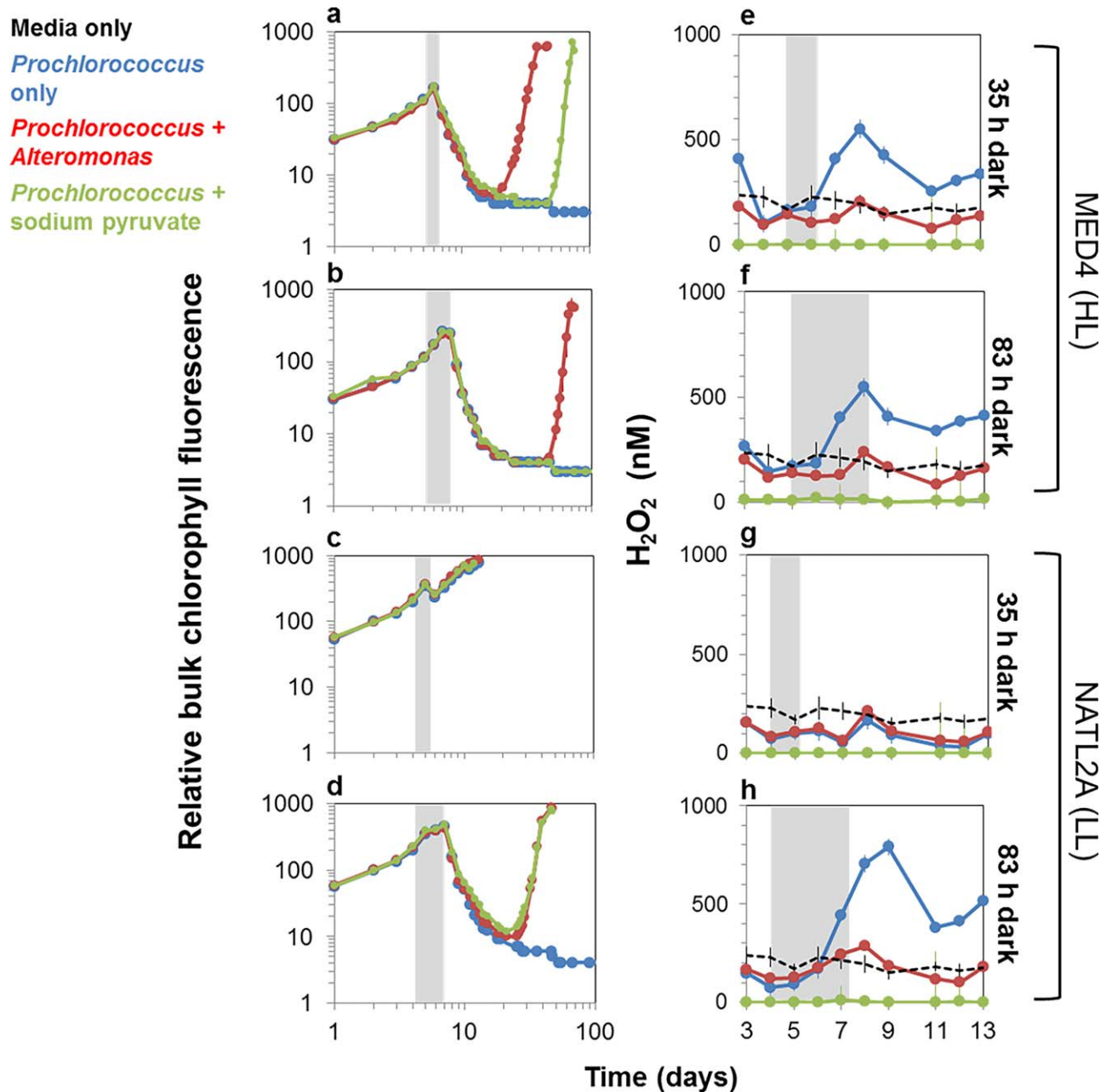
Fig. 3. Changes in cultures of two *Prochlorococcus* strains after extended dark treatment, as measured by bulk chlorophyll fluorescence (a–d) and hydrogen peroxide concentration (e–h); data are shown in the presence and absence of either *Alteromonas* (red) or sodium pyruvate (green). Cultures of high-light (HL)-adapted MED4 (a, b, e, f) and low-light (LL)-adapted NATL2A (c, d, g, h) were grown either alone, with *Alteromonas*, or with 5 mM sodium pyruvate and were placed into the dark (indicated by gray shading) for 35 (a, c, e, g) or 83 (b, d, f, h) h before re-exposure to light. "*Prochlorococcus* only" cultures (blue) were not amended with sodium pyruvate or *Alteromonas*. Peroxide measurements (panels e–h) were made in the cultures alongside a media control (black dashed line) and were taken during the critical time frame for the experiment (before extended dark, throughout the extended darkness, and after re-exposure).

4a); with glucose they can withstand 59 h in the dark, but no longer (Fig. 4b). When we added pyruvate to the cultures in addition to the glucose, the cells recovered even after 83 h of darkness (Fig. 4d). MED4 cells kept in the dark for 197 d in the presence of glucose and pyruvate, however, did not resume growth when returned to their original light

conditions; the cell abundance, bulk chlorophyll per cell, and bulk forward light scatter per cell results were similar to untreated MED4 shown in Fig. 1a–d (data not shown). It is important to note that there was no population growth in the dark when glucose and pyruvate are supplied, suggesting that organics can play a role in maintenance metabolism
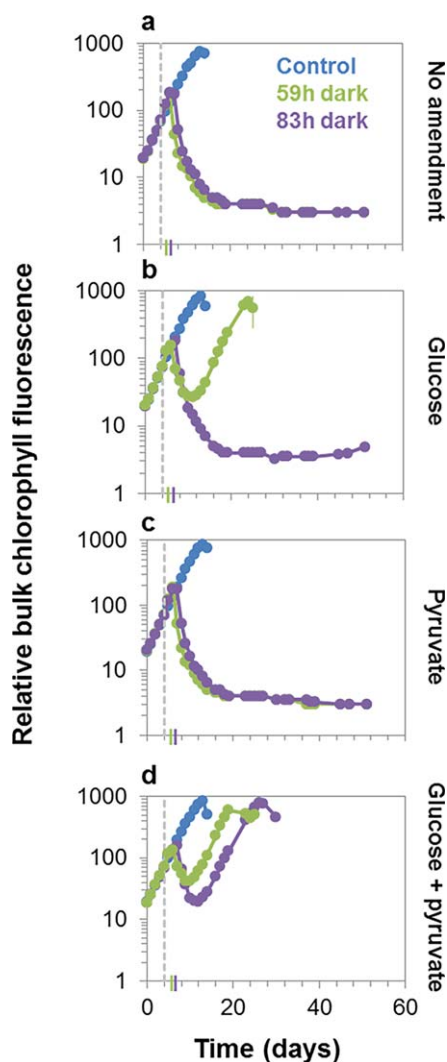
Fig. 4. Response of *Prochlorococcus* high-light (HL)-adapted MED4 to the presence of glucose, sodium pyruvate, or both, during extended dark exposure. Cultures were placed into the dark (grey dotted line) for 59 h or 83 h before re-exposure to light (colored ticks on axis). Cultures were monitored using bulk chlorophyll fluorescence to detect whether they could be revived when placed back in the light. Note that the *x* axis is a linear scale, unlike that of Figs. 2a–f, 3a–d.

and reduction of oxidative stress which facilitates survival and recovery after extended darkness. In an independent experiment, we examined whether results seen in Fig. 4 with MED4 were consistent with another *Prochlorococcus* strain, NATL2A, and indeed the combination of glucose and pyruvate enabled all cultures to recover (Supporting Information Fig. S2). We suggest that *Alteromonas* might play this dual role in its interactions with *Prochlorococcus*.

Cell viability and intra-population variability in response to stress

In the experiments described above we used bulk culture fluorescence as a measure of culture status. That is, we inter-

pret exponential increases in fluorescence when cells are placed in light after prolonged darkness to reflect the growth—or at least the metabolic activity—of surviving cells. It is well established, however, that bulk fluorescence does not reflect cell number when a culture is not in steady state exponential growth (Falkowski and Kiefer 1985; Olson et al. 1988; Thompson et al. 2011). Thus we explored what was happening to individual *Prochlorococcus* cells in a limited set of the dark-stressed cell populations using flow cytometry. Where relevant, we also monitored the population dynamics of *Alteromonas* in the co-cultures. Examining the effects of 83 h of darkness on MED4 alone and in co-culture with *Alteromonas*, we found that while bulk culture fluorescence plummeted after re-exposure to light in both the axenic and cocultures (Fig. 5a,b, blue line), the total *Prochlorococcus* cell abundance remained constant over the whole experiment (Fig. 5a,b, black line); however, only *Prochlorococcus* in the co-cultures with *Alteromonas* recovered (Fig. 5b). The abundance patterns of *Alteromonas* mimicked that of *Prochlorococcus*, at 10-fold lower concentration throughout dark and recovery time periods (Fig. 5b, red line)—likely due to the fact that *Alteromonas* is unable to grow in Pro99 medium alone and is dependent on the organic compounds produced by *Prochlorococcus* (Biller et al. 2014b).

Examination of the flow cytometry dot plots of *Prochlorococcus* from this experiment revealed complex intra-population dynamics (Fig. 5c–j). We observed flow cytometric evidence for *Prochlorococcus* differentiation into subpopulations which we defined as "High Fluorescence *Prochlorococcus*" (red circle) and "Total *Prochlorococcus*" (black outline), the latter a combination of the High Fluorescence population and the *Prochlorococcus* populations that decrease in fluorescence over time. In the *Prochlorococcus*-alone cultures, which were unable to recover (Fig. 5a), the mean fluorescence per cell of the entire population decreased steadily upon reintroduction to the light (Fig. 5c–f), even though total cell numbers remained constant (black line, Fig. 5a). In the presence of *Alteromonas* the population trajectory was similar to that of *Prochlorococcus*- alone cultures for 14 d after reintroduction to the light, but in this case a population of *Prochlorococcus* cells appeared after 38 d with fluorescence and light scatter values identical to those observed after 3 d in the dark (compare Fig. 5g,j, circled). We hypothesize that a very small subpopulation of cells began growing upon re-exposure to light and only became detectable after 38 d. It appears that this small subpopulation was the seed population that grew exponentially between days 50 and 65 and is evident in the bulk culture fluorescence values (Fig. 5b). Thus, we argue, the "rebound" of this culture has its origins in a small subset of the cells. If we assume this subpopulation began growing exponentially immediately upon re-exposure to light, the size of this "inoculum" is estimated to be around 83 cells mL^{-1}—or 0.00015% of the cells that were placed in the dark.

We note that the cell number data for *Alteromonas* reported in Fig. 5b were determined by staining a replicate set of
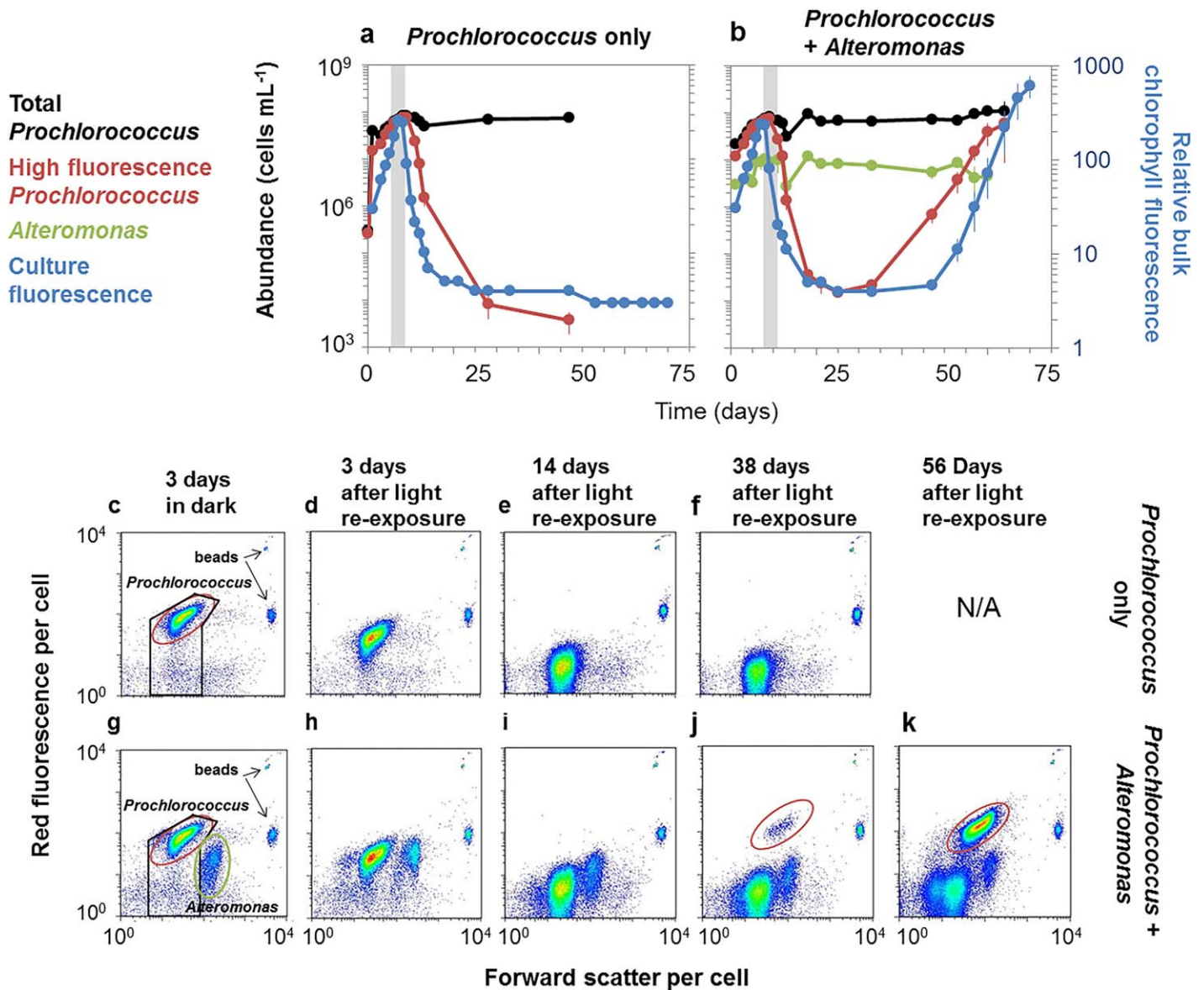
Fig. 5. Physiological changes of *Prochlorococcus* MED4 compared to the same strain in coculture with *Alteromonas* after 83 h of dark exposure. Cultures were grown with and without *Alteromonas* and placed into the dark for 83 h (solid gray bar) before re-exposure to light. Recovery response of *Prochlorococcus* (a, b) was measured by bulk chlorophyll fluorescence (blue lines) and cell abundance by flow cytometry (black, red, and green lines) to determine if cultures were able to revive after dark exposure. Selected snapshots of unstained flow cytometry dot plots (c–j) show physiological changes in red fluorescence and forward scatter over time and the emergence of sub populations. Total *Prochlorococcus* is delineated by the black outline and the High Fluorescence population is circled in red. *Alteromonas* abundance (b, green line) was measured by SYBR staining (see methods) on a parallel set of samples. We note that the *Alteromonas* populations seen in the dot plots (green circle) are not autofluorescent, and that this signal was found to be an artifact of preserving the samples in glutaraldehyde.

samples, not from the exact same populations identified in Fig. 5g–k. *Alteromonas* cells in the latter display red fluorescence under these conditions, as a by-product of glutaraldehyde staining. This same strain does not autofluoresce when grown in the organic media (ProMM) in which it is maintained and it does not influence the results presented here, as this population can be clearly separated from *Prochlorococcus*.

To this point, we have been defining dark-survival as the ability to grow upon re-exposure to light. One wonders, however, whether cells held in the dark for long periods of time might indeed be "viable," but we have simply not found the optimal conditions to allow them to actually grow and replicate while in the dark. A common way to address this type of question is through the use of a vital stain to determine what fraction of the population remains "viable"—i.e., alive—in the
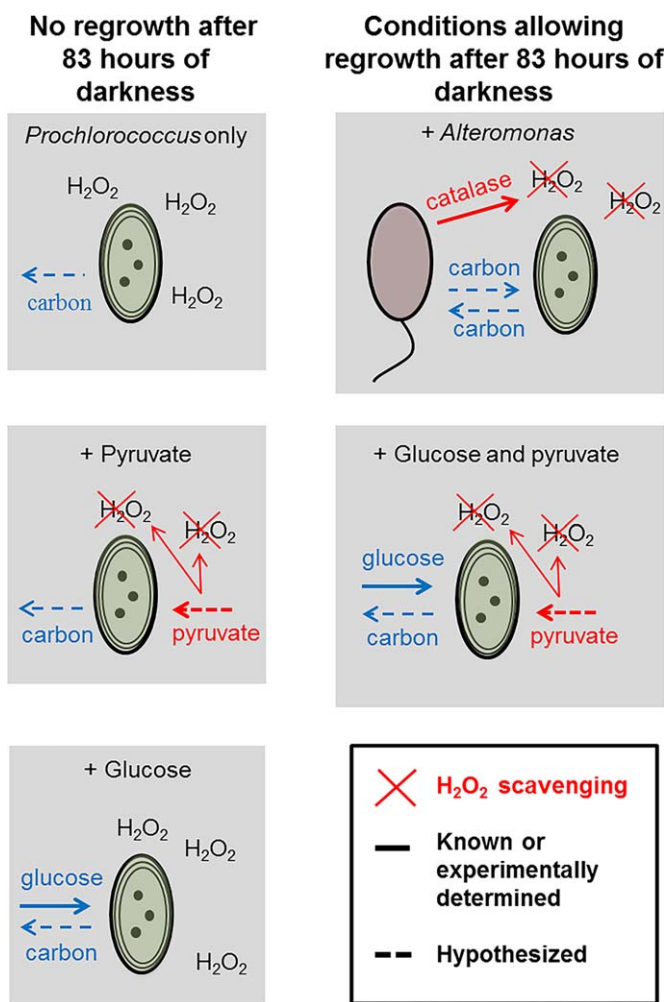
No regrowth after 83 hours of darkness

Prochlorococcus only

H_2O_2 H_2O_2 H_2O_2

carbon

+ Pyruvate

H_2O_2 H_2O_2

carbon pyruvate

+ Glucose

glucose

H_2O_2 H_2O_2

carbon H_2O_2

Conditions allowing regrowth after 83 hours of darkness

+ *Alteromonas*

catalase H_2O_2 H_2O_2

carbon carbon

+ Glucose and pyruvate

H_2O_2 H_2O_2

glucose

carbon pyruvate

✕ H_2O_2 scavenging

— Known or experimentally determined

- - Hypothesized

Fig. 6. Summary of the factors influencing survival and regrowth of *Prochlorococcus* MED4ax cultures (green ovals) after 83 h dark exposure. On the left are conditions that did not result in regrowth upon reintroduction to the light. On the right are shown the experimental treatments that result in regrowth, including the results in co-culture with *Alteromonas* (brown oval). H_2O_2 scavengers (red) indicate extracellular quenching of H_2O_2. Solid arrows represent functions either imposed by the experimental design, or for which there is experimental evidence, and dotted lines indicate hypotheses based on this work. Results indicate that if extracellular H_2O_2 concentrations are lowered by the addition of pyruvate, and a source of carbon (glucose) is supplied, *Prochlorococcus* is able to regrow after 83 h of darkness; it is unable to recover with either of these treatments on its own, or in the control with no treatment. Co-culture with *Alteromonas* yields similar results to those of the combined glucose and pyruvate additions, suggesting that *Alteromonas* could be providing *Prochlorococcus* with an alternative energy source.

dark. To examine the viability of *Prochlorococcus* in the dark, we treated cultures with the nucleic acid stain SYTOX Green—an asymmetrical cyanine dye with three positive charges that is typically excluded from live eukaryotic and prokaryotic cells. This compound has been previously shown to identify viability in *Prochlorococcus* cultures (Hughes et al. 2011; Morris et al.

2011) and we found that it was effective in separating live and dead (glutaraldehyde-treated) cells in cultures grown on the standard 13: 11 L: D cycle (Supporting Information Fig. S3a); as expected, glutaraldehyde-treated cells took up the stain while live ones did not. Surprisingly, after extended darkness, this assay failed to identify glutaraldehyde-treated cells as "dead" (Supporting Information Fig. S3b); the glutaraldehyde-killed cells excluded the stain, as would be expected for live cells (Supporting Information Fig. S2b, solid line). As a consequence of this intriguing phenotype, we were not able to use the SYTOX stain to differentiate "viable" and "dead" *Prochlorococcus* cells under extended darkness.

While it is possible that the extended-darkness cells were not easily killed by glutaraldehyde, we doubt this. It is possible, however, that extended darkness induces extensive physiological changes in *Prochlorococcus* that could influence the entry of the dye. It is also possible that the DNA in these cells has degraded and does not stain with this dye, as has been previously reported with other stressed cell cultures (Lebaron et al. 1998). We also tried other treatments as alternatives to dead-cell controls such as treating with glutaraldehyde for less time (10 min), or heat shocking under various conditions (60°C for 15 min or 30 min, 70°C for 15 min, and 80°C for 5 min), but none of these methods were as effective at killing exponentially growing *Prochlorococcus* as the one hour glutaraldehyde treatment. Although our attempts at determining viability were inconclusive, *Prochlorococcus* have been isolated into culture from untreated euphotic zone seawater samples that were held in the dark for up to 21 d (Biller et al. 2014b); this illustrates the potential for relatively long term survival of these phototrophs in the dark and recovery upon reintroduction into the light—though only when embedded in their indigenous microbial community.

Differences between *Prochlorococcus* and *Synechococcus*

Since *Prochlorococcus* always co-occurs with *Synechococcus*, we thought it of interest to compare the dark-survival times of the two genera in a limited set of experiments. Two *Synechococcus* strains (WH8102 and WH7803) were examined simply for their dark survival times in the absence of any treatments. WH8102 is typically found in oligotrophic open-ocean waters whereas WH7803 is a widely distributed oceanic clade (Scanlan et al. 2009). Both were able to recover even after 83 h of darkness, two days longer than any *Prochlorococcus* strains in the absence of any treatments (Table 1). While data is too limited for strong inference, the difference in survivability in the dark between *Prochlorococcus* and *Synechococcus* might be rooted, at least in part, in the differences between their circadian clock networks. *Prochlorococcus* has an incomplete circadian clock (Holtzendorff et al. 2008; Axmann et al. 2009; Mullineaux and Stanewsky 2009); while its cell division and gene expression synchronizes tightly to a diel light dark

cycle (Zinser et al. 2009; Waldbauer et al. 2012) when switched to continuous light it loses the rhythmicity quickly (Holtzendorff et al. 2008), suggesting that it needs light to reset its rudimentary circadian clock (Axmann et al. 2009). By contrast, *Synechococcus* has a complete circadian clock and displays free-running rhythmicity for several days when switched to continuous light (Holtzendorff et al. 2008). Perhaps its complete circadian clock allows *Synechococcus* to keep cellular functions coordinated over a few days in the dark, thus increasing their probability of survival when re-introduced into the light.

Another difference between *Prochlorococcus* and *Synechococcus* that may play a role in their relative abilities to survive extended darkness is their sensitivity to oxidative stress. While the catalase-peroxidase gene, *katG*, is absent in all *Prochlorococcus* genomes sequenced thus far (Regelsberger et al. 2002; Scanlan et al. 2009; Morris et al. 2012; Biller et al. 2014a), it is present in most strains of *Synechococcus* (Scanlan et al. 2009). That said, only one (WH7803) of the two *Synechococcus* strains we examined has *katG* yet both had similar dark survival characteristics –longer than any of the *Prochlorococcus* strains. Thus the presence or absence of *katG* cannot be the primary factor in the differences observed between these two genera.

Conclusions

We have shown that all axenic strains of *Prochlorococcus* tested can survive up to 35 h of darkness, but not beyond. Survival capacity was unrelated to whether or not a strain was high- or low-light adapted. In the presence of the heterotroph *Alteromonas macleodii* MIT1002, survival of *Prochlorococcus* in darkness is significantly enhanced by 3–11 d. This enhancement effect appears to be due at least in part to the reduction of oxidative stress by the heterotroph under extended darkness. Addition of glucose to the media also extends dark survival, and the combined effects of added glucose and pyruvate (which quenches hydrogen peroxide) mimics the effect of co-culture with *Alteromonas*. Two strains of *Synechococcus* were able to survive up to, but not limited to, 83 h of darkness in the absence of any treatments, longer than any *Prochlorococcus* strains examined under these conditions. Thus in addition to strain to strain variability, it is clear that one needs to consider the impact of biotic and abiotic milieu, the residence time in dark, the affect of their surrounding microbial community, and the unpredictable conditions of their reintroduction to the light in assessing the real limits of picocyanobacterial survival over extended dark periods.

While these results do not fully resolve one of the questions that motivated this study—i.e., whether or not *Prochlorococcus* cells observed below the euphotic zone are viable—they do point us in interesting directions vis-a-vis the role of oxidative stress in dark survival and the role of

metabolic exchange with heterotrophs that promote the survival under this particular stress condition (Fig. 6). Fruitful directions to pursue in future studies would be to identify the mechanisms through which co-culture with *Alteromonas* prolongs dark survival and to further explore the differences between *Prochlorococcus* and *Synechococcus* and their ability to tolerate extended darkness.

References

Axmann, I. M., U. Dühring, L. Seeliger, A. Arnold, J. T. Vanselow, A. Kramer, and A. Wilde. 2009. Biochemical evidence for a timing mechanism in *Prochlorococcus*. J. Bacteriol. **191**: 5342–5347. doi:10.1128/JB.00419-09

Berta-Thompson, J. W. 2015. *Prochlorococcus*: Life in light. Ph.D. thesis. Massachusetts Institute of Technology.

Berube, P. M., and others. 2015. Physiology and evolution of nitrate acquisition in *Prochlorococcus*. ISME J. **9**: 1195–1207. doi:10.1038/ismej.2014.211

Biller, S. J., and others. 2014a. Genomes of diverse isolates of the marine cyanobacterium *Prochlorococcus*. Sci. Data **1**: 140034. doi:10.1038/sdata.2014.34

Biller, S. J., F. Schubotz, S. E. Roggensack, A. W. Thompson, R. E. Summons, and S. W. Chisholm. 2014b. Bacterial Vesicles in Marine Ecosystems. Science **343**: 183–186. doi:10.1126/science.1243457

Biller, S. J., A. Coe, A.-B. Martin-Cuadrado, and S. W. Chisholm. 2015. Draft genome sequence of *Alteromonas macleodii* strain MIT1002, isolated from an enrichment culture of the marine Cyanobacterium *Prochlorococcus*. Genome Announc. **3**: e00967–15. doi:10.1128/genomeA.00967-15

Bunton, C. A. 1949. Oxidation of α-diketones and α-keto-acids by hydrogen peroxide. Nature **163**: 444.

Church, M. J., H. W. Ducklow, and D. M. Karl. 2004. Light dependence of [3H]leucine incorporation in the oligotrophic North Pacific ocean. Appl. Environ. Microbiol. **70**: 4079–4087. doi:10.1128/AEM.70.7.4079-4087.2004

Cooper, W. J., and R. G. Zika. 1983. Photochemical formation of hydrogen peroxide in surface and ground waters exposed to sunlight. Science **220**: 711–712. doi:10.1126/science.220.4598.711

Cottrell, M. T., and D. L. Kirchman. 2009. Photoheterotrophic microbes in the Arctic Ocean in summer and winter. Appl. Environ. Microbiol. **75**: 4958–4966. doi:10.1128/AEM.00117-09

Dehning, I., and M. M. Tilzer. 1989. Survival of *Scenedesmus acuminatus* (chlorophyceae) in darkness. J. Phycol. **25**: 509–515. doi:10.1111/j.1529-8817.1989.tb00256.x

DeLong, E. F., and others. 2006. Community genomics among stratified microbial assemblages in the ocean's interior. Science **311**: 496–503. doi:10.1126/science.1120250

Evans, C., P. R. Gómez-Pereira, A. P. Martin, D. J. Scanlan, and M. V. Zubkov. 2015. Photoheterotrophy of

bacterioplankton is ubiquitous in the surface oligotrophic ocean. Prog. Oceanogr. **135**: 139–145. doi:10.1016/j.pocean.2015.04.014

Falkowski, P., and D. A. Kiefer. 1985. Chlorophyll a fluorescence in phytoplankton: Relationship to photosynthesis and biomass. J. Plankton Res. **7**: 715–731. doi:10.1093/plankt/7.5.715

Gómez-Baena, G., A. López-Lozano, J. Gil-Martínez, J. M. Lucena, J. Diez, P. Candau, and J. M. García-Fernández. 2008. Glucose uptake and its effect on gene expression in *Prochlorococcus*. PLoS One **3**: e3416. doi:10.1371/journal.pone.0003416

Gómez-Pereira, P. R., and others. 2013. Comparable light stimulation of organic nutrient uptake by SAR11 and *Prochlorococcus* in the North Atlantic subtropical gyre. ISME J. **7**: 603–614. doi:10.1038/ismej.2012.126

Holleman, A. F. 1904. Notice sur l'action de l'eau oxygénée sur les acides α-cétoniques et sur les dicétones 1. 2. Recl. Trav. Chim. Pays-Bas Belg. **23**: 169–172. doi:10.1002/recl.19040230504

Holtzendorff, J., F. Partensky, D. Mella, J.-F. Lennon, W. R. Hess, and L. Garczarek. 2008. Genome streamlining results in loss of robustness of the circadian clock in the marine cyanobacterium *Prochlorococcus marinus* PCC 9511. J. Biol. Rhythms **23**: 187–199. doi:10.1177/0748730408316040

Huang, S., S. W. Wilhelm, H. R. Harvey, K. Taylor, N. Jiao, and F. Chen. 2012. Novel lineages of *Prochlorococcus* and *Synechococcus* in the global oceans. ISME J. **6**: 285–297. doi:10.1038/ismej.2011.106

Hughes, C., D. J. Franklin, and G. Malin. 2011. Iodomethane production by two important marine cyanobacteria: *Prochlorococcus marinus* (CCMP 2389) and *Synechococcus sp.* (CCMP 2370). Mar. Chem. **125**: 19–25. doi:10.1016/j.marchem.2011.01.007

Jiao, N., and others. 2013. Presence of *Prochlorococcus* in the aphotic waters of the western Pacific Ocean. Biogeosciences. **10**: 9345–9371. doi:10.5194/bgd-10-9345-2013

Johnson, K. S., S. W. Willason, D. A. Wiesenburg, S. E. Lohrenz, and R. A. Arnone. 1989. Hydrogen peroxide in the western Mediterranean Sea: A tracer for vertical advection. Deep Sea Res. Part Oceanogr. Res. Pap. **36**: 241–254. doi:10.1016/0198-0149(89)90136-2

Johnson, Z. I., E. R. Zinser, A. Coe, N. P. McNulty, E. M. S. Woodward, and S. W. Chisholm. 2006. Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients. Science **311**: 1737–1740. doi:10.1126/science.1118052

Laybourn-Parry, J. 2002. Survival mechanisms in Antarctic lakes. Philos. Trans. R. Soc. B Biol. Sci. **357**: 863–869. doi:10.1098/rstb.2002.1075

Lebaron, P., P. Catala, and N. Parthuisot. 1998. Effectiveness of SYTOX green stain for bacterial viability assessment. Appl. Environ. Microbiol. **64**: 2697–2700. doi:9647851

Lochte, K., and C. M. Turley. 1988. Bacteria and cyanobacteria associated with phytodetritus in the deep sea. Nature **333**: 67–69. doi:10.1038/333067a0

Malmstrom, R. R., R. P. Kiene, M. Vila, and D. L. Kirchman. 2005. Dimethylsulfoniopropionate (DMSP) assimilation by *Synechococcus* in the Gulf of Mexico and northwest Atlantic Ocean. Limnol. Oceanogr. **50**: 1924–1931. doi:10.4319/lo.2005.50.6.1924

Malmstrom, R. R., A. Coe, G. C. Kettler, A. C. Martiny, J. Frias-Lopez, E. R. Zinser, and S. W. Chisholm. 2010. Temporal dynamics of *Prochlorococcus* ecotypes in the Atlantic and Pacific oceans. ISME J. **4**: 1252–1264. doi:10.1038/ismej.2010.60

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. **17**: 10–12. doi:10.14806/ej.17.1.200

Martínez, A., M. S. Osburne, A. K. Sharma, E. F. DeLong, and S. W. Chisholm. 2012. Phosphite utilization by the marine picocyanobacterium *Prochlorococcus* MIT9301. Environ. Microbiol. **14**: 1363–1377. doi:10.1111/j.1462-2920.2011.02612.x

Mary, I., G. A. Tarran, P. E. Warwick, M. J. Terry, D. J. Scanlan, P. H. Burkill, and M. V. Zubkov. 2008. Light enhanced amino acid uptake by dominant bacterioplankton groups in surface waters of the Atlantic Ocean. FEMS Microbiol. Ecol. **63**: 36–45. doi:10.1111/j.1574-6941.2007.00414.x

Michelou, V. K., M. T. Cottrell, and D. L. Kirchman. 2007. Light-stimulated bacterial production and amino acid assimilation by cyanobacteria and other microbes in the North Atlantic Ocean. Appl. Environ. Microbiol. **73**: 5539–5546. doi:10.1128/AEM.00212-07

Moore, L., R. Goericke, and S. Chisholm. 1995. Comparative physiology of *Synechococcus* and *Prochlorococcus*: Influence of light and temperature on growth, pigments, fluorescence and absorptive properties. Mar. Ecol. Prog. Ser. **116**: 259–275. doi:10.3354/meps116259

Moore, L. R., G. Rocap, and S. W. Chisholm. 1998. Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. Nature **393**: 464–467. doi:10.1038/30965

Moore, L. R., and S. W. Chisholm. 1999. Photophysiology of the marine cyanobacterium *Prochlorococcus*: Ecotypic differences among cultured isolates. Limnol. Oceanogr. **44**: 628–638. doi:10.4319/lo.1999.44.3.0628

Moore, L. R., and others. 2007. Culturing the marine cyanobacterium *Prochlorococcus*. Limnol. Oceanogr.: Methods **5**: 353–362. doi:10.4319/lom.2007.5.353

Morris, J. J., R. Kirkegaard, M. J. Szul, Z. I. Johnson, and E. R. Zinser. 2008. Facilitation of robust growth of *Prochlorococcus* colonies and dilute liquid cultures by "helper" heterotrophic bacteria. Appl. Environ. Microbiol. **74**: 4530–4534. doi:10.1128/AEM.02479-07

Morris, J. J., Z. I. Johnson, M. J. Szul, M. Keller, and E. R. Zinser. 2011. Dependence of the cyanobacterium *Prochlorococcus* on hydrogen peroxide scavenging microbes for growth at the ocean's surface. PloS One **6**: e16805. doi:10.1371/journal.pone.0016805

Morris, J. J., R. E. Lenski, and E. R. Zinser. 2012. The Black Queen Hypothesis: Evolution of dependencies through adaptive gene loss. mBio **3**: e00036–12. doi:10.1128/mBio.00036-12

Mullineaux, C. W., and R. Stanewsky. 2009. The Rolex and the hourglass: A simplified circadian clock in *Prochlorococcus*? J. Bacteriol. **191**: 5333–5335. doi:10.1128/JB.00719-09

Muñoz-Marín Mdel, C., I. Luque, M. V. Zubkov, P. G. Hill, J. Diez, and J. M. García-Fernández. 2013. *Prochlorococcus* can use the Pro1404 transporter to take up glucose at nanomolar concentrations in the Atlantic Ocean. Proc. Natl. Acad. Sci. U. S. A. **110**: 8597–8602. doi:10.1073/pnas.1221775110

Olson, R. J., S. W. Chisholm, E. R. Zettler, and E. V. Armbrust. 1988. Analysis of *Synechococcus* pigment types in the sea using single and dual beam flow cytometry. Deep-Sea Res. Part Oceanogr. Res. Pap. **35**: 425–440. doi:10.1016/0198-0149(88)90019-2

Olson, R. J., S. W. Chisholm, E. R. Zettler, M. A. Altabet, and J. A. Dusenberry. 1990. Spatial and temporal distributions of prochlorophyte picoplankton in the North Atlantic Ocean. Deep-Sea Res. Part Oceanogr. Res. Pap. **37**: 1033–1051. doi:10.1016/0198-0149(90)90109-9

Palenik, B., and F. M. M. Morel. 1988. Dark production of H2O2 in the Sargasso Sea. Limnol. Oceanogr. **33**: 1606–1611. doi:10.4319/lo.1988.33.6part2.1606

Paoli, A., M. Celussi, P. Del Negro, S. Fonda Umani, and L. Talarico. 2008. Ecological advantages from light adaptation and heterotrophic-like behavior in *Synechococcus* harvested from the Gulf of Trieste (Northern Adriatic Sea). FEMS Microbiol. Ecol. **64**: 219–229. doi:10.1111/j.1574-6941.2008.00459.x

Partensky, F., W. R. Hess, and D. Vaulot. 1999. *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. Microbiol. Mol. Biol. Rev. **63**: 106–127.

Regelsberger, G., and others. 2002. Occurrence and biochemistry of hydroperoxidases in oxygenic phototrophic prokaryotes (cyanobacteria). Plant Physiol. Biochem. **40**: 479–490. doi:10.1016/S0981-9428(02)01405-5

Roberts, E. C., and J. Laybourn-Parry. 1999. Mixotrophic cryptophytes and their predators in the Dry Valley lakes of Antarctica. Freshw. Biol. **41**: 737–746. doi:10.1046/j.1365-2427.1999.00401.x

Rocap, G., D. L. Distel, J. B. Waterbury, and S. W. Chisholm. 2002. Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. Appl. Environ. Microbiol. **68**: 1180–1191. doi:10.1128/AEM.68.3.1180-1191.2002

Rocap, G., and others. 2003. Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. Nature **424**: 1042–1047. doi:10.1038/nature01947

Rose, A. L., A. Godrant, A. Godrant, M. Furnas, and T. D. Waite. 2010. Dynamics of nonphotochemical superoxide production in the Great Barrier Reef lagoon. Limnol. Oceanogr. **55**: 1521–1536. doi:10.4319/lo.2010.55.4.1521

Saito, M. A., J. W. Moffett, S. W. Chisholm, and J. B. Waterbury. 2002. Cobalt limitation and uptake in *Prochlorococcus*. Limnol. Oceanogr. **47**: 1629–1636. doi:10.4319/lo.2002.47.6.1629

Scanlan, D. J., and others. 2009. Ecological genomics of marine picocyanobacteria. Microbiol. Mol. Biol. Rev. **73**: 249–299. doi:10.1128/MMBR.00035-08

Sher, D., J. W. Thompson, N. Kashtan, L. Croal, and S. W. Chisholm. 2011. Response of *Prochlorococcus* ecotypes to co-culture with diverse marine bacteria. ISME J. **5**: 1125–1132. doi:10.1038/ismej.2011.1

Shi, Y., G. W. Tyson, J. M. Eppley, and E. F. DeLong. 2011. Integrated metatranscriptomic and metagenomic analyses of stratified microbial assemblages in the open ocean. ISME J. **5**: 999–1013. doi:10.1038/ismej.2010.189

Shibl, A. A., L. R. Thompson, D. K. Ngugi, and U. Stingl. 2014. Distribution and diversity of *Prochlorococcus* ecotypes in the Red Sea. FEMS Microbiol. Lett. **356**: 118–126. doi:10.1111/1574-6968.12490

Smetacek, V. S. 1985. Role of sinking in diatom life-history cycles: Ecological, evolutionary and geological significance. Mar. Biol. **84**: 239–251. doi:10.1007/BF00392493

Sohrin, R., and others. 2011. Distribution of *Synechococcus* in the dark ocean. Aquat. Microb. Ecol. **64**: 1–14. doi:10.3354/ame01508

Steglich, C., M. Futschik, T. Rector, R. Steen, and S. W. Chisholm. 2006. Genome-wide analysis of light sensing in *Prochlorococcus*. J. Bacteriol. **188**: 7796–7806. doi:10.1128/JB.01097-06

Thompson, A. W., K. Huang, M. A. Saito, and S. W. Chisholm. 2011. Transcriptome response of high- and low-light-adapted *Prochlorococcus* strains to changing iron availability. ISME J. **5**: 1580–1594. doi:10.1038/ismej.2011.49

Urakawa, H., W. Martens-Habbena, and D. A. Stahl. 2010. High abundance of ammonia-oxidizing Archaea in coastal waters, determined using a modified DNA extraction method. Appl. Environ. Microbiol. **76**: 2129–2135. doi:10.1128/AEM.02692-09

Vermilyea, A. W., S. Paul Hansard, and B. M. Voelker. 2010. Dark production of hydrogen peroxide in the Gulf of Alaska. Limnol. Oceanogr. **55**: 580–588. doi:10.4319/lo.2010.55.2.0580

Vila-Costa, M., R. Simó, H. Harada, J. M. Gasol, D. Slezak, and R. P. Kiene. 2006. Dimethylsulfoniopropionate Uptake by Marine Phytoplankton. Science **314**: 652–654. doi:10.1126/science.1131043

Vilibić, I., and D. Šantić. 2008. Deep water ventilation traced by *Synechococcus* cyanobacteria. Ocean Dyn. **58**: 119–125. doi:10.1007/s10236-008-0135-8

Waldbauer, J. R., S. Rodrigue, M. L. Coleman, and S. W. Chisholm. 2012. Transcriptome and proteome dynamics

of a light-dark synchronized bacterial cell cycle. PloS One **7**: e43432. doi:10.1371/journal.pone.0043432

West, N. J., and D. J. Scanlan. 1999. Niche-partitioning of *Prochlorococcus* populations in a stratified water column in the Eastern North Atlantic Ocean. Appl. Environ. Microbiol. **65**: 2585–2591. Doi:10347047

White, A. W. 1974. Growth of two facultatively heterotrophic marine centric diatoms. J. Phycol. **10**: 292–300. doi:10.1111/j.1529-8817.1974.tb02717.x

Yuan, J., and A. M. Shiller. 2004. Hydrogen peroxide in deep waters of the North Pacific Ocean. Geophys. Res. Lett. **31**: L01310. doi:10.1029/2003GL018439

Zepp, R. G., Y. I. Skurlatov, and J. T. Pierce. 1987. Algal-induced decay and formation of hydrogen peroxide in water: Its possible role in oxidation of anilines by algae, p. 215–224. In R. G. Zika and W. J. Cooper [eds.], Photochemistry of environmental aquatic systems. American Chemical Society.

Zhu, A., R. Romero, and H. R. Petty. 2010. A sensitive fluorimetric assay for pyruvate. Anal. Biochem. **396**: 146–151. doi:10.1016/j.ab.2009.09.017

Zinser, E. R., A. Coe, Z. I. Johnson, A. C. Martiny, N. J. Fuller, D. J. Scanlan, and S. W. Chisholm. 2006. *Prochlorococcus* ecotype abundances in the North Atlantic Ocean as revealed by an improved quantitative PCR method. Appl. Environ. Microbiol. **72**: 723–732. doi:10.1128/AEM.72.1.723-732.2006

Zinser, E. R., Z. I. Johnson, A. Coe, E. Karaca, D. Veneziano, and S. W. Chisholm. 2007. Influence of light and temperature on *Prochlorococcus* ecotype distributions in the Atlantic Ocean. Limnol. Oceanogr. **52**: 2205–2220. doi:10.4319/lo.2007.52.5.2205

Zinser, E. R., and others. 2009. Choreography of the transcriptome, photophysiology, and cell cycle of a minimal photoautotroph, *Prochlorococcus*. PloS One **4**: e5135. doi:10.1371/journal.pone.0005135

Zubkov, M. V., B. M. Fuchs, G. A. Tarran, P. H. Burkill, and R. Amann. 2003. High rate of uptake of organic nitrogen compounds by *Prochlorococcus* cyanobacteria as a key to their dominance in oligotrophic oceanic waters. Appl. Environ. Microbiol. **69**: 1299–1304. doi:10.1128/AEM.69.2.1299-1304.2003

Zubkov, M. V., G. A. Tarran, and B. M. Fuchs. 2004. Depth related amino acid uptake by *Prochlorococcus* cyanobacteria in the Southern Atlantic tropical gyre. FEMS Microbiol. Ecol. **50**: 153–161. doi:10.1016/j.femsec.2004.06.009

Zubkov, M. V., and Tarran, G. A. 2005. Amino acid uptake of *Prochlorococcus spp.* in surface waters across the South Atlantic Subtropical Front. Aquat. Microb. Ecol. **40**: 241–249. doi:10.3354/ame040241