Survival of Prochlorococcus in extended darkness

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

| Citation | Coe, Allison et al. “Survival of Prochlorococcus in Extended Darkness.” Limnology and Oceanography 61, 4 (May 2016): 1375–1388 © 2016 Association for the Sciences of Limnology and Oceanography |
| As Published | http://dx.doi.org/10.1002/lno.10302 |
| Publisher | Association for the Sciences of Limnology and Oceanography |
| Version | Author's final manuscript |
| Accessed | Sun Oct 28 02:29:38 EDT 2018 |
| Citable Link | http://hdl.handle.net/1721.1/111625 |
| Terms of Use | Creative Commons Attribution-Noncommercial-Share Alike |
| Detailed Terms | http://creativecommons.org/licenses/by-nc-sa/4.0/ |
Survival of Prochlorococcus in extended darkness

Allison Coe*, Julie Ghizzoni, Kristen LeGault, Steven Biller, Sara E. Roggensack, Sallie W. Chisholm*

Department of Civil and Environmental Engineering, Massachusetts Institute of Technology,
Cambridge, MA 02139, USA

* Correspondence: S.W. Chisholm, Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Bldg. 48-419, Cambridge, MA 02139, USA. Email: chisholm@mit.edu; A.Coe, Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Bldg. 48-305, Cambridge, MA 02139, USA. Email: a_coe@mit.edu

Running title: Prochlorococcus in extended darkness
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 hours 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 days. 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.
Introduction

Found primarily throughout the ocean’s euphotic zone between 45°N and 40°S, *Prochlorococcus* is the smallest (<1µm) 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; N. Jiao 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) 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; N. Jiao 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 300m in the Suruga Bay (Sohrin et al. 2011) have been observed to maintained their cell density for 30 days 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; Zubkov, Mikhail V. and Tarran, Glen A 2005; Malmstrom et al. 2005; Paoli et al. 2008) and appears to be widespread in the global oceans (Yelton et al submitted). 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
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; Mary et al. 2008; Gómez-Baena 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 0.2µm filtered sterile Sargasso Sea water amended with Pro99 nutrients prepared as previously described (Moore et al. 2007). *Prochlorococcus* cells starting at a concentration of \(5 \times 10^6\) to \(1 \times 10^7\) 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
mid-day, remaining at peak light for 4 hours, and then decreasing light to sunset over the course of 13 hours. 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 hours of their last “natural” L:D cycle, these cultures were in the dark for a total of 35, 59, 83, 107, 179, or 275 hours, which amounts to an additional 1, 2, 3, 4, 7, or 11 days 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, CA) 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, CA) 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 $5 \times 10^5$ to $1 \times 10^6$ cells mL$^{-1}$ were added at the onset of the experiments and maintained approximately 10 fold lower abundance than Prochlorococcus during exponential growth. 5 mM stock solutions of glucose and sodium pyruvate (99% ultrapure, Sigma Aldrich, St. Louis, MO) were prepared with Type I ultrapure water and sterilized with a 0.2µm Supor syringe filter (Pall Corporation, Port Washington, NY) 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, NJ, USA) with 2µm blue and red excited reference beads. Cells were excited with a blue 488nm laser analyzed for chlorophyll fluorescence (692/40nm), SYBR Green I stained DNA fluorescence content (530/40nm), 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, PA, USA) as previously described (Olson et al. 1990). Co-culture samples were also stained with 1x SYBR Green I (Invitrogen, Grand Island, NY) and incubated for 10min 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 1000m depths were extracted from frozen filters (100mL raw seawater filtered onto a 0.2µm polycarbonate filter) using a phenol:chloroform based method (Urakawa et al. 2010), and 1ng of DNA was used to generate sequencing libraries using the Nextera XT DNA library prep kit (Illumina, San Diego, CA). Libraries were sequenced on the Illumina NextSeq platform at the MIT BioMicro center, yielding ~20-30 million 150+150nt 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 1µM SYTOX Green (Invitrogen, Carlsbad, CA) to the cells for 10 minutes 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 hour. Cells were excited with a blue 488nm laser and analyzed for chlorophyll fluorescence (692/40nm) and size (forward scatter). Using events within the Prochlorococcus defined gate, dead cells were determined by examining SYTOX Green content (580/30nm) 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 50µM (instead of 100µM as suggested). All Prochlorococcus cultures were grown in a medium buffered with 3.75mM 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 0.22µm Multiscreen HTS GV sterile filter plate (MSGVS2210, EMD Millipore, Billerica, MA). The filtrate was collected and transferred to black walled plates with H₂O₂ standard curves (0-2.5µm), 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-H₂O₂ control by adding 1mM sodium pyruvate (a
H₂O₂ 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 (Bunton, 1949;
Holleman, 1904), 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-
H₂O₂ 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 530/25nm excitation and 590/20nm 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; N. Jiao 2013; Shibl et al. 2014),
we found Prochlorococcus and Synechococcus across a gradient of depths up to 1000m at station
ALOHA in the Pacific, as determined by both flow cytometric and metagenomic approaches.
Prochlorococcus and Synechococcus concentrations were relatively consistent between 600 and
1000m depth, at 3-4x10³ and 2-6x10² cells mL⁻¹, respectively (Table S1). These deep
populations were dominated by HL adapted Prochlorococcus ecotypes (Table S1) as was
observed for deep water samples from the Luzon Straight off China (N. Jiao 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 1000m might be and to address this indirectly, we
subjected a *Prochlorococcus* MED4 culture to complete darkness for 200 days, and measured its
flow cytometry signature over this period (Fig. 1). Cell abundance remained roughly the same
during the entire experiment (Fig. 1 a), but the relative chlorophyll per cell and cell size (as
inferred from forward scatter) decreased gradually (Fig. 1 b, c). Flow cytometry dot plots after
197 days in the dark revealed a population that remained well above the noise level and
recognizable as an intact population (Fig. 1 d). 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 hours (1, 2, and 3 extra days of darkness beyond their 11 hour 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 hours of darkness, but none were able to after 59 or 83 hours
in the dark. The 3 strains unable to tolerate even 35 hours 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 hours 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 oxidative 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
reintroduced 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 (Fig. S1), survival of
MED4 (a HL –adapted strain) and NATL2A (a LL- adapted strain) in the dark was significantly
enhanced by its presence (Fig. 2 a,d and b,e respectively). When alone, Prochlorococcus MED4
was unable to survive even 35 hours of continuous darkness (Fig. 2 a), but in co-culture it could
survive an additional 6 days (Fig. 2 d). Similarly, Prochlorococcus NATL2A was able to
survive an additional 10 days of darkness when with Alteromonas (Fig. 2 e).

For MIT9313 (LLIV clade), the presence of Alteromonas extended the dark survival time from
35 to 83 hours, but could not mediate recovery for longer dark periods (Fig. 2 c,f). Furthermore,
its recovery was much slower relative to that of MED4 and NATL2A for the same period of
darkness (Fig. 2 d,e,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, JW
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$_2$O$_2$ 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$_2$O$_2$ could explain the ability of Alteromonas to partially mitigate the
stress of prolonged darkness for Prochlorococcus.
We measured hydrogen peroxide levels in MED4 (Fig. 3 a-d) and NATL2A (Fig. 3 e-h) cultures during extended darkness and upon re-exposure to light, with and without additions of *Alteromonas* (Fig. 3 a-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 hours in the dark (Fig. 3 a,b); NATL2A cells were able to tolerate 35 hours of darkness but no longer (Fig. 3 e-f). In all the untreated cultures that were unable to recover, hydrogen peroxide concentrations peaked 3-4 days after the cells were placed in the dark (Fig. 3 e,f,h). In the untreated NATL2A culture subjected to 35 hours of darkness (Fig. 3 g) that did recover, hydrogen peroxide levels remained near media background levels throughout. In the 83 hour dark treatment of both strains, hydrogen peroxide concentration increased before the cells were re-introduced into the light (Fig. 3 f, 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 3 e-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 3. a-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. 3 a-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\textsubscript{2}O\textsubscript{2} in all the cultures to zero (Fig. 3 a-d, green lines).

The presence of pyruvate in the Prochlorococcus NATL2A cultures extended their dark survival times from 35 to 83 hours (Fig. 3 c,d), mimicking the effect of Alteromonas. While the addition of pyruvate allowed MED4 to survive 35 hours 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. 3 a). Furthermore, Alteromonas could facilitate MED4’s survival of 83 hours of darkness, while pyruvate could not (Fig. 3b). This suggests that its role in promoting dark survival does not lie solely in H\textsubscript{2}O\textsubscript{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 5mM glucose and subjected cultures to 59, 83, or 107 hours of darkness (Fig. 4 a-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. 4 a); with glucose they can withstand 59 hours in the dark, but no longer (Fig. 4
b). When we added pyruvate to the cultures in addition to the glucose, the cells recovered even
after 83 hours of darkness (Fig. 4 d). MED4 cells kept in the dark for 197 days in the presence of
glucose and pyruvate, however, also 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 Figure 1 a-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 and reduction of oxidative
stress which facilitates survival and recovery after extended darkness. In an independent
experiment, we examined whether results seen in Figure 4 with MED4 were consistent with
another Prochlorococcus strain, NATL2A, and indeed the combination of glucose and pyruvate
enabled all cultures to recover (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 interpret 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 hours 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
cultures (Fig. 5 a-b, blue line), the total *Prochlorococcus* cell abundance remained constant
over the whole experiment (Fig. 5 a-b, black line); however, only *Prochlorococcus* in the co-
cultures with *Alteromonas* recovered (Fig. 5 b). The abundance patterns of *Alteromonas*
mimicked that of *Prochlorococcus*, at 10 fold lower concentration throughout dark and recovery
time periods (Fig. 5 b, 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. 5 c-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. 5 a), the mean fluorescence per cell of the entire population decreased steadily
upon re-introduction to the light (Fig. 5 c-f), even though total cell numbers remained constant
(black line, Fig. 5 a). In the presence of *Alteromonas* the population trajectory was similar to that
of *Prochlorococcus*- alone cultures for 14 days after reintroduction to the light, but in this case a
population of *Prochlorococcus* cells appeared after 38 days with fluorescence and light scatter
values identical to those observed after 3 days in the dark (compare Fig. 5 g and j, circled). We
hypothesize that a very small subpopulation of cells began growing upon re-exposure to light and
only became detectable after 38 days. 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. 5 b). 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 samples, not from the exact same populations identified in Fig 5 g-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 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 (Morris et al. 2011; Hughes 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 (Fig. S3 a); 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’ (Fig. S3 b); the glutaraldehyde-killed cells excluded the stain, as would be expected for live cells (Fig. S2 b, 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 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 days (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 hours 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 hours 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 days. 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 hours 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


doi:10.1177/0748730408316040

Huang, S., S. W. Wilhelm, H. R. Harvey, K. Taylor, N. Jiao, and F. Chen. 2012. Novel lineages of
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).


Niche partitioning among Prochlorococcus ecotypes along ocean-scale environmental gradients.
Science 311: 1737–1740. doi:10.1126/science.1118052


Lebaron, P., P. Catala, and N. Parthuisot. 1998. Effectiveness of SYTOX Green Stain for Bacterial


Chisholm. 2010. Temporal dynamics of Prochlorococcus ecotypes in the Atlantic and Pacific
oceans. ISME J. 4: 1252–1264. doi:10.1038/ismej.2010.60

assimilation by Synechococcus in the Gulf of Mexico and northwest Atlantic Ocean. Limnol.


Shibl, A. A., L. R. Thompson, D. K. Ngugi, and U. Stingl. 2014. Distribution and diversity of 
doi:10.1111/1574-6968.12490

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

Smetacek, V. S. 1985. Role of sinking in diatom life-history cycles: ecological, evolutionary and 


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

Archaea in Coastal Waters, Determined Using a Modified DNA Extraction Method. Appl. 

Vermilyea, A. W., S. Paul Hansard, and B. M. Voelker. 2010. Dark production of hydrogen peroxide in 

doi:10.1126/science.1131043


Acknowledgements

The authors thank the members of the Chisholm Lab for support and comments, especially Jessica Berta-Thompson for her valuable input. The authors would also like to thank Keven Dooley, Anna Rasmussen, Chris Marx, Lisa Moore, Jose Cruz, and Meghan O’Dell for help with obtaining preliminary data, and Erik Zinser and the HOT crew for collecting samples from the HOT 152 cruise. This research was supported by grants from the Gordon and Betty Moore Foundation (grant GBMF495), Simons Foundation (LIFE-337262), and the National Science Foundation (OCE-1153588, OCE-1356460 and DBI-0424599, the NSF Center for Microbial Oceanography Research and Education).
Figure 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 days of darkness) reveals that the population maintained its distinct flow cytometric signature, slightly shifted, even after 197 days in darkness. Populations in the upper right corner (d) are 2µm diameter internal standard beads. Error bars represent the standard deviation from triplicate samples and are smaller than the data points when not visible.
Figure 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 co-cultured 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 hours beyond the normal ‘night’ (for a total of 35, 83, 179, or 275 hours 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 days 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 two months, but no growth was observed. Note that both axes are on a log scale.
Figure 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 5mM sodium pyruvate and were placed into the dark (indicated by gray shading) for 35 (a, c, e, g) or 83 (b, d, f, h) hours 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).
Figure 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 or 83 hours 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 2 (a-f) and 3 (a-d).
Figure 5. Physiological changes of *Prochlorococcus* MED4 compared to the same strain in co-culture with *Alteromonas* after 83 hours of dark exposure. Cultures were grown with and without *Alteromonas* and placed into the dark for 83 hours (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.
Figure 6. Summary of the factors influencing survival and regrowth of *Prochlorococcus* MED4ax cultures (green ovals) after 83hr 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). \( \text{H}_2\text{O}_2 \) scavengers (red) indicate extracellular quenching of \( \text{H}_2\text{O}_2 \). 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 \( \text{H}_2\text{O}_2 \) concentrations are lowered by the addition of pyruvate, and a source of carbon (glucose) is supplied, *Prochlorococcus* is able to regrow after 83 hours 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.
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 hours (for a total of 35, 59, or 83 hours) 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clade</th>
<th>Growth following time in dark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>35hr</td>
</tr>
<tr>
<td>MED4</td>
<td>HLI</td>
<td>-</td>
</tr>
<tr>
<td>MIT9202</td>
<td>HLII</td>
<td>-</td>
</tr>
<tr>
<td>MIT9301</td>
<td>HLII</td>
<td>+</td>
</tr>
<tr>
<td>AS9601</td>
<td>HLII</td>
<td>+</td>
</tr>
<tr>
<td>MIT9312</td>
<td>HLII</td>
<td>+</td>
</tr>
<tr>
<td>NATL2A</td>
<td>LLI</td>
<td>+</td>
</tr>
<tr>
<td>NATL1A</td>
<td>LLI</td>
<td>+</td>
</tr>
<tr>
<td>MIT9211</td>
<td>LLII/III</td>
<td>-</td>
</tr>
<tr>
<td>MIT9303</td>
<td>LLIV</td>
<td>+</td>
</tr>
<tr>
<td>MIT9313</td>
<td>LLIV</td>
<td>+</td>
</tr>
<tr>
<td>WH8102</td>
<td>III, 5.1A</td>
<td>+</td>
</tr>
<tr>
<td>WH7803</td>
<td>V, 5.1B</td>
<td>+</td>
</tr>
</tbody>
</table>
Supplemental Table 1. Presence of *Prochlorococcus* and *Synechococcus* in deep water samples from the Pacific Ocean (Station ALOHA, October 2003). Cell concentrations were quantified by flow cytometry. Metagenomic sequencing of samples from the same depths confirmed the presence of both HL- and LL-adapted *Prochlorococcus* DNA below the euphotic zone. The *Prochlorococcus* and *Synechococcus* % values represent the percent of total reads mapping to *Prochlorococcus* and *Synechococcus* genomes. Data for the % of the total *Prochlorococcus* reads mapping to HL- or LL-adapted genomes is shown in the HL or LL *Prochlorococcus* % columns.

<table>
<thead>
<tr>
<th>Dept h (m)</th>
<th>Flow cytometry data</th>
<th>Metagenomic data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synechococcus s cells mL⁻¹</td>
<td>Prochlorococcus s cells mL⁻¹</td>
</tr>
<tr>
<td>50</td>
<td>530</td>
<td>98644</td>
</tr>
<tr>
<td>600</td>
<td>220</td>
<td>3160</td>
</tr>
<tr>
<td>1000</td>
<td>633</td>
<td>3745</td>
</tr>
</tbody>
</table>
Supplemental Figure 1. Abundance of *Prochlorococcus* grown alone, and in co-culture with *Alteromonas*. Cultures of MED4 (a), NATL2A (b), and MIT9313 (c) were grown with and without *Alteromonas*. The lack of change in abundance of both *Prochlorococcus* was determined by flow cytometry.
Supplemental Figure 2. Response of *Prochlorococcus* low-light (LL)-adapted strain NATL2A in the presence of 5mM glucose and 5mM sodium during extended dark exposure. Cultures were placed into the dark (solid grey bar) for 107 hours before re-exposure to light. 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 2 (a-f) and 3 (a-d).
Supplemental Figure 3. Fraction of *Prochlorococcus* NATL2A cells identified as ‘viable’ by the SYTOX-Green assay, which uses a fluorescent stain that is expected to be impermanent to live cells but which can permeate compromised membranes (a characteristic of dead cells). All cultures were grown on a 13:11 L:D cycle, which was maintained in the control cultures (a) while the experimental cultures were placed in constant darkness (b) after 7 days. Dark periods are shown as grey shading.