

1 ***Prochlorococcus* rely on microbial interactions rather than on chlorotic resting stages to**
2 **survive long-term stress**

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14

15 **Abstract**

16 Many microorganisms produce resting cells with very low metabolic activity that allows them to survive
17 phases of prolonged stress conditions. Using axenic lab cultures, we show that *Prochlorococcus*, the
18 dominant phytoplankton lineage in large regions of the nutrient-poor ocean, cannot survive extended
19 nutrient starvation alone. Under starvation conditions some cells retain metabolic activity, measured as
20 single-cell C and N uptake, but these cultures do not re-grow when transferred into new media.
21 Nevertheless, co-cultures with a heterotrophic bacterium enabled *Prochlorococcus* to survive nutrient
22 starvation for months. We extend these observations to natural conditions, suggesting that up to 10% of
23 the *Prochlorococcus* cells in the oceans live under conditions of light starvation, utilizing organic matter
24 produced by other organisms. We propose that reliance on co-occurring heterotrophic bacteria or on
25 the organic matter they produce, rather than the ability to survive extended starvation as resting cells,
26 underlies the ecological success of *Prochlorococcus*.

27

28 **Introduction**

29 Not all microbial cells living in natural environments are equally active. In aquatic environments, up to
30 90% of the cells do not exhibit measurable metabolic activity (“vitality”), based on dyes (e.g. that assess
31 electron transport) or on uptake assays with labeled substrates (Del Giorgio and Gasol 2008). Several

32 possible and non-exclusive explanations have been proposed for this heterogeneity. First, inherent
33 differences in activity between genetically-different organisms, e.g. due to variations in maximum
34 growth rate or the ability to utilize the specific substrate tested. Second, cells might be at different
35 physiological states, e.g. exponentially growing, starved or dying, and thus less active
36 metabolically (Anderson et al. 2015, Jørgensen et al. 2015, Bergkessel et al. 2016). Third, cells show
37 stochastic fluctuations in their activity, due to noise in gene expression or regulatory networks (Engl
38 2018). Finally, some organisms respond to environmental stress by producing resting stages or spores.
39 Such cells often exhibit very low (or undetectable) metabolic activity, yet are viable, namely able to
40 return to an active state and reproduce when environmental conditions return to favorable (Harms et al.
41 2016). The presence of such resting stages, together with a fluctuating activity at the single-cell level and
42 the genetic variability found within natural populations, are suggested to promote the survival of the
43 population as a whole (Lennon and Jones 2011, Bergkessel et al. 2016) .

44 Understanding the factors affecting the metabolic activity (vitality) of phytoplankton is of special
45 interest. These microbial primary producers perform about one-half of the photosynthesis on Earth,
46 providing energy through carbon fixation at the base of the aquatic ecosystem. At the same time, low
47 nutrient concentrations due to uptake by phytoplankton may constrain the growth of co-occurring
48 organisms. Phytoplankton viability, e.g. their ability to survive under conditions of nutrient stress, has
49 also been extensively studied, especially for organisms that produce massive blooms that emerge and
50 decline rapidly. For example, some bloom-forming cyanobacteria such as *Aphanizomenon* species
51 produce morphologically-distinct spores that show very little photosynthetic activity, yet remain viable
52 in the sediment for long periods of time, providing the inoculum for the next growth season (Sukenik et
53 al. 2015). In laboratory cultures of *Synechococcus elegantus* PCC7942 and *Synechocystis* PCC6803, two
54 unicellular freshwater cyanobacteria, nitrogen starvation results in a programmed process where cells
55 enter a resting stage, enabling them to survive prolonged periods of stress (Sauer et al. 2001, Klotz et al.
56 2016). As part of this process, cells degrade their photosynthetic apparatus in a controlled manner,
57 resulting in a loss of chlorophyll autofluorescence and culture bleaching (a process termed chlorosis).
58 However, the observation that chlorotic cells are viable resting stages is not universal. Chlorotic cultures
59 of *Microcystis aeruginosa* PCC 7806 were shown to contain a small population of non-chlorotic cells with
60 high chlorophyll autofluorescence (described throughout this study as “high-fl”). Only these high-fl cells
61 were suggested to revive after the re-addition of a nitrogen source, while the low-fl cells are presumably
62 dead (de Abreu Meireles et al. 2015). Chlorotic cells were also observed in eukaryotic phytoplankton

63 albeit it is not yet clear to what extent such cells remain viable as it may depend on the specific
64 organism and stress conditions (Behrenfeld and Falkowski 1997, Franklin et al. 2006).

65 *Prochlorococcus* is a pico-cyanobacterium that is extremely abundant in the oligotrophic
66 oceans, performing an estimated ~8.5% of global ocean photosynthesis (Flombaum et al. 2013), and
67 providing fixed carbon for up to 75% of the co-occurring heterotrophic community (Ribalet et al. 2015).
68 *Prochlorococcus* cells in the oceans exhibit extremely high genetic diversity (Kashtan et al. 2014), and
69 some of this diversity has been linked with their ability to grow under conditions of extreme nutrient
70 limitation (e.g. Martiny et al. 2006, Thompson et al. 2011). It has therefore been suggested that this
71 genetic diversity enables *Prochlorococcus* as a group to thrive across a wide variety of oceanic conditions
72 (Biller et al. 2014). While the physiological and transcriptional responses of multiple *Prochlorococcus*
73 lineages to short-term nutrient starvation have been extensively studied (e.g. Steglich et al. 2001, Martiny
74 et al. 2006, Tolonen et al. 2006, Thompson et al. 2011, Krumhardt et al. 2013), little is known about their
75 ability to survive more than a few days under such conditions. A recent study on the response of
76 *Prochlorococcus* strains to extended darkness (i.e. C starvation) has shown that these organisms can
77 survive light starvation only for a limited time (Coe et al. 2016). In these experiments, low-fl cell
78 populations reminiscent of chlorotic cells in other cyanobacteria appear after the light-starved cultures
79 were re-exposed to light, regardless of whether these cultures could continue growing (Coe et al. 2016).
80 We therefore asked: i) Do *Prochlorococcus* respond to long-term nutrient starvation by producing
81 chlorotic cells? ii) If so, are such cells metabolically active (vital) and are they able to reproduce and
82 grow when stress conditions end (viable)? iii) Can chlorotic *Prochlorococcus* cells be observed in nature?
83 To address these questions, we used fluorescence-activated cell sorting (FACS) to obtain distinct
84 chlorotic sub-populations from axenic and unialgal laboratory cultures of *Prochlorococcus* which were
85 pre-incubated with isotopically-labelled tracers for photosynthesis (H^{13}CO_3) and nutrient uptake ($^{15}\text{NH}_4^+$)
86 and we visualized their activity using Nanoscale Secondary Ion Mass Spectrometry (NanoSIMS). This
87 method enabled us to measure photosynthesis and N uptake at a single cell resolution by quantifying
88 the change in isotopic ratios (Gao et al. 2016, Berthelot et al. 2018). Our results show that while
89 *Prochlorococcus* do undergo a chlorosis-like process, with some of the chlorotic cells still
90 photosynthesizing and taking up NH_4^+ , the chlorotic cells are unable to re-grow and thus do not
91 represent resting stages. Instead, co-culture with heterotrophic bacteria in the lab and mixotrophy in
92 the ocean, two aspects of microbial interactions, enable *Prochlorococcus* to survive long-term stress
93 even without producing resting stages.

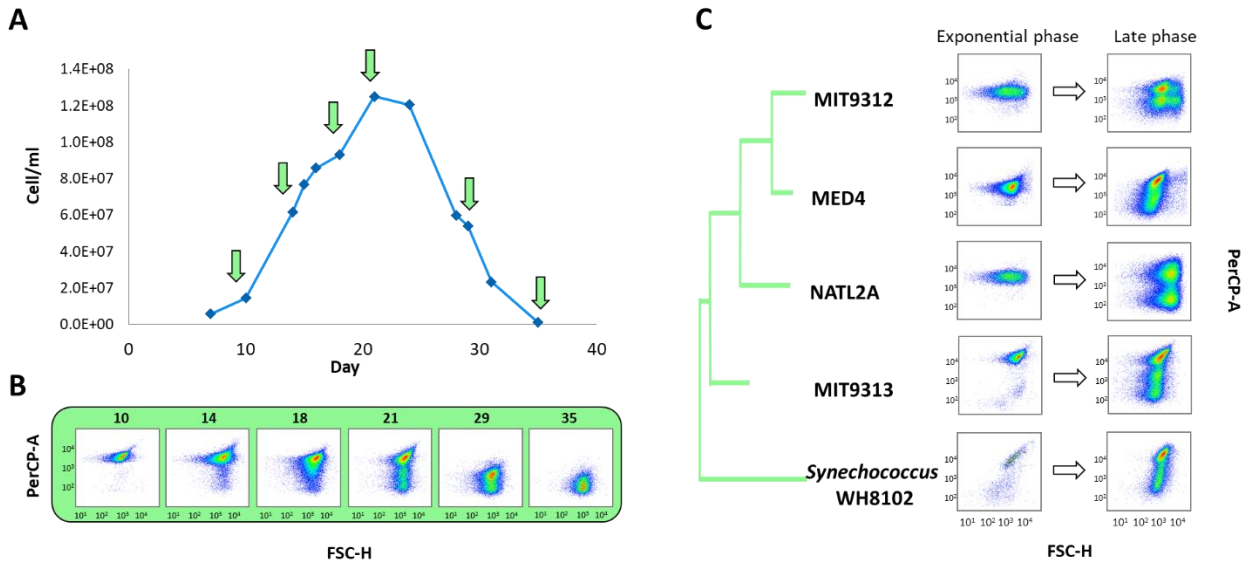
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95 **Results and discussion**

96 **Emergence of chlorotic sub-populations in *Prochlorococcus* cultures**

97 As *Prochlorococcus* batch cultures reach stationary stage and start declining, the green color of the
98 cultures disappears, and sub-populations of cells emerge with lower chlorophyll autofluorescence that
99 can be identified by flow cytometry (Figure 1A, B). This phenomenon is observed in strains from all
100 major cultured ecotypes, as well as in *Synechococcus* WH8102 (Figure 1C). In *Prochlorococcus* strain
101 MIT9312, lower chlorophyll populations emerged in batch cultures that reached stationary stage due to
102 both N and P limitation, although the timing of sub-population emergence and the forward light scatter
103 and chlorophyll autofluorescence (analyzed by flow cytometry) were different (Supplementary Fig. 1A,
104 B). Cells with lower chlorophyll autofluorescence also appeared in populations of another strain,
105 MIT9313, when these cultures were inhibited in a co-culture with high cell densities of the heterotrophic
106 bacterium *Alteromonas* HOT1A3 (Supplementary Fig. 1C, D (Aharonovich and Sher 2016)). Thus, the
107 emergence of populations of cells with lower chlorophyll autofluorescence under a variety of stress
108 conditions is a pervasive phenomenon across marine pico-cyanobacteria. We therefore focused on
109 *Prochlorococcus* strain MIT9313, which has been extensively studied (e.g. (Rocap et al. 2003, Martiny et
110 al. 2006, Tolonen et al. 2006, Thompson et al. 2011, Voigt et al. 2014)), as in this strain three clearly
111 separate sub-populations can be observed (Figure 1B, referred to throughout the study as high-, mid-
112 and low-fl populations).

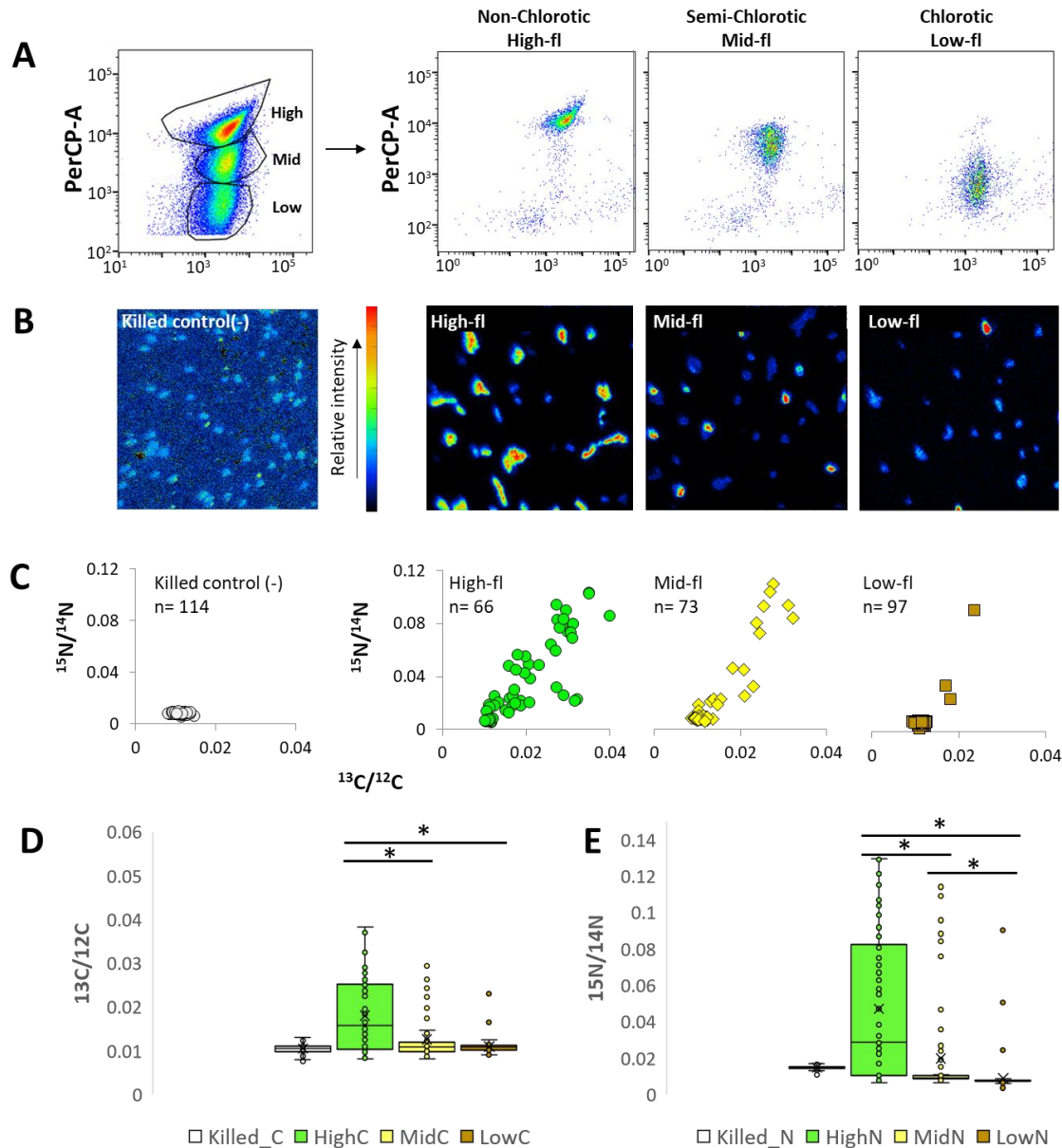
113 In addition to differing in their chlorophyll autofluorescence, the high-, mid- and low-fl cell
114 populations also differ by their forward and side light scatter properties, which are related to cell size
115 and (in larger cells) morphological complexity (Supplementary Fig 2A, B). In agreement with these
116 observations, cells sorted from the high-fl population and observed by SEM (Scanning Electron
117 Microscopy) were larger than those from the mid- and low-fl populations (Sup Fig 2C, D).



118 **Figure 1. Emergence of chlorotic sub-populations in *Prochlorococcus* batch cultures as measured by**
 119 **flow cytometry.** A) A representative growth curve of MIT9313. The arrows mark the days shown in
 120 panel B. B) Flow cytometry scattergrams at the marked time-points from the MIT9313 culture. The x-axis
 121 is Forward Scatter (FSC, a proxy for cell size), the y-axis is the chlorophyll autofluorescence of the cells
 122 (Per-CP). The emergence of chlorotic sub-population observed from the late exponential phase (Day 18).
 123 C) Chlorotic sub-population observed in ageing batch cultures of *Prochlorococcus*, belonging to different
 124 ecotypes: High-Light adapted MED4 (HLI), MIT9312 (HLII) and Low-Light adapted NATL2A (LLI) and
 125 MIT9313 (LLIV). In all strains, the chlorotic cells begin to emerge at late exponential stage, becoming
 126 dominant in declining cultures, while in the exponential phase only one population can be observed.

127 **Assessing the metabolic activity of sorted chlorotic sub-populations**

128 We next asked whether the high, mid- and low-fl populations differ in their vitality, measured here as
 129 their photosynthesis and nutrient uptake rates (incorporation of $\text{H}^{13}\text{CO}_3^-$ and $^{15}\text{NH}_4^+$, respectively). The
 130 uptake ratio of labeled versus unlabeled nutrients were then used to calculate the metabolic activity of
 131 the sorted cells (Table 1). As shown in Figure 2 and Table 1, the mean uptake of both $\text{H}^{13}\text{CO}_3^-$ and $^{15}\text{NH}_4^+$
 132 was highest in the high-fl population, followed by the mid and low-fl populations, with the latter
 133 population indistinguishable from the control, i.e. glutaraldehyde-killed cells. We have repeated the
 134 entire workflow in an independent experiment, and the results are in striking correspondence
 135 (Supplementary Fig. 3, Table 1).



136 **Figure 2. Metabolic activity of sorted sub-populations by NanoSIMS** A) Flow cytometry scatterplots
 137 before and after sorting of three distinct sub-populations (high, mid and low-fl) of an aging
 138 *Prochlorococcus* MIT9313 culture, detected by flow cytometry. The cultures were grown for 30 days in
 139 Pro99 and labeled with $\text{H}^{13}\text{CO}_3^-$ and $^{15}\text{NH}_4^+$ for 18h. B) NanoSIMS images of $^{15}\text{N}/^{12}\text{C}$ analysis of killed cells
 140 (negative control) and high, mid and low-fl cells after sorting. C) Scatterplot of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios
 141 obtained from NanoSIMS analysis of each sub-population. D, E) Boxplots of the $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$
 142 enrichment in each sub-population. Lines represent the median, X represents the mean, box borders are
 143 1st quartiles and whiskers represent the full range.

Strain and growth stage	Sub-population	Illumination	V^C (fg cell ⁻¹ day ⁻¹)	V^N (fg cell ⁻¹ day ⁻¹)
Batch culture				
MIT9313, exponential growth*	High	Constant light 27 μ E	12.92 \pm 11.93	2.74 \pm 2.43
	High		2.67- 2.77 \pm 2.89- 3.57	0.62- 0.69 \pm 0.54- 0.63
MIT9313, old cultures **	Mid	Constant light 27 μ E	0.75- 0.79 \pm 1.73- 1.78	0.25- 0.32 \pm 0.42- 0.35
	Low		0.09- 0.14 \pm 0.52- 0.67	0.08- 0.14 \pm 0.15- 0.12
	High	Photo-period	1.01 \pm 2.67	0.32 \pm 0.33
MIT9313, old culture***	Mid	12:12 L/D, 27 μ E	0.48 \pm 2.47	0.20 \pm 0.20
	Low		0.13 \pm 0.31	0.14 \pm 0.11
Natural samples				
115m	High	Natural cycle	1.04 \pm 0.6	1.17 \pm 0.20
115m	Low	approximately 14:10 L/D,	1.14 \pm 0.69	1.15 \pm 0.38
125m	High	2-5 μ E	1.21 \pm 0.89	1.28 \pm 0.21

144 **Table 1: Calculated mean C and N uptake rates from the experiments performed with *Prochlorococcus***
145 **MIT9313 and natural samples.** The means and standard deviation were calculated from the uptake rates values
146 of single cells in each experiment.

147 *The results for MIT9313 exponential growth refers to the experiment presented in Supplementary Fig. 5.

148 **The results for old MIT9313 cultures under constant light refer to the two experiments presented in Fig. 2 and
149 Supplementary Fig. 3.

150 *** The results for Old MIT9313 cultures under L/D refer to the experiment presented in Supplementary Fig. 4.

151 The mean uptake rates for glutaraldehyde killed cells (control) were 0.06 \pm 0.15 for C and 0.18 \pm 0.02 for N, and most
152 likely depict the absorption of the label by non-specific binding or diffusion. Unlabeled-unsorted cells showed C
153 values of 0.18 \pm 0.39.57 and N values of 0.41 \pm 0.14, the higher C and N values were probably due to non-sorted
154 conditions and the higher density of the cells.

155

156 Within each of the populations, cell-cell heterogeneity was observed in both ¹³C and ¹⁵N uptake
157 (Fig. 2, Supplementary Fig. 3). Within all of the populations (including the high-fl, “healthy” one), some
158 cells were inactive, and this could not be explained by the limited purity of the FACS-sorting procedure

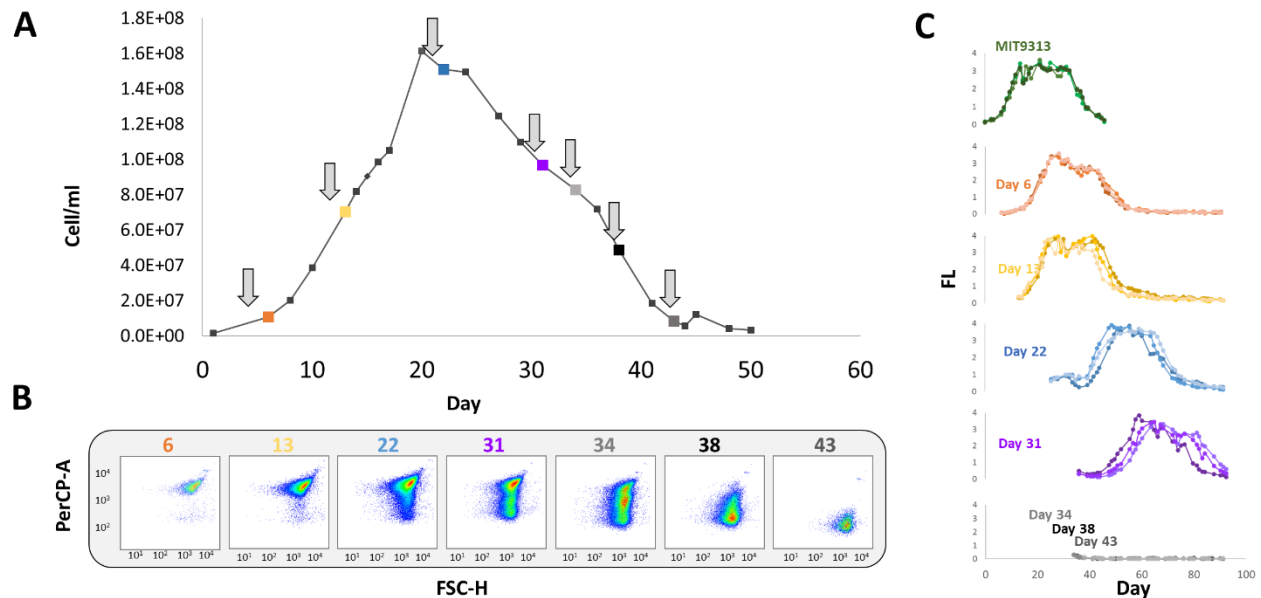
159 (Supplementary Tables 1,2). The coefficients of variation in C and N uptake rates were within the range
160 shown for other organisms, or higher (Supplementary Table 3, (Matantseva et al. 2016, Berthelot et al.
161 2018)). Similar levels of heterogeneity (primarily in N uptake) were also seen in cells grown under a
162 12:12 light-dark cycle, where the *Prochlorococcus* cell-cycle follows a diel rhythm, suggesting that this
163 heterogeneity is not due to different stages of the cell cycle or the diel cycle (Supplementary Table 3,
164 Supplementary Fig. 4). Cell-cell heterogeneity was also observed in cells from an exponentially-growing,
165 nutrient-replete culture (Supplementary Figure 5, Table 3), suggesting that this heterogeneity is not
166 exclusively limited to ageing or stressed cells. This is in accordance with studies assessing the vitality of
167 *Prochlorococcus* cells using various dyes, which consistently show that a significant fraction of the cells
168 in laboratory cultures are inactive or potentially dead (Agusti and Sanchez 2002, Hughes et al. 2011).

169 **Evaluating the viability of sub-populations**

170 To determine whether the low-fl *Prochlorococcus* cells are viable resting stages, we tested the ability of
171 cells from an MIT9313 culture to grow upon transfer to new growth media at different times during
172 exponential growth and upon culture decline. As shown in Figure 3 and Supplementary Fig. 6, only cells
173 from cultures where the high-fl cells were dominant could grow when transferred to new growth media.
174 No growth was observed upon transfer of cells from stationary or declining cultures where no high-fl
175 cells were observed. Intriguingly, the presence of high-fl cells was not enough to ensure culture growth
176 (e.g. day 34 in Figure 3). This is consistent with a previous study showing that cells belonging to a
177 different *Prochlorococcus* strain, MED4, that were incubated for three days in the dark, were unable to
178 resume growth after return to light despite showing no clear difference in the chlorophyll
179 autofluorescence (Coe et al. 2016). The probability of growth after transfer did not depend on the
180 number of transferred cells (Morris et al. 2008), with as many as 2.5×10^7 cells/ml failing to grow after
181 transfer during culture decline (cells at $\sim 1/10$ of this density grew after being transferred during
182 exponential stage). Thus, non-chlorotic cells (defined as being within the range of chlorophyll
183 autofluorescence exhibited by exponentially-growing cells) are not necessarily viable.

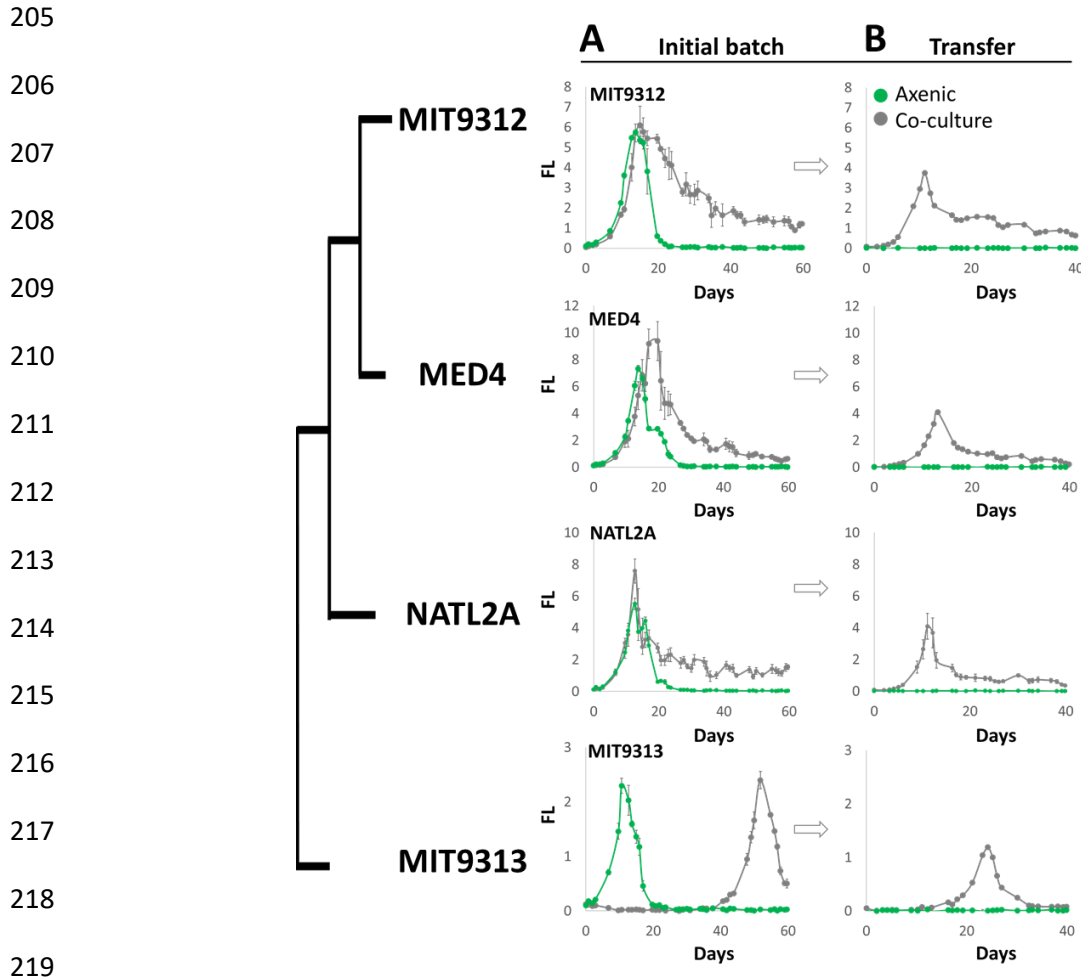
184 As shown in Figure 4, the inability to survive prolonged nitrogen starvation is not unique to
185 MIT9313, but rather is common to the four tested *Prochlorococcus* strains, covering the major cultured
186 ecotypes. This is in marked contrast to the ability of (presumably axenic) cultures of two freshwater
187 cyanobacteria, *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803, to revive after extended N
188 starvation (Sauer et al. 2001, Klotz et al. 2016). However, when co-cultured with a heterotrophic

189 bacterium, *Alteromonas* HOT1A3, all *Prochlorococcus* strains were able to re-grow after 60 days of N
190 stress. Interestingly, strain MIT9313, which was initially inhibited by this *Alteromonas* strain (Figure 4A,
191 (Sher et al. 2011, Aharonovich and Sher 2016)), was also able to survive long-term starvation in co-
192 culture, suggesting that fundamentally different interactions occur during exponential growth compared
193 to long-term, presumably nutrient-limited growth. These results are consistent with the ability of
194 heterotrophic bacteria to extend the survival time of different *Prochlorococcus* strains under conditions



195 of constant darkness (albeit for only several days, (Coe et al. 2016)) and with the ability of different
196 heterotrophic bacteria to support the long-term viability of batch cultures of *Synechococcus* WH7803
197 (Christie-Oleza et al. 2017).

198 **Figure 3: Time-dependent changes in viability of cells transferred into fresh media at different life**
199 **cycle stages of a batch culture.** A) Growth curve of an MIT9313 culture, grown in Pro99, similar to the
200 experiments shown in Figure 2 and Supplementary Fig. 3, 4 and 7. Arrows indicate the time points at
201 which triplicate 1ml samples were transferred into fresh media. B) Flow cytometry scatterplots of the
202 culture shown in panel A. C) Growth curves of cells being transferred at different times to new, nutrient-
203 replete media (assessed via bulk culture fluorescence). Cells could not re-grow when transferred after
204 day 34, suggesting that not all high-fl cells are viable, and that mid- and low-fl cells are non-viable.



220 **Figure 4: Co-culture with a heterotrophic bacterium, *Alteromonas* HOT1A3, enables multiple**
221 ***Prochlorococcus* strains to survive long-term N starvation.** Panel A: 10^6 axenic *Prochlorococcus* cells/ml
222 from different strains were incubated alone (green line) or with the addition of 10^7 *Alteromonas*
223 HOT1A3 cells/ml in low-N media (grey line). Bulk culture fluorescence was recorded as a proxy for cell
224 growth, and 1ml from each culture was transferred into fresh media after 60 days. Panel B: The
225 transferred cultures were recorded for additional 40 days. Error bars are standard deviation from
226 triplicate cultures. The late growth of MIT9313 in co-culture is the “delayed growth” phenotype
227 described in (Sher et al. 2011, Aharonovich and Sher 2016).

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231 **Exploring the metabolic activity of naturally occurring sub-populations of *Prochlorococcus* at different**
232 **depths in the eastern Mediterranean**

233 While chlorotic cells consistently emerge under nutrient starvation in all tested *Prochlorococcus* strains
234 (Fig 1), environmental conditions in laboratory cultures greatly differ from those in the oligotrophic sea.
235 For example, cell densities and nutrient concentrations are typically orders of magnitude higher in the
236 lab than in the nutrient-poor ocean. Indeed, little is known about the prevalence of chlorosis under
237 natural conditions for phytoplankton in general. Thus, we wondered whether chlorotic *Prochlorococcus*
238 cells (defined as low-fl and low C and N uptake) can be identified also in nature. We focused on the deep
239 euphotic zone (100-140m, above the nutricline) where both inorganic nutrient concentrations and light
240 availability are low. At this depth, “double” *Prochlorococcus* populations, differing by their chlorophyll
241 auto-fluorescence, have repeatedly been observed (e.g. (Campbell and Vaulot 1993, Moore et al. 1998)).
242 Previously, these double populations have been shown to contain genetically-different cells belonging to
243 the High-Light adapted (HL, low chlorophyll) and Low-Light adapted (LL, high chlorophyll) clades (Moore
244 et al. 1998), However, a recent study using genetic tools suggested that each of the double populations
245 consists of both HL and LL cells, implying that phenotypic heterogeneity (acclimation) can also
246 contribute to this phenomenon (Thompson et al. 2018). We therefore asked whether the double
247 population could also be due to the presence of chlorotic cells, e.g. if LL cells were mixed above the
248 nutricline, became exposed to nutrient starvation, and subsequently underwent chlorosis. To test this
249 hypothesis, we characterized the *Prochlorococcus* population structure and single-cell activity (carbon
250 fixation and NH_4^+ uptake) during late summer in the ultra-oligotrophic Eastern Mediterranean Sea
251 (Figure 5A, B). At the time of sampling, the water column was highly stratified and nutrients were
252 depleted down to around 130m, with a peak in NH_4^+ concentrations slightly above the nutricline (15nM
253 at 115m and 125m, Fig. 5B). *Prochlorococcus* were the numerically dominant phytoplankton (Fig 5C),
254 exhibiting a double population at 115m, but not at samples collected above or below this depth (Fig 5E).
255 Amplicon sequencing of the Internal Transcribed Spacer between the 16S and 23S genes (ITS,
256 (Thompson et al. 2018)) revealed that between ~100-137m LL cells (belonging primarily to the LL-I and
257 LL-IV clades) gradually replace the HL cells, consistent with previous studies (e.g. (Malmstrom et al.
258 2010)) but contrasting with the sharp delineation between high-chlorophyll and low-chlorophyll
259 *Prochlorococcus* populations (Fig. 5E, (Thompson et al. 2018)).

260 Mean $\text{H}^{13}\text{CO}_3^-$ and $^{15}\text{NH}_4^+$ uptake rates for the majority of the cells sorted from each of the
261 double *Prochlorococcus* populations at 115m depth were not statistically different, nor were they

262 different from cells collected at greater water depths (Figure 5. F, G, Kruskal-Wallis test, $p=0.7$ for C and
263 $p=0.07$ for N). Essentially all of the cells from the Eastern Mediterranean were active, although we did
264 observe some cells with lower C and N uptake rates in the low-fl population at 115m depth (6/45 cells,
265 Figure 5F). This contrasts with the observation from our lab experiments, where inactive cells were
266 observed in all populations, and formed the majority of the cells in the chlorotic (mid- and low-fl)
267 populations. The observation that essentially all of the *Prochlorococcus* cells in natural samples are
268 active is consistent with a similar study in the North Pacific (Berthelot et al. 2018).

269 At a depth of 115m light intensity was low (5-6 μ E during the afternoon), potentially enough to
270 support the growth of some LL strains but not sufficient for active growth under laboratory conditions of
271 most HL strains, including those that are present based on the ITS sequences (Moore and Chisholm
272 1999). However, previous studies based on cell cycle analysis and on 14 C incorporation into divinyl-
273 chlorophyll a have suggested that, even at this depth, *Prochlorococcus* cells divide every 4-7 days
274 (Goericke and Welschmeyer 1993, Vaultot et al. 1995, Binder et al. 1996, Partensky et al. 1996). The C
275 uptake rates observed in the cells from 115 and 137m were well below the rate required to support this
276 division rate (~ 1 fg cell $^{-1}$ day $^{-1}$, Table 1). Additionally, unlike the laboratory cultures, in which C
277 incorporation rates were 3-6 times larger than N-incorporation rates, in our samples from the
278 Mediterranean Sea the measured C:N uptake ratio was about 1. Thus, the measured C and N uptake
279 rates suggest that, most likely, *Prochlorococcus* obtain most of the carbon needed for cell growth from
280 non-photosynthetic sources, i.e. through mixotrophy.

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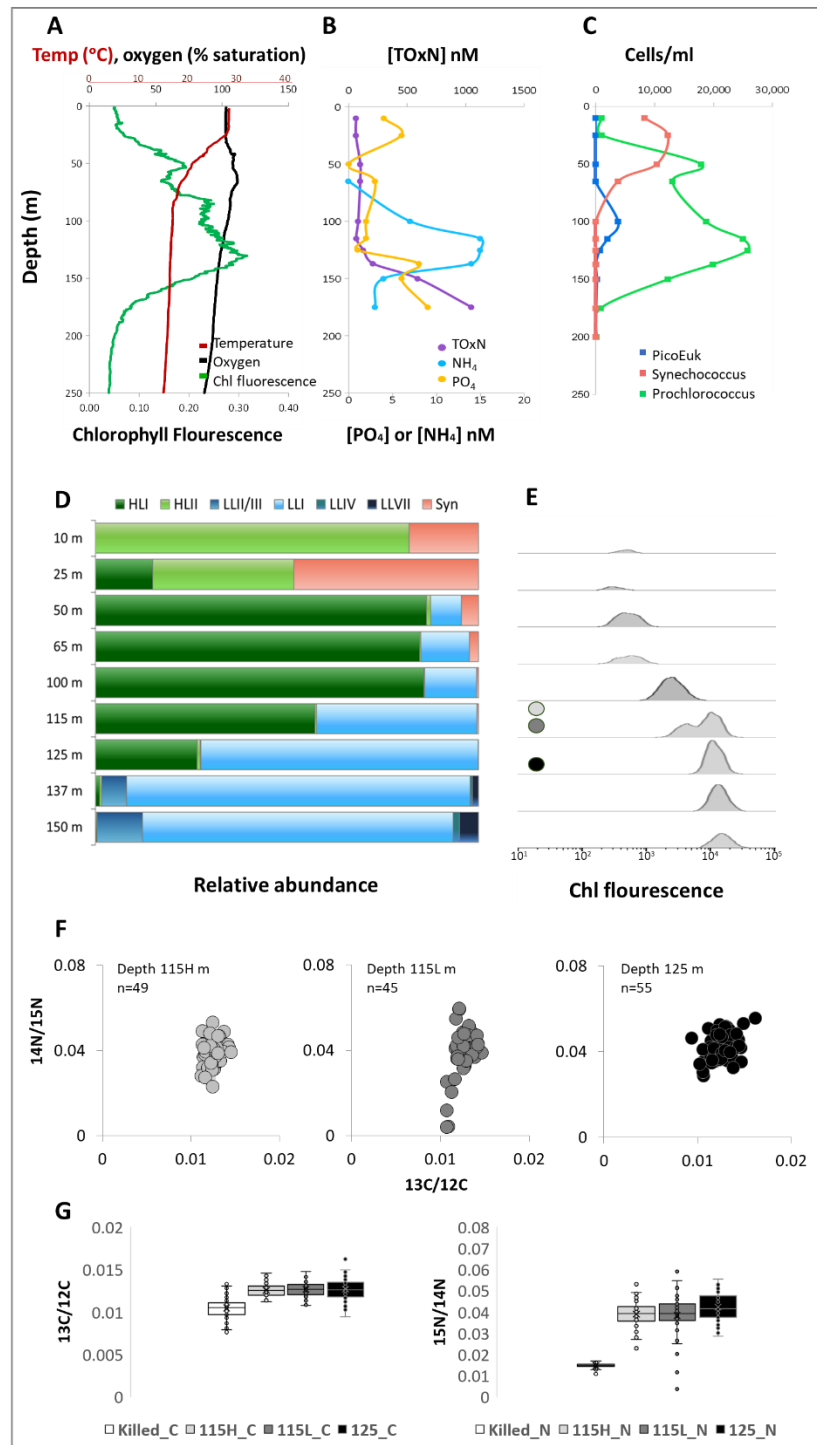
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309 **Figure 5: Nutrient uptake of naturally occurring *Prochlorococcus* populations at the Eastern**
310 **Mediterranean Sea. A-C) Oceanic parameters at the sampling site: Temperature, Oxygen and**

311 Chlorophyll (A); Nutrient concentrations (B); Cell counts by flow cytometry (C). D) Relative abundance of
312 different *Prochlorococcus* clades across the water column, determined by ITS sequencing. E) Histograms
313 of chlorophyll autofluorescence, analyzed from flow cytometry of picoplankton throughout the water
314 column. Note the double population at 115m. F) Scatterplot of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios obtained from
315 NanoSIMS analysis of each sorted sub-population from 115m and the single population from 125m. G)
316 Boxplots of the $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ enrichment in each sub-population. Lines represent the median, X
317 represents the mean, box borders are 1st quartiles and whiskers represent the full range. The three
318 populations did not statistically differ (Kruskal-Wallis test, $p < 0.001$).

319 **Stress survival in pico-cyanobacteria: why is *Prochlorococcus* different?**

320 In this study, we demonstrate that phenotypic heterogeneity between clonal *Prochlorococcus* cells
321 occurs at multiple “scales”. In exponentially growing axenic laboratory cultures C and N uptake rates
322 differ significantly between individual cells. This variation is independent of genetic variability.
323 Additionally, as axenic cultures become stressed, a larger phenotypic change occurs as cells lose their
324 chlorophyll auto-fluorescence and become chlorotic. Under these experimental conditions, most cells
325 are inactive (primarily in the low-fl population), although, due to the level of sensitivity of the
326 NanoSIMS, we cannot rule out that even low-fl cells still retain a residual level of activity. Some cells
327 from the chlorotic populations retain at least part of their photosynthetic capacity, and indeed can fix
328 carbon and take up NH_4 . Yet, in our experiments, they do not re-grow when condition become more
329 favorable. In *Synechococcus elegantus* PCC 7942, chlorotic cultures retain approximately 0.01% of their
330 photosynthetic activity, as well as a residual level of protein translation, although it remains unclear
331 whether this is a process shared by all cells in the culture or whether this activity is only due to a small
332 subset of more active cells (Sauer et al. 2001). The clear difference between the ability of axenic
333 *Synechococcus elegantus* PCC 7942 and *Synechocystis* PCC6803 to survive long-term N starvation, and
334 the inability of axenic *Prochlorococcus* cultures to do so, suggests an inherent difference in the
335 physiology and genomic functional capacity between these unicellular cyanobacteria.

336 Entry into chlorosis in *Synechocystis* is a regulated process that involves the organized degradation of
337 the phycobilisomes in parallel with an increase in the storage products glycogen and
338 polyhydroxybutyrate (PHB) (Klotz et al. 2016). The photosynthesis apparatus of *Prochlorococcus* is
339 different from that of other cyanobacteria, using unique chlorophyll a2/b2 binding proteins rather than
340 phycobilisomes (Ting et al. 2002), and indeed they lack orthologs of the nblA gene required for

341 phycobilisome degradation during chlorosis (Klotz et al. 2016). Additionally, while *Prochlorococcus* likely
342 use glycogen as a C storage pool (Lichtlé et al. 1995), they lack the *phaA-C* and *phaE* genes required for
343 PHB biosynthesis and which are induced in *Synechocystis* PCC 6803 under chlorosis (although these
344 genes are not required for revival from chlorosis (Klotz et al. 2016)). Taken together, these differences
345 suggest that *Prochlorococcus* lack the genetic toolkit employed by *Synechocystis* PCC6803 and
346 *Synechococcus elegantus* PCC7942 to enter into a resting stage.

347 If *Prochlorococcus* are indeed incapable of producing resting stages in response to nutrient or light
348 starvation, what are the evolutionary drivers of this phenotype, and what are the consequences for the
349 dynamics of *Prochlorococcus* populations in the ocean? While the open oligotrophic ocean is often
350 considered a relatively stable environment, nutrient concentrations do fluctuate, and phytoplankton
351 (including *Prochlorococcus*) inhabiting these waters show multiple signs of nutrient stress (Moore et al.
352 2013, Saito et al. 2014). Many of the microbes that live in such environments comprising a large fraction
353 of the surface ocean have small, highly streamlined genomes (Yooseph et al. 2010) and this has been
354 suggested to be an adaptation to low nutrient concentrations (Yooseph et al. 2010, Biller et al. 2014,
355 Giovannoni 2017). It is possible that the lack of resting stages is a result of this genome streamlining (the
356 genomes of *Synechococcus elegantus* PCC7942 and *Synechocystis* PCC6803 are ~3.2mbp and ~4mbp
357 with their plasmids, respectively, compared to ~1.4-2.5 mbp for *Prochlorococcus* strains).

358 **Surviving nutrient stress “with a little help from my friends”.**

359 Despite the clear effect of nutrient stress on *Prochlorococcus* in laboratory culture, manifesting in
360 chlorosis and reduction of vitality, we observed relatively few less-active (potentially chlorotic)
361 *Prochlorococcus* cells in the deep euphotic zone of the Eastern Mediterranean (approximately 5% of the
362 population at 115m, Figure 5F). It is possible that cell stress and chlorosis change as a function of the
363 diel cycle, and indeed previous studies have suggested *Prochlorococcus* cell mortality increases during
364 the night (Llabrés et al. 2011, Ribalet et al. 2015). As the sampling of natural population in the Eastern
365 Mediterranean Sea took place during the day (~12:00-16:00) we cannot exclude this hypothesis.
366 Alternatively, *Prochlorococcus* may actually never experience nutrient starvation in the oceans. While
367 physiological and molecular analyses of field populations of *Prochlorococcus* suggest that the cells are
368 nutrient-limited (e.g. (Saito et al. 2014, Szul et al. 2019)), cell growth and death are usually balanced,
369 suggesting these cells are not experiencing acute starvation (e.g. (Ribalet et al. 2015)). The ability of
370 *Prochlorococcus* to thrive under conditions of extreme nutrient limitation is often explained by their

371 small cell size (increasing their biomass-specific diffusion), their generally low nutrient requirements,
372 and their specific metabolic strategies to minimize the per-cell elemental quotas (Van Mooy et al. 2006,
373 Gilbert and Fagan 2011, Read et al. 2017). We propose that interactions with co-occurring
374 microorganisms, e.g. through the recycling of inorganic nutrients or the exchange of organic
375 compounds, enable *Prochlorococcus* to survive when these nutrient-saving mechanisms are not
376 sufficient. Indeed, the observation that *Prochlorococcus* can compete with heterotrophic bacteria for
377 amino acids (Zubkov et al. 2004), carbohydrates (Muñoz-Marín et al. 2013) and perhaps DMSP (Vila-
378 Costa et al. 2006, Becker et al. 2019) suggests that mixotrophy is prevalent and important in natural
379 communities. This is supported by genomic analyses (Rocap et al. 2003, Yelton et al. 2016). The
380 importance of mixotrophy is not limited to organic forms of N, P or Fe, but can be extended to light (or
381 resulting C) stress. Illustrating the potential magnitude of this effect, an average of ~8-10% of the
382 *Prochlorococcus* cells at HOT and BATS (Hawaii and Bermuda time series study sites, respectively
383 (Malmstrom et al. 2010)), are found under conditions where the average integrated illumination is not
384 enough to support their growth under laboratory conditions (Sup Fig. S7, see supplementary text for
385 more details (Moore and Chisholm 1999)). This includes the vast majority of LL adapted ecotypes.
386 Previous studies have shown that uptake of glucose by *Prochlorococcus* cells in the central Atlantic
387 Ocean can support up to ~20% of their carbon requirements, supporting the notion of mixotrophy
388 (Muñoz-Marín et al. 2013, Muñoz-Marín et al. 2017). However, glucose uptake is light dependent and
389 occurs at higher rates in the surface ocean, suggesting that some other form of DOC is likely important
390 in the deep euphotic zone. Amino acids provide another potential DOC form, which can supply both N
391 and C to the cells. Indeed, cell-specific amino acid uptake rates of *Prochlorococcus* from the southern
392 Atlantic Ocean increase with depth, and high-fl cells take up more than low-fl cells, although to what
393 extent this supports the C and N needs of the cells is unknown (Zubkov et al. 2004). Regardless of the
394 specific forms of dissolved organic carbon being utilized by the cells, the lack of any mechanism for the
395 production of resting stages by *Prochlorococcus* may be considered another manifestation of the “Black
396 Queen Hypothesis”, which states that microorganisms “outsource” essential survival mechanisms such
397 as detoxification of reactive oxygen species to the surrounding microbial community (Morris et al. 2012).
398 These forms of microbial interactions likely affect the distribution and activity of *Prochlorococcus* on a
399 global scale (Hennon et al. 2017, Ma et al. 2017).

400

401

402 **Material and Methods**

403 **Prochlorococcus growth and Stable Isotope Incubations**

404 Axenic *Prochlorococcus* strains were grown in Pro99 media under constant cold white light (27 μ E) at
405 22 °C. Bulk chlorophyll fluorescence (FL) (ex440; em680) was measured almost daily using a
406 Fluorescence Spectrophotometer (Cary Eclipse, Varian). In parallel, samples for flow cytometry were
407 taken for cell numbers. When three distinct sub-populations appeared in the flow cytometry, the
408 cultures were labeled with 1mM Sodium bicarbonate-¹³C and 1mM Ammonium-¹⁵N chloride (Sigma-
409 Aldrich, USA) for 18-24 hours. The optimal incubation time based on preliminary isotope labeling
410 experiments with *Prochlorococcus* MED (Supplementary figure 8). Incubations were stopped by fixing 2
411 ml of the culture with 2X EM grade glutaraldehyde (2.5% final concentration) and subsequently storing
412 at 4 °C until the sorting analysis. Non-labeled cells that were killed before labeling (by adding 2.5%
413 glutaraldehyde) were used as a negative control.

414

415 **Cell Sorting and Filtration**

416 Sorting of sub-population was carried out using a BD FACSAria III sorter (BD Biosciences) at the Life
417 Sciences and Engineering Infrastructure Center, Technion, Israel. Each sample was sorted for 3 sub-
418 populations: Non-chlorotic (High-fl), Semi chlorotic (Mid-fl) and Chlorotic (Low-fl) (Figure 2A). The
419 sorting gates for each sub-population were determined from the population observed in forward scatter
420 (FSC, a proxy for cell size) and auto-fluorescence (PerCP, chlorophyll auto-fluorescence). After sorting,
421 the sorted sub-population were gently filtered on 13 mm diameter polycarbonate filters (GTTP, 0.2 μ M
422 pore size, Millipore, MA), washed twice with sterile sea water and air-dried. The filters were stored at 4
423 °C until nanoSIMS analyses.

424

425 **Nanoscale secondary ion mass spectrometry (nanoSIMS) and data analysis**

426 The samples were coated with a layer of ca. 30 nm gold with a Cressington 108 auto sputter coater
427 (Watford, United Kingdom). Random spots were employed for NanoSIMS analyses. SIMS imaging was
428 performed using a NanoSIMS 50L instrument (Cameca, Paris, France) at the Leibniz-Institute for Baltic
429 Sea Research Warnemünde (IOW). A ¹³³Cs⁺ primary ion beam was used to erode and ionize atoms of the
430 sample. Images of secondary electrons, ¹²C⁻, ¹³C⁻, ¹²C¹⁴N⁻ and ¹²C¹⁵N⁻ were recorded simultaneously using
431 mass detectors equipped with electron multipliers (Hamamatsu). The mass resolving power was
432 adjusted to be sufficient to suppress interferences at all masses allowing, e.g. the separation of ¹³C from
433 interfering ions such as ¹²C¹H⁻. Prior to the analysis, sample areas of 50x50 μ m were sputtered for 2 min

434 with 600 pA to erode the gold, clean the surface and reach the steady state of secondary ion formation.
435 The primary ion beam current during the analysis was 1 pA; the scanning parameters were 512×512
436 pixels for areas of 30x30 to 48x48 μm, with a dwell time of 250 μs per pixel. 60 planes were analysed.
437

438 **Analyses of NanoSIMS measurements**

439 All NanoSIMS measurements were analysed with the Matlab based program look@nanosims (Polerecky
440 et al., 2012). Briefly, the 60 measured planes were checked for inconsistencies and all usable planes
441 accumulated, regions of interest (ROI's) (i.e. Prochlorococcus cells and filter regions without organic
442 material for background measurements) defined based on ¹²C/¹⁴N mass pictures, and ¹³C/¹²C as well as
443 ¹²C/¹⁵N/¹²C/¹⁴N ratios calculated from the ion signals for each region of interest.

444 **Uptake rate calculation**

445 Uptake rate was estimated using the following equation, based on that of (Legendre and Gosselin 1997),
446 as follows:

$$447 \quad V = \frac{(\%P_t^* - \%P_0^*) Q}{(\%D_i^* - \%D_0^*) t}$$

448 Where %P_t^{*} is the concentration (atom %) of the heavy isotope in the particulate matter at the end of
449 the incubation, %D_i^{*} is the concentration of the dissolved tracer added to the incubation (and assumed
450 not to change over the short incubation time), and %P₀^{*} and %D₀^{*} are the natural heavy isotope
451 concentrations in the particulate and dissolved matter, respectively. We estimated Q, the cell quota (in
452 fg cell⁻¹) of C or N, based on measurements of the biomass of MED4 and MIT9313 (66 fg cell⁻¹ and 158 fg
453 cell⁻¹, respectively, (Cermak et al. 2016)) and assuming that C comprises 50% and N comprises 7.5% of
454 the cell biomass. For heavy isotopes concentration in the particulate and dissolved phases before
455 incubation we used the natural values for isotopic ratios of ¹³C and ¹⁵N (1.12% and 0.37% respectively).
456 For the experiment shown in Supplementary Fig. 8, we measured the NH₄⁺ concentration in the media
457 and added the ¹⁵N tracer to 50% final concentration. Since all other experiments were performed in
458 declining cultures we assumed that the NH₄⁺ was depleted from the media, and thus %D_t^{*} was defined
459 as 90%, based on previous measurements of NH₄⁺ concentrations in old cultures. We used a value of
460 50% for the initial percentage of ¹³C, based on dissolved inorganic carbon (DIC) measurements
461 (Grossowicz et al. 2017). For the terminal concentrations of ¹⁵N and ¹³C in the particulate phase (%P_t^{*}) we
462 used the values of ¹³C/¹²C and ¹⁵N/¹⁴N that were obtained from the NanoSIMS analysis of the cells.

463 $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ below the natural values resulted with negative uptake values, and were treated as
464 zero uptake.

465 Mean and standard deviation of C and N uptake rates were calculated from the uptake rate values of
466 individual cells (Table 1). The uptake rate values were not corrected for negative control (killed cells),
467 which are presented for comparison in table 1. Since $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ values of individual cells were
468 not normally distributed, for significance analysis we used non-parametric tests (Mann-Whitney and
469 Kruskal-Wallis tests) performed using the Real Statistics Resource Pack software (Release 5.4 [www.real-](http://www.real-statistics.com)
470 [statistics.com](http://www.real-statistics.com)).

471 **Isotope labelling and phylogenetic analysis of a natural marine bacterioplankton population at sea**

472 **Water collection and labelling experiment procedure**

473 Mediterranean seawater was collected during August 2017 (station N1200, 32.45 degrees N, 34.37
474 degrees E) from 11 depths by Niskin bottles and divided into triplicates of 250 ml polycarbonate bottles.
475 Two bottles from each depth were labeled with 1mM Sodium bicarbonate- ^{13}C and 1mM Ammonium- ^{15}N
476 chloride (Sigma-Aldrich, USA) and all 3 bottles (2 labelled and 1 control) were incubated at the original
477 depth and station at sea for 3.5 hours on day time. After incubation, bottles were brought back on board
478 and the incubations were stopped by fixing with 2X EM grade glutaraldehyde (2.5% final concentration)
479 and stored at 4 °C until sorting analysis.

480

481 **DNA collection and extraction from seawater**

482 Samples for DNA collected on a 0.22 μm sterivex filters (Millipore). Excess water was removed using a
483 syringe and 1 ml Lysis buffer (40 mM EDTA, 50 mM Tris pH 8.3, 0.75 M sucrose) was added and both
484 ends were closed with parafilm. Samples were kept at -80°C until extraction. DNA extracted by using a
485 semi-automated protocol includes manually chemical cell lysis before the automated steps. The manual
486 protocol began with thawing the samples, then the storage buffer was removed using a syringe and 170
487 μl lysis buffer added to the filter. 30 μl of Lysozyme (20 mg/ml) added to filters and incubate at 37°C for
488 30 min. After incubation, 20 μl proteinase K and 200 μl Buffer AL added to the tube for 1 hour at 56°C
489 (with agitation). Then, the supernatant transferred to a new tube, which subjected to the QIAcube
490 automated system (at the BioRap unit, Faculty of Medicine, Technion) following the manufacturer's
491 instructions using QIAamp DNA Mini Protocol: DNA Purification from Blood or Body Fluids (Spin
492 Protocol) from step 6. All DNA samples were eluted in 100 μl DNA free distilled-water.

493

494 **16S and ITS PCR amplification**

495 PCR amplification of the ITS was carried out with specific primers for *Prochlorococcus* CS1_16S_1247F
496 (5'-ACACTGACGACATGGTTCTACACGTACTACAATGCTACGG) and Cs2_ITS_Ar (5'-
497 TACGGTAGCAGAGACTTGGTCTGGACCTCACCTTATCAGGG) (Thompson et al. 2018). The first PCR was
498 performed in triplicate in a total volume of 25 µl containing 0.5 ng of template, 12.5 µl of MyTaq Red
499 Mix (Bioline) and 0.5 µl of 10 µM of each primer. The amplification conditions comprised steps at 95°C
500 for 5 min, 28/25 (16S/ITS) cycles at 95°C for 30 sec, 50°C for 30 sec and 72°C for 1 min followed by one
501 step of 5 min at 72°C. All PCR products validated on 1% agarose gel and triplicates were pooled.
502 Subsequently, a second PCR amplification was performed to prepare libraries. These were pooled and
503 after a quality control sequenced (2x250 paired-end reads) using an Illumina MiSeq sequencer. Library
504 preparation and pooling were performed at the DNA Services (DNAS) facility, Research Resources Center
505 (RRC), University of Illinois at Chicago (UIC). MiSeq sequencing was performed at the W.M. Keck Center
506 for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign (UIUC).

507

508 **ITS Sequence processing** Paired-end reads, in the format of fastq files were analyzed by the DADA2
509 pipeline (<https://www.nature.com/articles/nmeth.3869>). Quality of the sequences per sample was
510 examined using the Dada2 'plotQualityProfile' command. Quality filtering was done using the Dada2
511 'filterAndTrim' command with parameters for quality filtering were truncLen=c(290,260), maxN=0,
512 maxEE=c(2,2), truncQ=2, rm.phix=TRUE, trimLeft=c(20,20). Following error estimation and dereplication,
513 the dada algorithm was used to correct sequences. Merging of the forward and reverse reads was done
514 with minimum overlap of 4 bp. Detection and removal of suspected chimera was done with command
515 'removeBimeraDenovo'. In total, 388,417 sequences in 484 amplicon sequence variants (ASVs) were
516 counted. The ASVs were aligned in MEGA6 (Tamura et al. 2013) and the first ~295 nucleotides,
517 corresponding to the 16S gene, were trimmed. The ITS sequences were then classified using BLAST
518 against a custom database of ITS sequences from cultured *Prochlorococcus* and *Synechococcus* strains
519 as well as from uncultured HL and LL clades.

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528

529 **Author contributions**

530 DRR, DA, TLK, AV, MV and DS designed experiments, DRR, DA, TLK, LZ, NN and DS performed
531 experiments and field analyses, DRR, DA, TLK, AV, and FE performed NanoSIMS analyses, DRR, DA, TLK,
532 AV, LZ, FE, NN, HPG, MV and DS analyzed results, DRR, DA, TLK and DS wrote manuscript with
533 contributions from all authors.

534

535 **Competing interests**

536 The authors declare no competing interests

537

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540

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725 **Supplementary data**

726 ***Prochlorococcus* rely on microbial interactions rather than on chlorotic resting stages to**
727 **survive long-term stress**

728

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740 **Supplementary text**

741 **Can mis-sorted cells explain the presence of non-active cells in the high-fl population, and of**
742 **active cells in the low- and mid-fl populations?**

743 FACS-sorting high-, mid- and low-fl populations from *Prochlorococcus* cultures resulted in
744 samples that are highly enriched in the sorted populations. However, the sorted populations
745 are not completely pure, with some incorrectly sorted cells observed when the sorted
746 populations were re-analyzed by flow cytometry (e.g. mid- or low-fl cells in the sorted high-fl
747 population, Supplementary Table 1). These incorrectly sorted cells could affect the
748 interpretation of the single-cell uptake rates, for example, the presence of inactive cells in the
749 high-fl population could be interpreted either as a real biological phenomenon (cells with high
750 autofluorescence that are nevertheless inactive) or as the result of incorrectly sorted cells
751 belonging to the mid- or low-fl populations (assuming the mid- and low-fl cells are inactive). To
752 test this hypothesis, we first determined, for each cell in the NanoSIMS analysis, whether it was

753 active or inactive, defining inactive cells as those with C and N uptake rates in the range of the
754 control, i.e. glutaraldehyde killed cells (Supplementary Table 2, Killed cells $C_{\max} = 0.765$
755 fg/cell/day and $N_{\max} = 0.215$ fg/cell/day). We then compared the observed number of active
756 cells to the expected number of active cells, based on the number of high-, mid- and low-fl cells
757 after FACS sorting, and assuming only the high-fl cells are active. For the datasets shown in
758 Figure 2 and Supplementary Figure 3, the hypothesis that the number of active cells could be
759 explained by the number of high-fl cells after sorting was rejected (X^2 test, $DF=2$, $p<0.01$,
760 $X^2=24.4$ and $n=236$ cells for Figure 2, $X^2=336.1$ and $n=415$ cells for Supplementary Figure 3).
761 There were fewer active cells in the high-fl populations than expected (68% and 64% active cells
762 Supplementary Tables 2, compared to 94% and 84.5% high-fl cells, respectively, in the high-fl
763 subpopulation Supplementary Tables 1), suggesting that some high-fl cells are inactive.
764 Conversely, there were more active cells than expected in the mid- and low-fl populations (22%
765 and 51% active cells Supplementary Tables 2, compared to 7.9% and 7.5% high-fl cells,
766 respectively, in the high-fl subpopulation Supplementary Tables 1), suggesting that some cells in
767 these populations can be active.

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769 **Estimating the fraction of *Prochlorococcus* cells living mixotrophically**

770 A rough estimate of the fraction of *Prochlorococcus* cells living mixotrophically can be obtained
771 by determining how many cells are found under conditions where the light intensity is not
772 sufficient to support active growth. We used the experimentally-determined minimal light
773 requirement for active growth of high-light and low-light adapted strains in the lab (10 μE and
774 2.8 μE during a 14:10 day-night cycle for HL and LL strains, respectively) in the lab (Moore and
775 Chisholm 1999), converting these to integrated daily PAR levels (in mole quanta $m^{-2} day^{-1}$,
776 eMIT9313 is treated as a LL strain in this analysis despite the limited light levels analyzed for the
777 MIT9313 strain). The threshold light levels based on this calculation were 0.50 mol Q day^{-1} for
778 HL strains and 0.18 mol Q day^{-1} for LL strains. We then determined the number of
779 *Prochlorococcus* cells found at depths where the integrated daily PAR is lower than the minimal
780 light requirement (i.e. below the photic depth for *Prochlorococcus*). For this analysis, we used

781 the qPCR estimates of the cell abundance of each ecotype from a 5-year time series of the
 782 *Prochlorococcus* ecotypes at the Hawaii and Bermuda time series study sites (HOT and BATS,
 783 (Malmstrom et al. 2010)). In our analysis, however, we consider only the time of the year when
 784 the water column is stratified (defined here as a mixed layer depth that is shallower than the HL
 785 *Prochlorococcus* photic depth), because at other times cells below the photic depth but still
 786 within the upper mixed layer could be mixed closer to the surface and therefore receive
 787 sufficient light. During these times, the estimated photic depth for HL ecotypes was between
 788 83-114m at HOT and 81-110m at BATS, and for LL ecotypes between 101-140m at HOT and
 789 103-135m at BATS. An average of ~8-10% of the *Prochlorococcus* cells at HOT and BATS,
 790 respectively, are found below these depths (range ~1.4-37.5% at HOT and ~0.5-43% at BATS),
 791 including most of the LL cells (Supplementary Figure 7). Multiple studies have shown that
 792 *Prochlorococcus* cells undergo division at these depths (growth rates of 0.1-0.2 day have been
 793 recorded down to depths of 150m; e.g. (Vaulot et al. 1995, Binder et al. 1996, Liu et al. 1997)),
 794 and thus at least some cells observed at these depths are not quiescent or dead. Future studies
 795 are needed in order to better constrain these rough estimates of the percent of mixotrophic
 796 *Prochlorococcus*, taking into account processes leading to mixing of cells below the mixed layer
 797 (e.g. internal waves) and also accounting for other *Prochlorococcus* ecotypes not analyzed here.

798 **Supplementary Table 1: Cell counts and purity of the sorted cells**

		Sorted sub-populations *MIT9313 old			Sorted sub-populations **MIT9313 old		
		High	Mid	Low	High	Mid	Low
% Purity and number of sorted cells	High	3388 (94%)	239 (7.9%)	19 (1%)	3109 (84.5%)	301 (7.5%)	100 (2.9%)
	Mid	174 (4.8%)	2673 (88.2%)	172 (9.1%)	514 (14%)	3639 (91%)	685 (19.6%)
	Low	59 (1.6%)	118 (3.9%)	1699 (90%)	56 (1.5%)	57 (1.4%)	2715 (77.6%)
	Total cells	3621	3030	1890	3621	3030	1890

799 *The result refers to the experiment presented in Fig. 2

800 ** The result refers to the experiment presented in Supplementary Fig. 3

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805 **Supplementary Table 2: Number of active and inactive cells in each of the sorted sub-populations.**

	Sorted sub-populations *MIT9313 old			Sorted sub-populations **MIT9313 old		
	High	Mid	Low	High	Mid	Low
% and number of active cells	45 (68%)	16 (22%)	3 (3.1%)	75 (64%)	107 (51%)	5 (6%)
% and number of inactive cells	21 (31.8%)	57 (78%)	94 (97%)	43 (36%)	101 (49%)	83 (94%)
Total cells	66	73	97	118	208	88

806 *The result refers to the experiment presented in Fig. 2

807 ** The result refers to the experiment presented in Supplementary Fig. 3

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809 **Supplementary Table 3: Coefficient of variation**

Strain and growth stage	Sub-population	Illumination	Number of cells	C _{cov}	N _{cov}
Batch culture					
MIT9313, exponential growth*	High	Constant light 27 μ E	158	0.92	0.89
MIT9313, old culture**	High		66	1.08-1.29	0.92-0.87
	Mid		73	2.19-2.37	1.63-1.09
	Low		97	3.77-7.11	1.83-0.85
MIT9313, old culture***	High	Photo-period 12:12 L/D, 27 μ E	86	2.65	1.02
	Mid		171	5.15	0.99
	Low		73	0.69	2.33
Natural samples					
115m	High	Natural cycle approximately 14:10 L/D, 2-5 μ E	55	0.73	0.16
115m	Low		45	0.61	0.33
125m	High		49	0.58	0.17
Killed cells			114	2.63	0.09

810 *The results for MIT9313 exponential growth refer to the experiment presented in Supplementary Fig.

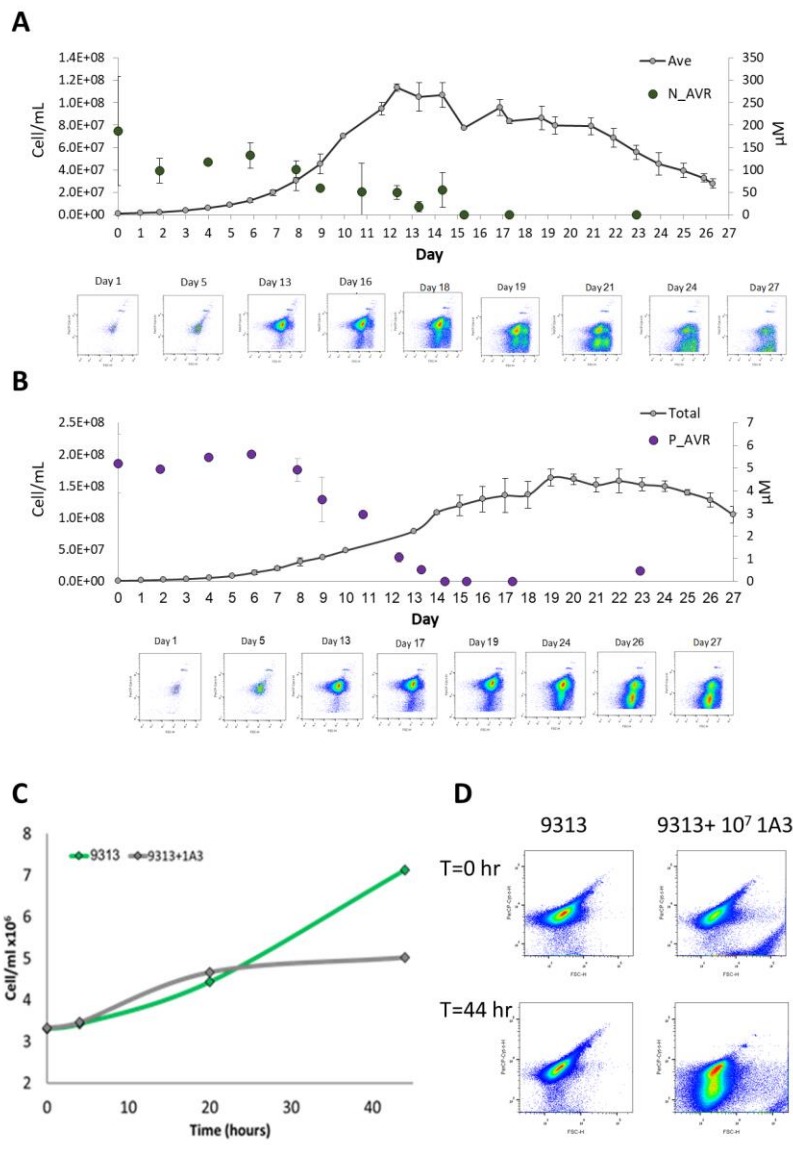
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812 **The results for old MIT9313 cultures under constant light refer to the two experiments presented in
813 Fig. 2 and Supplementary Fig. 3.

814 *** The results for Old MIT9313 cultures under L/D refer to the experiment presented in Supplementary
815 Fig. 4.

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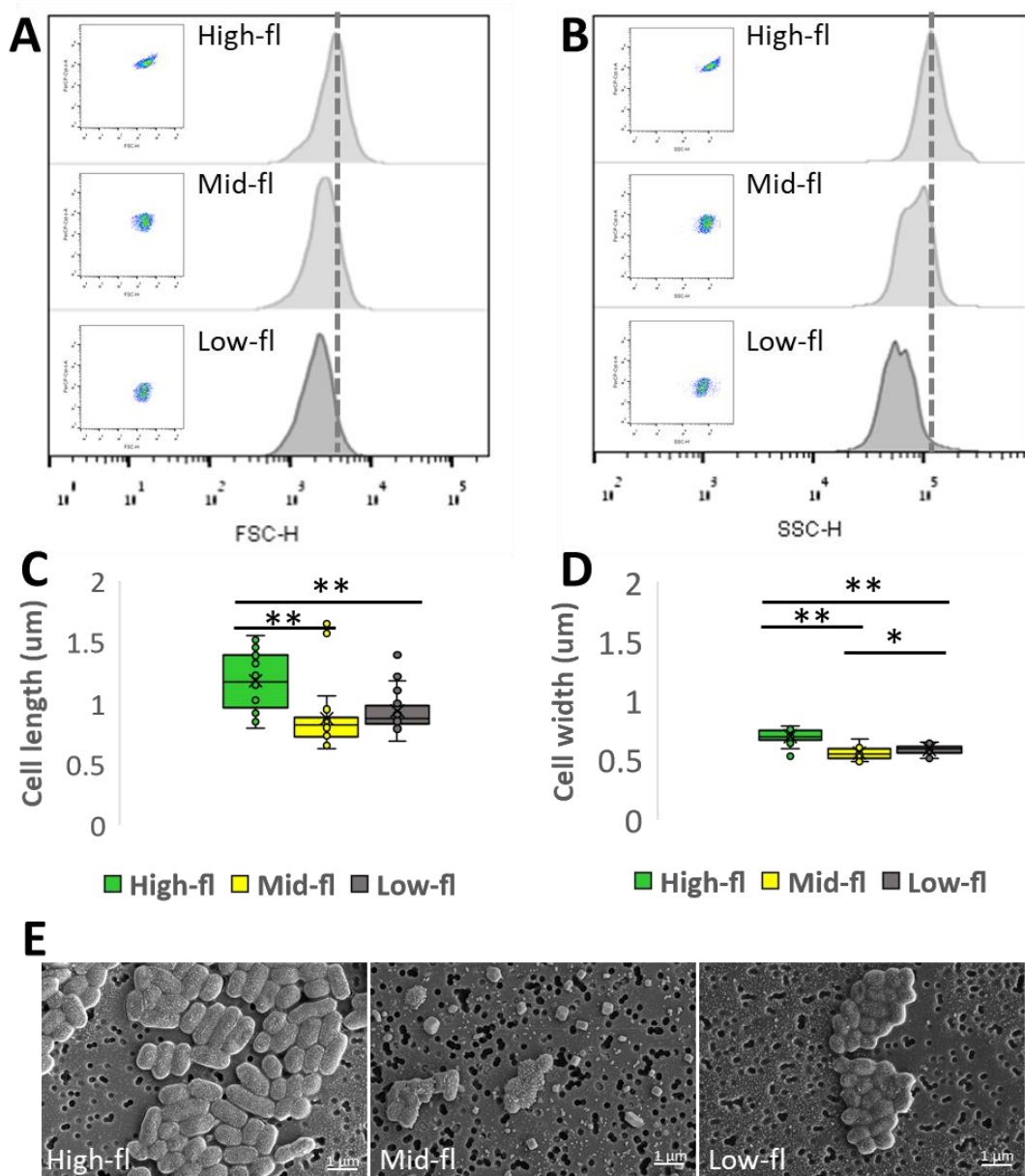
817 Supplementary figures



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819 **Supplementary Figure 1:** Sub-populations of low-chl cells emerge in *Prochlorococcus* cultures under N
820 and P starvation, and when inhibited by heterotrophic bacteria. A, B) Batch cultures of strain MIT9312

821 grown under conditions where stationary phase is induced by N and P starvation (panels A and B,
822 respectively). The data shown are from (Grossowicz et al. 2017). C) Batch cultures of *Prochlorococcus*
823 MIT9313 grown axenically and in co-culture with a heterotrophic bacterium, *Alteromonas* HOT1A3. D)
824 FCM of *Prochlorococcus* populations at two time points ('0' and '44' hours). Data are from Aharonovich
825 and Sher (Aharonovich and Sher 2016).

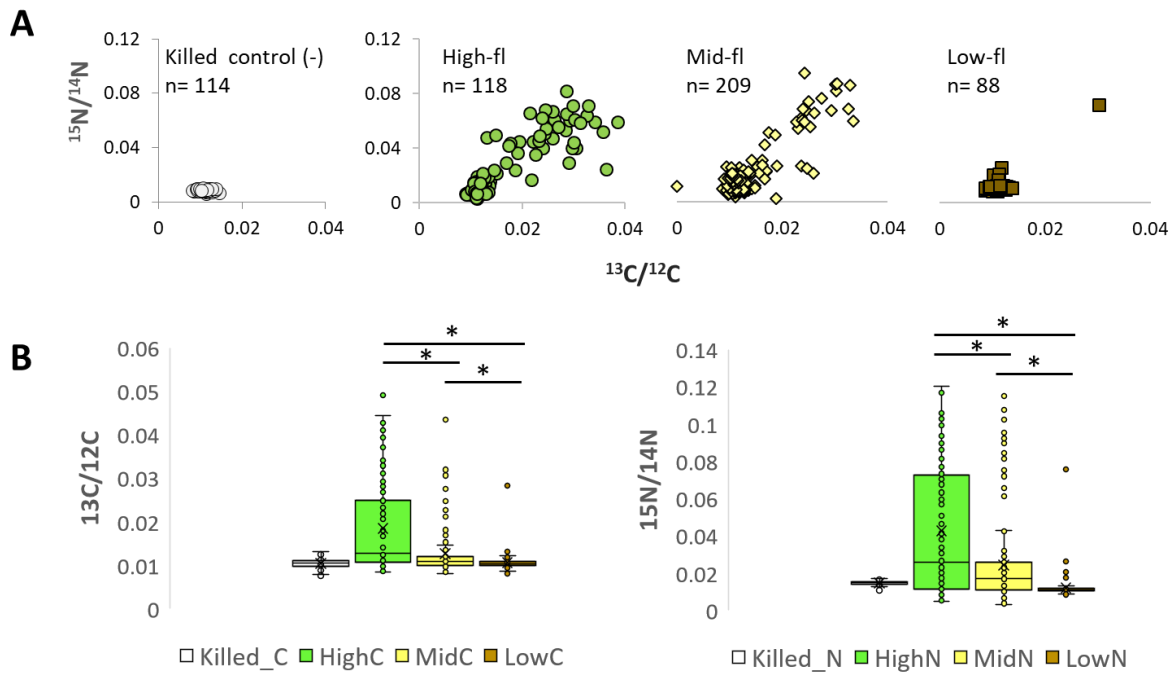


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827 **Supplementary Figure 2: Sorted cells belonging to different sub-populations of *Prochlorococcus***
828 **MIT9313 vary in size.** A late-exponential phase MIT9313 culture was fixed using glutaraldehyde,

829 analyzed using flow cytometry, and the three different sub-populations sorted and observed by SEM. A,
830 B) Histograms representing changes in cell size (Forward scatter, FSC, panel A) and complexity (Side
831 Scatter, SSC, panel B) as measured by flow cytometry. Boxplots of variations in cell length (C) and cell
832 width (D) as measured from SEM images of sorted populations. High-fl (n=27), Mid-fl (n=23), Low-fl
833 (n=24). The three subpopulations were statistically different (Kruskal-Wallis test, $p < 0.001$). Significant
834 differences between each of the two populations (Mann-Whitney U test) are shown (** - $p < 0.001$, * -
835 $p < 0.05$). Lengths values from Mid and Low were not significantly different. E) SEM image of sorted sub-
836 populations, scale bar is 1 μ m. The small square objects in the middle panel are salt crystals.

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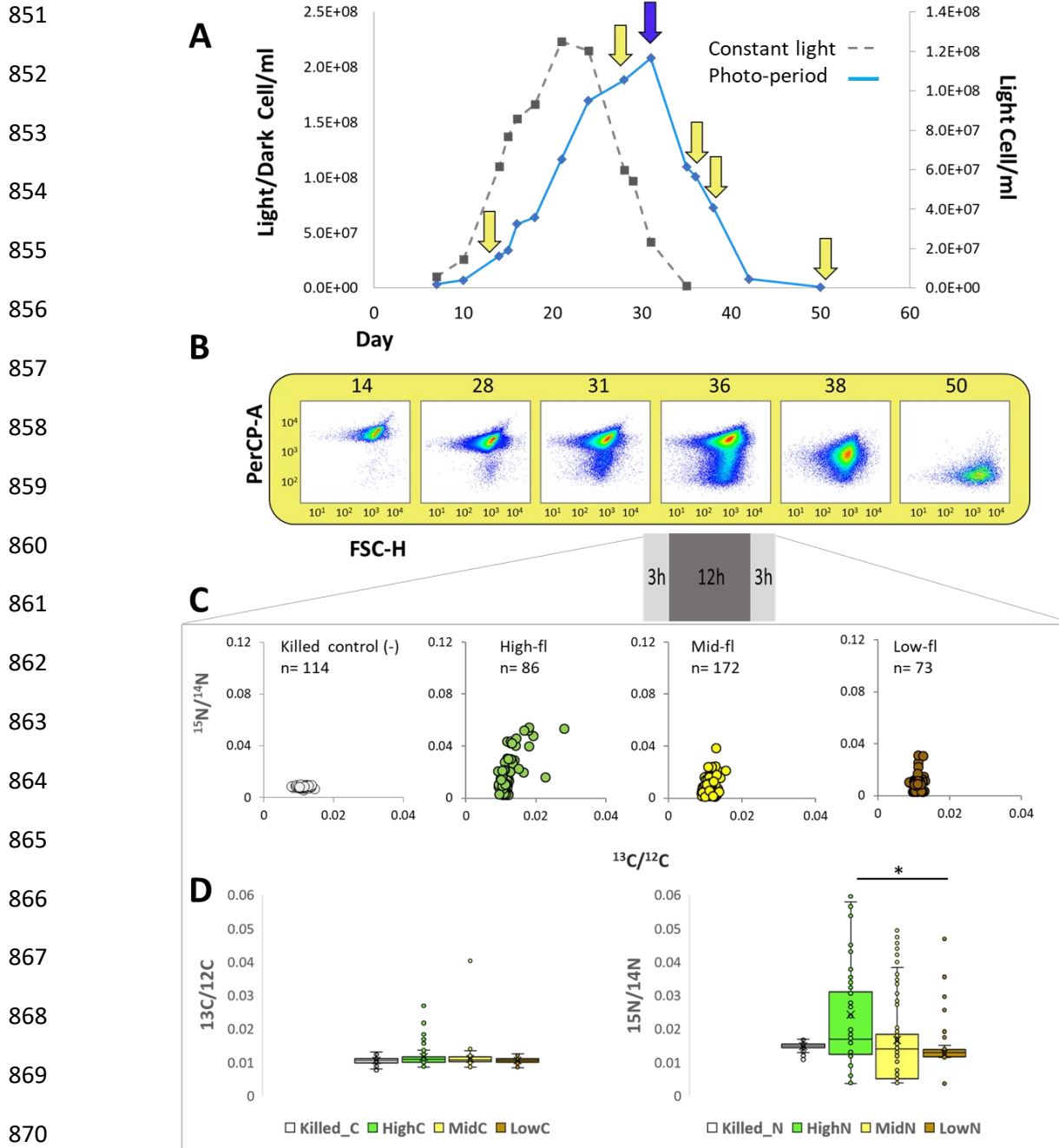
840 **Supplementary Figure 3. An independent experiment confirming differences in the metabolic activity**
841 **of sorted sub-populations of MIT9313 by NanoSIMS.** *Prochlorococcus* MIT9313 cultures were grown for
842 44 days in Pro99 and labeled with $\text{H}^{13}\text{CO}_3^-$ and $^{15}\text{NH}_4^+$ for 18h. (A) Scatterplot of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios
843 obtained from NanoSIMS analysis of each subpopulation (indicating the number of events detected
844 from multiple fields). (B) Boxplot represent the variances of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ in each sub-population.

845 The three populations were statistically different (Kruskal-Wallis test, $p < 0.001$, asterisks show significant
846 differences in comparisons between each of the two populations using the Mann-Whitney U test,
847 $p < 0.001$).

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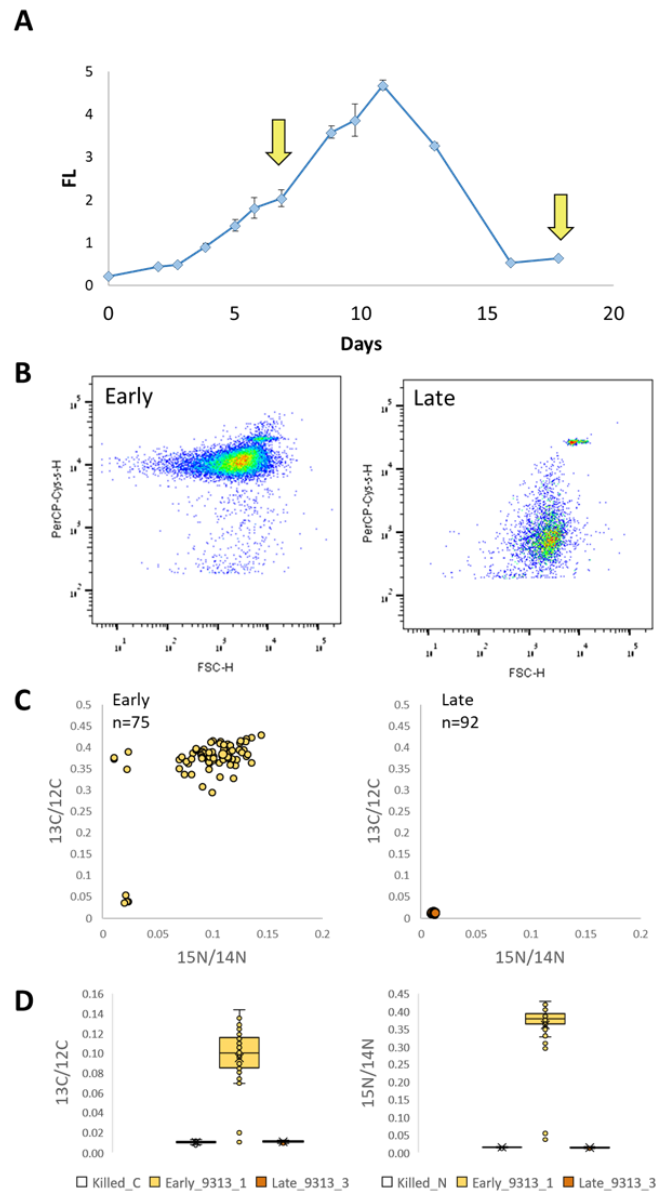
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871 **Supplementary Figure 4. NanoSIMS analysis for metabolic activity of *Prochlorococcus* chlorotic sub-**
 872 **populations under light/dark growth conditions.** For *Prochlorococcus*, cell physiology is strongly
 873 entrained by the diel cycle, with cells typically replicating early during the night (Zinser et al. 2009). As
 874 the cultures grown for these experiments presented in Figure 2 and Supplementary Fig 3 were grown
 875 under conditions of constant light, it was possible that heterogeneity in C and N uptake rates was due to
 876 the presence of cells at different cell cycle stages. To test this hypothesis, we repeated this experiment
 877 using an MIT9313 culture grown under 12:12 light/dark conditions. The C uptake rate, as well as the cell-

878 cell heterogeneity, were lower under these conditions, potentially because the cells were in darkness for
879 12 out of the 18 hours of labeling, including the “evening” and “morning” periods when the cell’s
880 photosynthetic machinery is not running at its maximal capacity (Zinser et al. 2009). In contrast, the N
881 uptake rate remained high under the light-dark cycle. This suggests that NH_4 uptake in *Prochlorococcus*
882 is decoupled from photosynthesis and occurs during both light and dark periods, unlike amino acid
883 uptake which occurs in *Prochlorococcus* primarily during the day (Mary et al. 2008). A) MIT9313 growth
884 curve under 12 h light and 12 h dark (The arrows mark the days shown in panel B.) B) A time series of
885 flow cytometry scattergrams from the tested MIT9313 culture. The x-axis is Forward Scatter (FSC, a
886 proxy for cell size), the y-axis is the chlorophyll autofluorescence of the cells. The appearance of
887 chlorotic sub-population observed from the late exponential phase (Day 31). C) Scatterplot of $^{13}\text{C}/^{12}\text{C}$
888 and $^{15}\text{N}/^{14}\text{N}$ ratios obtained from NanoSIMS analysis following 18 h incubation 3h L/12h D/3h L on day
889 36. D) Boxplot of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ enrichment in each subpopulation. Glutaraldehyde killed cells used
890 as a negative control. Lines represent the median, X represents the mean, box borders are 1st quartiles
891 and whiskers represent the full range. The three populations were statistically different for N uptake
892 (Kruskal-Wallis test, $p < 0.001$) but not for C uptake ($p = 0.06$). Significant differences between each two
893 populations (Mann-Whitney U test) are shown (* - $p < 0.001$).

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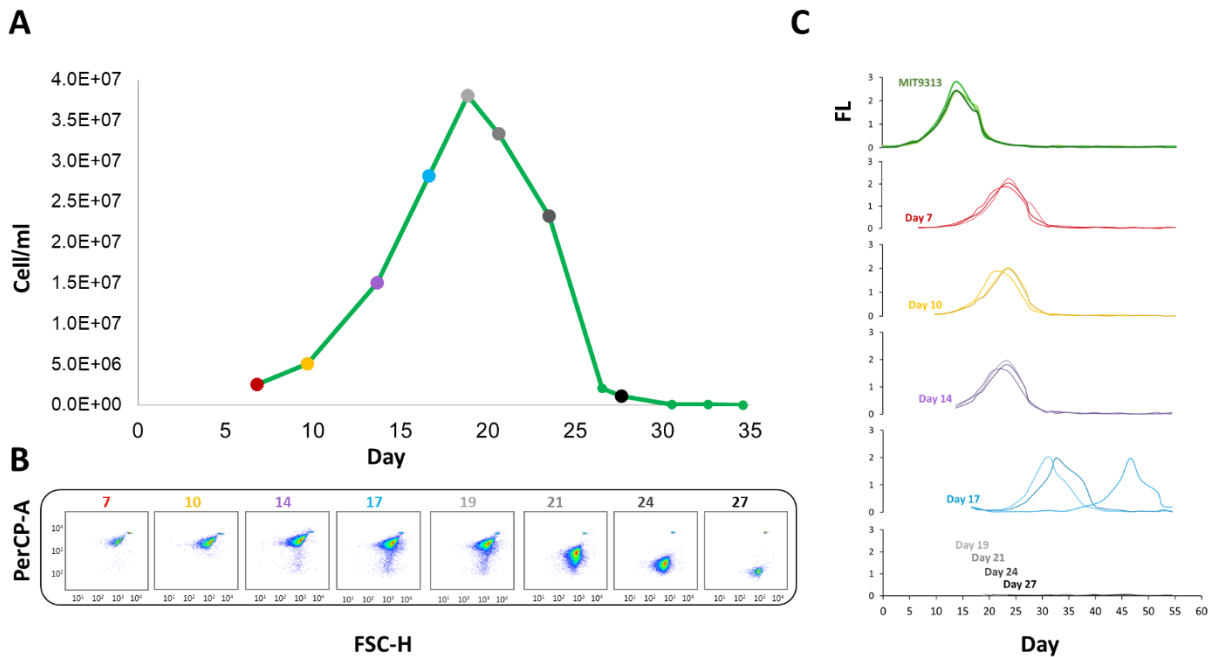
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899 **Supplementary Figure 5. NanoSIMS analysis of *Prochlorococcus* MIT9313 from exponentially-growing,**
900 **nutrient-replete cultures compared to late, post-decline stage in batch culture. We measured the N**

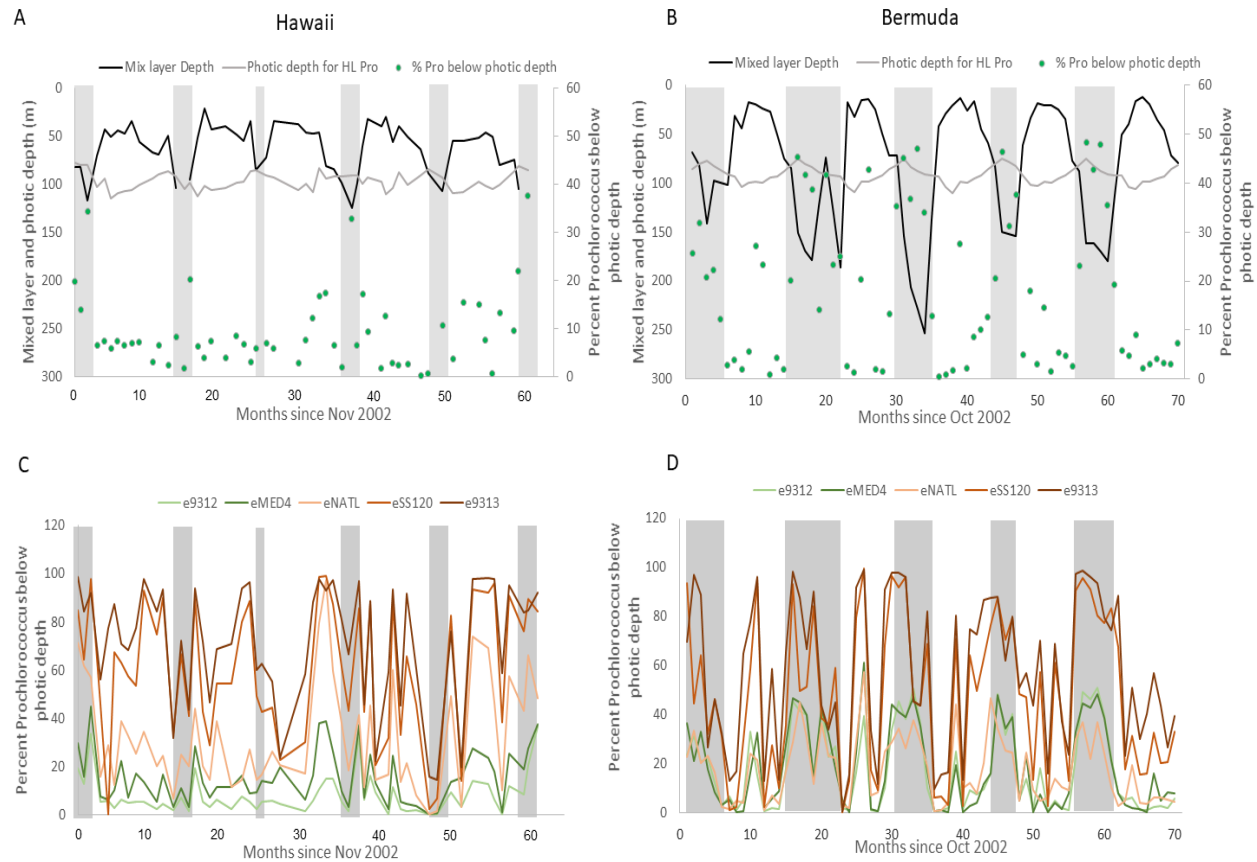
901 and C uptake rates in MIT9313 cultures for which the stationary stage is induced by N starvation (low-N
902 Pro99, N:P ratio = 2, (Grossowicz et al. 2017)). A) Growth curve monitored via the chlorophyll auto-
903 fluorescence. Arrows indicate the heavy-nutrient labeling time points (days 7 and 18). B) FCM
904 scatterplots of populations of 24hr post labelling. C) Scatterplot of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios obtained
905 from NanoSIMS analysis, representing single cell uptake. D) Boxplot of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ enrichment in
906 each population, compared to killed cells in the control.

907



908 **Supplementary Figure 6: Time-dependent changes in viability of cells transferred into fresh media at**
909 **different life cycle stages of a batch culture. A)** A growth curve of a MIT9313 culture, under conditions
910 where stationary stage is induced by nitrogen starvation (2:1 N/P ratio in the growth media, (Grossowicz
911 et al. 2017)). Colored circles indicate the times point at which triplicate 1ml samples were transferred
912 into fresh media. B) Flow cytometry scatterplots of the culture shown in panel A. Note that, under
913 nitrogen starvation, the cultures shift rapidly from being comprised primarily of high-fl cells (day 17,
914 early stationary phase) to mainly mid-fl cells, with essentially no high-fl cells (day 21). C) Growth curves
915 of cells being transferred at different times to new, nutrient-replete media (assessed via bulk culture
916 fluorescence). Cells could not re-grow when transferred after more than 17 days of nitrogen starvation
917 suggesting that not all high-fl cells are viable, and that low-fl cells are non-viable. Similar results were
918 observed in MIT9313 cultures grown in Pro99 media, where lag phase is longer and mid-fl cells are also
919 observed (Figure 3).

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922 **Supplementary figure 7: Estimating the number of *Prochlorococcus* cells found below their photic zone**

923 **at Hawaii and Bermuda.** A, B) The percent of total *Prochlorococcus* cells found below their photic zone

924 at Hawaii (A) and Bermuda (B), defined as the integrated illumination level supporting the growth of

925 representative strains in laboratory cultures (Moore and Chisholm 1999) (grey line shows this depth for

926 HL strains). The black line shows the mixed layer depth (MLD), the grey areas are non-stratified

927 conditions where cells may be mixed from depth to the surface. C, D) The percentage of each

928 *Prochlorococcus* ecotype below its photic depth. For more information see supplementary text. The

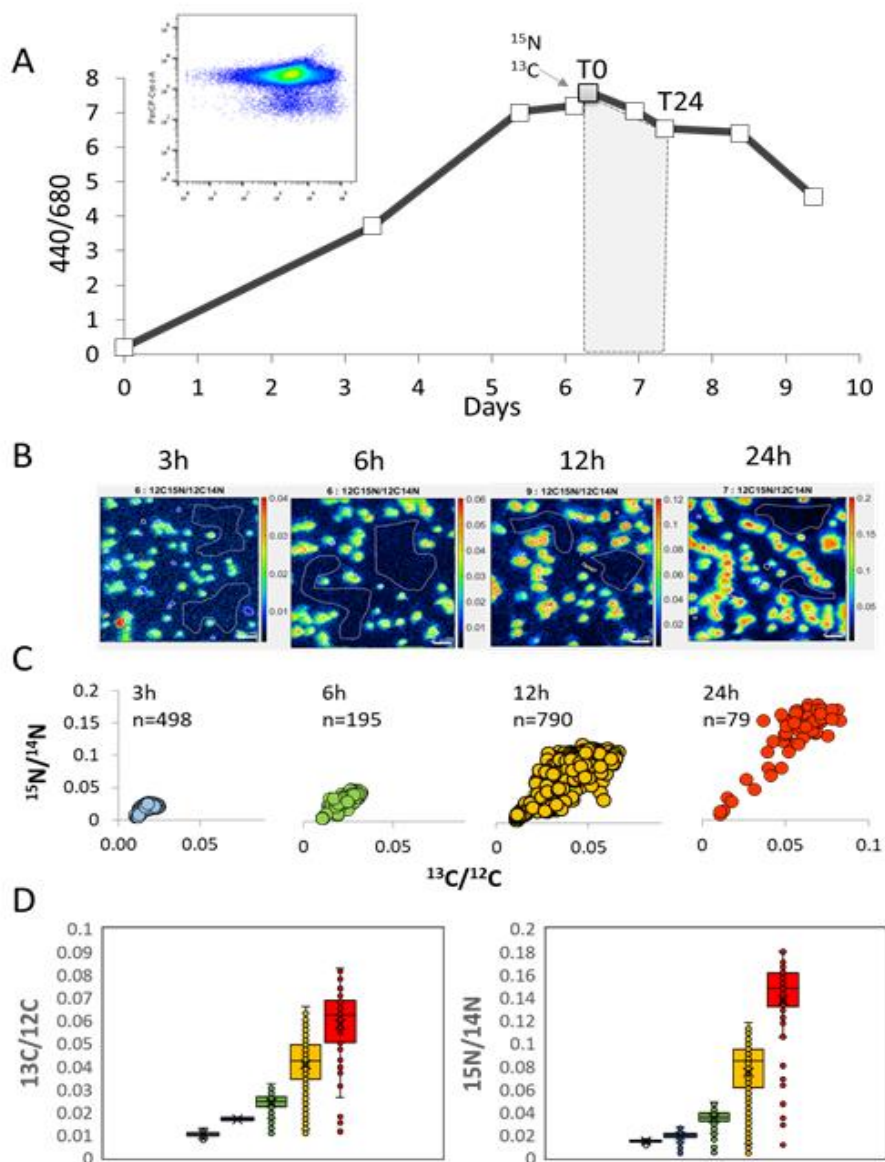
929 data are taken from (Malmstrom et al. 2010).

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951 **Supplementary Figure 8: Optimization for measuring metabolic activity of *Prochlorococcus* by**
952 **NanoSIMS** A) Growth curve of *Prochlorococcus* MED4 measured by fluorescence 440/680nm. Labeled
953 nutrients ($\text{H}^{13}\text{CO}_3^-$ and $^{15}\text{NH}_4^+$) were added after day 6 and sampled for NanoSIMS at 3h, 6h, 12h, and
954 24h. Insert shows the cell population at the time of labeling (T0) by FCM. B) NanoSIMS images of
955 $^{15}\text{N}/^{12}\text{C}$ analysis of cell for measuring metabolic activity. C) Scatterplot of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios
956 obtained from NanoSIMS analysis at each time point (indicating the number of events detected from
957 multiple fields). D) Boxplots represent the variances of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ at each time point.

958

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