Phototroph-heterotroph interactions during growth and long-term starvation across *Prochlorococcus* and *Alteromonas* diversity

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Running title: *Prochlorococcus*-*Alteromonas* interactions across diversity

Abstract

Interactions among microorganisms are ubiquitous, yet to what extent strain-level diversity affects interactions is unclear. Phototroph-heterotroph interactions in marine environments have been studied intensively, due to their potential impact on ocean ecosystems and biogeochemistry. Here, we characterize the interactions between five strains each of two globally abundant marine bacteria, *Prochlorococcus* (a phototroph) and *Alteromonas* (a heterotroph), from the first encounter between individual strains and over more than a year of subsequent co-culturing. *Prochlorococcus*-*Alteromonas* interactions affected primarily the dynamics of culture decline, which we interpret as representing cell mortality and lysis. The shape of the decline curve and the carrying capacity of the co-cultures were determined by the phototroph and not the heterotroph strains involved. Comparing various models of culture mortality suggests that death rate increases over time in mono-cultures but decreases in co-cultures, with cells potentially becoming more resistant to stress. During 435 days of co-culture, mutations accumulated in one *Prochlorococcus* strain (MIT9313) in genes involved in nitrogen metabolism and the stringent response, indicating that these processes occur during long-term nitrogen starvation. Our results suggest potential mechanisms involved in long-term starvation survival in co-culture, and highlight the information-rich growth and death curves as a useful readout of the interaction phenotype.

Keywords: Microbial interactions, growth curves, machine learning, mortality, co-culture

35 Introduction

36 Interactions among microorganisms occur in every known ecosystem. Positive (synergistic)
 37 interactions allow organisms to exchange resources, potentially increasing biodiversity, whereas
 38 negative interactions such as competition, allelopathy and predation may lead to organisms
 39 displacing one another (recently reviewed by (Fritts et al., 2021; Gralka et al., 2020)). Detailed
 40 studies of the interactions between selected model organisms (often in laboratory co-cultures)
 41 have begun to reveal the diversity of molecular mechanisms whereby organisms interact with
 42 each other (Fritts et al., 2021; Harrington et al., 2021; Weisskopf et al., 2021). However, it is
 43 currently unknown to what extent the studied interactions are conserved across organisms,
 44 growth stages or environmental conditions. For example, while broad-scale phylogenetic patterns
 45 are often observed in microbial interactions, closely related bacteria may differ in the way they
 46 interact with other organisms, likely as a result of the significant genetic diversity observed in
 47 many microbial clades (e.g. (Cordero et al., 2012; Long & Azam, 2001)). Additionally, the same
 48 pair of interacting organisms can synergize or compete depending on the composition of the
 49 culture media and the growth stage of (co)-culture (e.g. (Grossart & Simon, 2007; Segev et al.,
 50 2016; Seyedsayamdost et al., 2011)). Finally, both the coarse-grained ecological classification of
 51 microbial interactions (e.g. positive/negative) and the high-resolution mechanistic view obtained
 52 using advanced physiology and omics approaches are difficult to translate into quantitative,
 53 predictive models of organismal growth and decline (Antoniewicz, 2020; Gralka et al., 2020;
 54 Zoccarato et al., 2020). Therefore, we still face fundamental challenges in interpreting and
 55 understanding the increasingly diverse and mechanistic view of microbial interactions and
 56 translating these insights into conceptual and mathematical models of cell growth and death.

57 Growth curves from batch (co)-cultures are information-rich views of microbial growth and
 58 mortality, which can provide an important link between the phenotypic outcome of microbial
 59 interactions and the underlying mechanism. Growth curves can be divided into discrete phases
 60 (lag, exponential, stationary, decline and long-term stationary phases), and can be used to extract
 61 quantitative parameters such as growth rates, lag times, etc. (Sher et al., 2011; Warringer et al.,
 62 2008). An extra layer of more subtle information may exist in the shapes of the growth curves,
 63 providing hints of important shifts in the physiology of the growing organisms, as classically
 64 demonstrated for diauxic growth in *E. coli* (Monod, 1949). Notably, while many studies of
 65 bacterial interactions focus on the exponential growth stage or on culture yield at a specific time-
 66 point (e.g. (Foster & Bell, 2012; Pacheco et al., 2021; Vetsigian et al., 2011)), fewer studies look
 67 at the shape and dynamics of the decline phases, which can provide important hints regarding
 68 the effect of interactions on the process of microbial mortality (e.g. (Christie-Oleza et al., 2017;
 69 Grossart, 1999; Ratzke & Gore, 2017)).

70 Here, we explore to what extent intra-clade diversity affects the outcome of microbial
 71 interactions, using growth curves as the major phenotypic “readout”. Our model organisms are
 72 two globally abundant clades of marine bacteria: a cyanobacterial primary producer
 73 (*Prochlorococcus*) and a heterotrophic γ -proteobacterium (*Alteromonas*). Interactions between
 74 marine phototrophs (phytoplankton, including cyanobacteria) and heterotrophic bacteria have
 75 been studied intensively, as phytoplankton are responsible for about one-half of the
 76 photosynthesis on Earth (e.g. (Cirri & Pohnert, 2019; Cole, 1982; Field et al., 1998; Ramanan et

al., 2016; Seymour et al., 2017)). Thus, phytoplankton-bacteria interactions may strongly affect community structure and function on scales from microns to thousands of kilometers (Hennon et al., 2018; J. Jeffrey Morris et al., 2012). Our model primary producer, *Prochlorococcus*, is found throughout the euphotic zone, the sunlit upper portion of the ocean. There are multiple *Prochlorococcus* clades, broadly partitioned into high-light (HL) and low light (LL) adapted ecotypes, which differ in their photosynthetic parameters and occupy different niches in the ocean (surface vs deep water, reviewed by (Biller et al., 2015)). Strains differ also in traits such as the capacity to utilize different forms of inorganic nutrients and organic matter, as well as in their interactions with heterotrophic bacteria and phage. *Alteromonas* is a clade of free-living marine bacteria, which are also partitioned into surface and deep groups (*A. macleodii* and *A. mediterranea*, respectively) (López-Pérez & Rodriguez-Valera, 2016). *Alteromonas* strains also exhibit diverse capabilities to utilize carbohydrates, acquire iron and in motility (Koch et al., 2020). Interactions between individual strains of *Prochlorococcus* and *Alteromonas* have been characterized in some detail (Aharonovich & Sher, 2016; Diner et al., 2016; Hennon et al., 2018; Hou et al., 2018; J. J. Morris et al., 2011; Roth-Rosenberg et al., 2020; Sher et al., 2011). While the phenotype and gene expression patterns during interactions vary between strains, this variability has not been explored systematically (Aharonovich & Sher, 2016; Biller et al., 2016; Sher et al., 2011). Notably, strain- and condition-dependent phytoplankton-heterotroph interactions are observed also in other systems, including *Synechococcus*, a close relative of *Prochlorococcus* (Christie-Oleza et al., 2017; Zheng et al., 2020), as well as eukaryotic microalgae (e.g. coccolithophores and diatoms, (Amin et al., 2015; Grossart & Simon, 2007; Segev et al., 2016; Seyedsayamdost et al., 2011)).

We characterized the interactions between five strains each of *Prochlorococcus* and *Alteromonas*, from the first encounter between previously-axenic strains (i.e., grown in mono-culture) and over up to 435 days of co-culture (25 phototroph-heterotroph combinations across ~1.2 years, Fig 1a, Fig S1). The culturing period spanned multiple cycles of exponential growth, culture decline and long-term nitrogen starvation (Roth-Rosenberg et al., 2020). Nitrogen stress or starvation occurs across wide swaths of the global ocean, and affects a significant fraction of the *Prochlorococcus* diversity (Saito et al., 2014; Ustick et al., 2021). We focused our analysis on *Prochlorococcus* growth and decline, which is easily measured using bulk culture fluorescence in a non-invasive manner (e.g. (Coe et al., 2016; Sher et al., 2011)). Using this dataset of 429 growth curves, as well as associated cell number measurements and genome resequencing, we ask: i) How do the interactions between *Prochlorococcus* and *Alteromonas* vary across the diversity of each organisms? ii) Do the interactions change over time (i.e. do the organisms adapt to “living together”)? iii) When, during the life-cycle of a *Prochlorococcus* batch culture, do microbial interactions have the largest impact on growth and death, and can this impact be quantified? iv) Can the combination of growth curves, cell counts and genomic data provide hints as to which potential metabolic pathways are affected by the long-term interactions?

Results and Discussion

Differences in co-culture phenotype are related to *Prochlorococcus* but not *Alteromonas* strains

The axenic cultures and the co-cultures differed primarily in their decline stage, and this was observed already from the first encounter between previously-axenic *Prochlorococcus* and *Alteromonas* (Experiment 1). In experiment 1, all co-cultures and axenic controls grew exponentially (Fig 1a, b). However, all axenic cultures showed a rapid and mostly monotonic decrease in fluorescence starting shortly after the cultures stopped growing, reaching levels below the limit of detection after ~20-30 days. In contrast, the decline of co-cultures rapidly slowed, and in some cases was interrupted by an extended “plateau” or second growth stage (Fig 1b). Across multiple experiments, 92% of the co-cultures contained living cells for at least 140 days, meaning that they could be revived by transfer into fresh media. In contrast, none of the axenic cultures were able to re-grow when transferred into fresh media after 60 days (Fig 1a, Fig S1). These results are consistent with previous studies of *Prochlorococcus* (Roth-Rosenberg et al., 2020) and *Synechococcus* (Christie-Oleza et al., 2017). Similarly, it has previously been shown that *Prochlorococcus* MIT9313 is initially inhibited by co-culture with *Alteromonas* HOT1A3, while MED4 is not (Aharonovich & Sher, 2016; Sher et al., 2011). This “delayed growth” phenotype was observed here too, was specific to MIT9313 (not observed in other *Prochlorococcus* strains) and occurred with all *Alteromonas* strains tested (Fig 1b).

Fig 1: Overview of the dynamics of *Prochlorococcus*-*Alteromonas* co-cultures from first encounter and over multiple transfers. a. Mean growth curves of the five *Prochlorococcus* strains over multiple cycles of exponential growth, culture decline and long-term starvation. The top row shows the transfer of axenic strains from experiment 1 after 60 days, with no subsequent growth observed. The bottom row shows the transfer of co-cultures from experiment 1 after 100 days (experiment 4) and additional transfer after 140 days (experiment 6), with subsequent growth. Shaded areas indicate standard error. b. High reproducibility and strain-specific dynamics of the initial contact between *Prochlorococcus* and *Alteromonas* strains (experiment 1). Three biological replicates for each mono-culture and co-culture are shown.

A visual inspection of the growth curves in Fig 1b suggested subtle yet consistent differences in the shape of the growth curve, and especially the decline phase, between different *Prochlorococcus* strains. To test this, we used the growth curves as input for a Principal Component Analysis (PCA), revealing that the growth curves from each *Prochlorococcus* strain clustered together, regardless of which *Alteromonas* strain they were co-cultured with (Fig 2). The growth curves of all high-light adapted strains (MED4, MIT9312 and MIT0604) were relatively similar, the low-light I strain NATL2A was somewhat distinct, and the low-light IV strain MIT9313 was a clear outlier, consistent with this strain being the only one initially inhibited in all co-cultures. This strain (MIT9313) stands out even when the impact of growth timing and magnitude was removed by performing the ordination after scaling and aligning the growth times. The survival of *Prochlorococcus* after extended nitrogen starvation and the clustering of the growth curves by *Prochlorococcus* but not *Alteromonas* strains were observed also in multiple subsequent transfers into fresh media, and are thus robust to the time in co-culture and the initial cell densities (Supplementary text S1).

MIT9313 belongs to the low-light adapted clade IV (LLIV), which are relatively distant from other *Prochlorococcus* strains and differ from them in multiple physiological aspects including the structure of their cell wall (Ting et al., 2007), the use of different (and nitrogen-containing) compatible solutes (Klähn et al., 2010) and the production of multiple peptide secondary metabolites (lanthipeptides, (Cubillos-Ruiz et al., 2017; Li et al., 2010)). LLIV cells also have larger genomes, and are predicted to take up a higher diversity of organic compounds such as sugars and amino acids (Yelton et al., 2016). It is intriguing that this strain, which has higher predicted metabolic and regulatory flexibilities (Kettler et al., 2007), differs from all other *Prochlorococcus* strains in the phenotype of co-culture and is the only one initially inhibited in co-culture with *Alteromonas*.

The differences between *Prochlorococcus* strains are most evident in the decline and long-term starvation stages

We next used Random Forest Classification to quantitatively determine which stages of the co-culture were most different between the *Prochlorococcus*-*Alteromonas* pairs. The algorithm accurately predicted, from the growth curves, the *Prochlorococcus* strain (10x cross validation accuracy 0.92 ± 0.17). However, it performed much more poorly when trying to predict the *Alteromonas* strain (accuracy 0.62 ± 0.58) (Fig S4a), providing further support to the observation from the PCA in Fig 2 that the shape of the growth and decline curves was driven by the specific *Prochlorococcus* strain and not by the *Alteromonas*. The classification of the *Prochlorococcus* strains relied heavily on days during the decline phase, as these times had the highest Random Forest feature importance, in agreement with the visual inspection of the growth curves (Fig S4b). Similar results were obtained in all subsequent transfers (Supplementary text S1, Fig S4a).

Focusing on the decline stage, we asked whether the differences in growth curves during long-term co-culture represent differences in the number of cells. This is because physiological differences such as in the amount of chlorophyll per cell, or the fraction of chlorotic *Prochlorococcus* cells, could also affect the growth curves (Coe et al., 2016; Roth-Rosenberg et al., 2020). Flow cytometry counts during the decline stage (after 60, 100 and 140 days) revealed a decrease in the number of *Prochlorococcus* cells over time (Fig 3a), yet the number of cells in the co-cultures was still 3-4 orders of magnitude higher than in the axenic cultures (Fig S5). This is consistent with the growth curves (Fig 1). The number of *Alteromonas* cells also declined with time in the co-cultures (Fig 3a), but the difference between the axenic cultures and the co-cultures was much less pronounced than in *Prochlorococcus* (up to ~4-5-fold after 100 days, Fig S5c). This may be due to the ability of *Alteromonas* to utilize the organic carbon from the natural-seawater-

based media, in addition to the dissolved organic matter (DOM) produced by *Prochlorococcus* (Pedler et al., 2014).

We next asked whether the carrying capacity of the long-term co-cultures, defined as the sum of the concentrations of *Prochlorococcus* and *Alteromonas* cells, depends on the *Prochlorococcus* or the *Alteromonas* strain. Put differently, one could hypothesize that the total number of cells (and hence the carrying capacity) of the co-culture could depend on the photosynthetically-fixed C provided by *Prochlorococcus*, or alternatively on the recycling capacity of the different *Alteromonas* strains. As shown in Fig 3b and 3c, on days 60 and 100, the number of both *Prochlorococcus* and *Alteromonas* cells clustered depending on the *Prochlorococcus* strain (Adonis, $p = 0.001$), but not on the *Alteromonas* strain (Adonis, $p > 0.05$). In terms of carrying capacity, there are clear differences between *Prochlorococcus* strains (Fig S6a), but not *Alteromonas* strains (Fig S6b). Interestingly, two of the *Prochlorococcus* strains, MIT9312 and MIT9313 have similar (and lower) carrying capacity. These two strains belong to different ecotypes but were isolated from the same drop of water. Taken together, these results suggest that it is the dynamics of the *Prochlorococcus* strains, including potentially the amount or composition of fixed and released organic C, which determine the total community carrying capacity.

By day 140, the cell numbers had declined significantly, and there was no longer a correlation between the number of *Prochlorococcus* and *Alteromonas* cells (Fig 3). This suggests that the system is not in steady state, with a slow yet constant reduction in carrying capacity evident by the decrease in cell numbers. Indeed, the co-cultures did not survive indefinitely - in a subsequent experiment, only 16/30 cultures could be transferred after 195 days (Fig S7a, b), and only 3/75 cultures could be transferred after 250 days (Fig S7c). Notably, MIT0604 was the strain most likely to survive transfer after these extended periods, and was also the most abundant strain after 140 days (Fig 3b). While we do not currently have an explanation for the higher survival of this strain, it is noteworthy that it is the only strain to utilize nitrate (Hackl et al., 2021).

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217 Modeling the effect of co-culture on *Prochlorococcus* mortality

218 Given that the most striking effect of co-culture was on the decline phase of the co-cultures, we
 219 asked whether we could quantify this effect, and whether we could identify an appropriate model
 220 for mathematically describing culture decline. Importantly, while the growth of bacteria has been
 221 extensively studied and modelled, the decline of bacterial cultures, representing bacterial

mortality, is much less studied, and mortality is rarely represented in ecological or biogeochemical models of microbial dynamics (Shoemaker et al., 2021). Bacterial mortality has, however, often been modelled in the context of food safety and genome evolution, using either mechanistic or descriptive approaches (Brouwer et al., 2017; Crane & Moore, 1986; Finkel & Kolter, 1999; Patra & Klumpp, 2013; Shoemaker et al., 2021) We chose to focus on four previously-described models which are relatively simple and have a clear biological interpretation (Fig 4a, Table 1). The Exponential model is the simplest and most commonly used one (Pruitt & Kamau, 1993). The Bi-exponential model is slightly more complex, representing two separate subpopulations in the community, each with its own death rate (Crane & Moore, 1986). Weibull model is a probabilistic model, modeling heterogeneous population with a diverse stress tolerance (Shoemaker et al., 2021; Van Boekel, 2002) and finally the Harmonic model employs a quadratic rate of decline which is often associated with predator-prey interaction or encounter rate (Pruitt & Kamau, 1993). When fitting each of these models to the decline phase of the growth curves, the Weibull model stands out as it has a low error for both axenic and co-cultures (Table 1, Fig 4b). Based on this model, and assuming that culture fluorescence is related to the number of non-lysed cells in the media, axenic *Prochlorococcus* cells die more than ten-fold faster than cells in co-culture (2-decimal reduction time, td_2 , is 12.58 ± 3.85 days for axenic cultures and 316 ± 337 days for co-cultures).

242 **Table 1: Mathematical description and biological interpretation of four models used to describe**
 243 **bacterial mortality**

Mathematical model	Derivative	# Params ¹	Physiological Interpretation	Axenic RMSE ² (n=13)	Co-culture RMSE (n=343)	Reference
Exponential $X = X_0 e^{-a t}$	$\frac{dX}{dt} = -aX$	1	Constant ratio of the population dies in each time point	0.26 ± 0.11	0.29 ± 0.21	(Pruitt & Kamau, 1993)
Bi-Exponential $X = X_0(f e^{-a_1 t} + (1-f) e^{-a_2 t})$	$\frac{dX}{dt} = -f a_1 X_1 - (1-f) a_2 X_2$	3	Two subpopulations with different persistence under stress and death rates	0.26 ± 0.11	0.16 ± 0.12	(Crane & Moore, 1986)
Harmonic $X = X_0 \frac{1}{1+a t}$	$\frac{dX}{dt} = -a X^2$	1	Quadratic mortality rate – dependence on cell-cell encounters	0.50 ± 0.19	0.19 ± 0.13	(Pruitt & Kamau, 1993)
Weibull $X = X_0 e^{-\frac{t^n}{a}}$	$\frac{dX}{dt} = -c t^{n-1} X^*$	2	Probabilistic model with a heterogeneous distribution of stress tolerance	0.14 ± 0.08	0.18 ± 0.11	(Van Boekel, 2002)

¹ # Params – number of free parameters

² RMSE - Root Mean Square Error

In the Weibull model, the ‘shape parameter’ (n) represents the change over time in the susceptibility of the bacterial community to stress. A shape parameter above one represents an increasing probability that cells will die as time increases (e.g. due to the accumulation of damage), whereas a shape parameter below one suggests that, over time, the cells become more resistant to damage. Axenic cultures have high shape value of 2.1 ± 0.9 , suggesting an accumulation of cell damage leading to increasing death rate, whereas that of co-cultures is 0.5 ± 0.3 (student t-test, $p < 0.001$) (Fig 4c). This suggests that, in contrast to mono-cultures, in each cycle of co-culture the *Prochlorococcus* cells are acclimating over time to the nutrient stress conditions.

While the Weibull model is useful in quantifying mortality rates and raising the hypothesis that *Prochlorococcus* cells are acclimating to starvation over time, none of the tested models was able to fully capture the intricate dynamics of culture decline (Fig 4b). In most co-cultures involving *Prochlorococcus* strains NATL2A, MED4 and MIT0604, culture decline was not monotonic, and was interrupted by a second growth phase about 40-50 days after the cultures started declining. We propose that this “second breath” represents either a consistent shift in the physiology of one of the interacting partners (e.g. by activating pathways for the salvage of previously-exuded DOM) or a change in the availability of labile DOM, e.g. following a period of mortality and lysis of *Prochlorococcus* and/or *Alteromonas*. Because the second growth phase was mostly absent in MIT9312 and MIT9313, we speculate that this phenomenon, too, is driven primarily by the specific strain of *Prochlorococcus* cells in co-culture. Notably, the Weibull model, while best representing the experimental results, is less appropriate for ecological/biogeochemical models, because the time from peak is an inherent part of the differential equation. In such cases, the harmonic model formulation may be more appropriate, as it represents mortality of co-cultures better than exponential decline with the same number of parameters, and quadratic expressions for mortality are already used in such models (Dutkiewicz et al., 2001; S. K. Moore et al., 2006; Murray A.G. and Parslow J.S., 1999).

Accumulated mutations in *Prochlorococcus* suggest a role for nitrogen use and the stringent response

During the experiments described above, we co-cultured strains of *Prochlorococcus*, which had each been previously maintained in axenic mono-cultures for hundreds or thousands of generations. We also exposed these parental strains, which are routinely maintained under conditions of exponential growth, to repeated cycles of growth, mortality and long-term survival under N starvation. To identify whether these shifts in life-style could result in genetic changes in the co-cultured populations, we re-sequenced the genomes of duplicate cultures of *Prochlorococcus* MIT9313 co-cultured with *Alteromonas* HOT1A3 after a total of 435 days in co-culture (including 3 cycles of growth, mortality and survival, Fig 5a, Fig S1). We also re-sequenced the parental strain used in these experiments, which itself had been maintained as an axenic culture for ~16 years since originally being sequenced (Rocap et al., 2003). Since the read coverage for *Alteromonas* was low, we focus here on *Prochlorococcus*.

Over the course of 435 days in co-culture (~220 generations), the two replicate MIT9313 co-cultures accumulated 32 and 39 observed mutations in protein-coding genes, compared to 57 mutations accumulated in the parental strain over ~16 years of growth at similar maximum population sizes (~2,900 generations, 15-20ml cultures, Fig 5, Fig S8 and File S1). This suggests that, during the repeated cycles of growth, decline and starvation, the mutation rate of MIT9313 in co-culture increased roughly 7-9-fold compared to the parental axenic strain growing primarily exponentially (Supplementary text S2). Strikingly, many of the mutations were observed to occur in genes involved in nitrogen utilization and stress responses. Three genes involved in nitrogen metabolism each had two or more mutations associated with them – the nitrite reductase gene *nirA* and two genes in the operon involved in the production and transport of glycine betaine (Fig 5b, c).

The parental MIT9313 strain had a frameshift mutation in the N'-terminal reductase domain of *nirA*, observed in 100% of the sequencing reads, which likely led to a truncated, inactive protein. This frameshift is not found in the evolved strain, after ~220 generations in co-culture. Since nitrite was not added to the (co)-cultures, and the *Alteromonas* genome does not contain the genes for nitrification (the ammonia monooxygenase gene), this raises the question of what are the sources of nitrite that is presumably being utilized by the evolved strain. We provide a speculative answer in Fig 5d. It has previously been shown, in the freshwater cyanobacterium *Phormidium*, that nitrite can be produced by the enzymatic dismutation of hydroxylamine (NH₂OH) in the presence of hydrogen peroxide, H₂O₂ (reaction 3 in Fig 5d, the other product is NH₃ (Bagchi & Kleiner, 1990, 1991)). NH₂OH, in turn, can be produced by the peroxidation of glutamine to glutamate (reaction 2 in Fig 5d, (Bagchi & Kleiner, 1991)). The H₂O₂ required for these reactions is produced by multiple metabolic reactions in cyanobacteria (Latifi et al., 2009), and it has previously been shown that oxidative stress is enhanced during nitrogen starvation in *Prochlorococcus* (Mcdonagh et al., 2012). Thus, an active nitrite reductase may enable MIT9313 to recycle NH₂OH, produced when the cells are under nitrogen starvation conditions (Fig 5d). Intriguingly, H₂O₂ is also produced during amino acid oxidation, the process in which cells degrade amino acids to obtain N and carbon backbones (e.g. by several types of L-amino acid oxidases, reaction 1 in Fig 5d, (Schriek et al., 2007)). It is currently unclear to what extent these amino acid degradation pathways are present and active in *Prochlorococcus*, as the streamlined genomes of these organisms lack several known enzymes (Gau et al., 2007; Schriek et al., 2007), although we note that a putative L-aspartate oxidase had a non-synonymous mutation in the evolved strains. If, however, nitrogen starvation induces amino acid oxidation and the production of H₂O₂ in *Prochlorococcus*, this would provide a mechanistic explanation for the inability of these organisms to survive long-term N starvation alone (J. Jeffrey Morris et al., 2008, 2012) Further studies are required to test this hypothesis.

Three genes in the operon for the production and import of the compatible solute glycine betaine accumulated mutations – the glycine-sarcosine methyltransferase *gsmt*, and two subunits of the glycine-betaine ABC transporter (Fig 5c). None of the mutations occurred in residues known to be important to the activity of these proteins. Under N starvation, it is possibly advantageous for the cells to reduce the production of glycine betaine, which contains N atoms, in favor of other compatible solutes such as sucrose (Klähn et al., 2010). However, during the long experiments described here, it is also likely that the osmotic strength of the growth media increased (due to

both cell lysis and the evaporation of water), and this could lead to an increased requirement for glycine betaine (Klähn et al., 2010). Further work is needed in order to differentiate between these two hypotheses. Importantly, glycine betaine provides both carbon (energy) and N to co-occurring bacteria, including SAR11 (Becker et al., 2019). Additional genes involved in N metabolism that accumulated non-synonymous mutations (one for each gene) include enzymes involved in amino acids production or degradation (the L-aspartate oxidase mentioned above, biosynthetic arginine decarboxylase and cysteine synthase) and a putative aminotransferase (Fig 5b, File S1). Genetic changes occurred in additional genes not directly related to nitrogen metabolism, but known to be involved in the nitrogen stress response in cyanobacteria. These include the glycogen synthase gene *glgA* (Tolonen et al., 2006) as well as genes involved in the synthesis of chlorophyll and other proteins from the photosynthetic apparatus.

Finally, the bacterial “alarmone” (p)ppGpp is a master regulator of bacterial stress responses, including the “stringent response” to carbon or amino acid starvation (recently reviewed by (Steinchen & Bange, 2016)). Two mutations were observed in a gene homologous to the *relA*/*spoT* bifunctional (p)ppGpp synthase/hydrolase. The product of this gene both activates the stringent response by synthesizing (p)ppGpp from GTP and represses it, by hydrolyzing the alarmone back to GTP (Hogg et al., 2004; Steinchen & Bange, 2016). Both of the mutations were in the hydrolase domain, and one of them (P95T) occurs in a residue which is predicted to take part in Mn coordination in the active site of the enzyme, potentially leading to a hydrolase-incompetent enzyme (Hogg et al., 2004; Steinchen & Bange, 2016). We speculate that this mutant may be deficient in its ability to “turn off” the stringent response, potentially leading to enhanced survival under stress.

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Fig 5: Mutations arising during co-culture in *Prochlorococcus* MIT9313. a. Schematic illustration of resequencing setup. b. Location of accumulated mutations (SNPs or indels) on the MIT9313 genome. Bars represent the number of mutations (changes between the parental and evolved populations) per 20,000 bases in both biological duplicates. Dark purple bars represent non-synonymous mutations; light grey bars represent synonymous mutations. c. Type and location of mutations in genes involved in Glycine-betaine, ppGpp and Nitrite reductase metabolism. The bar below the spoT gene shows the domain structure. d. Schematic diagram of the proposed role of nitrite reductase during long-term starvation in MIT9313. The

suggested pathway leading to the production of nitrite (NO_2^-) through hydroxylamine (NH_2OH) is shown in long term starvation compared to exponential growth. The balanced equations for this pathway are: Reaction 1 - amino acid oxidation by L-amino acid oxidases (e.g. Alanine + $\text{H}_2\text{O} + \text{O}_2 \rightarrow \text{Pyruvate} + \text{NH}_4^+ + \text{H}_2\text{O}_2$); Reaction 2 - Peroxidation of glutamine to glutamate ($\text{C}_5\text{H}_{10}\text{N}_2\text{O}_3 + \text{H}_2\text{O}_2 \rightarrow \text{C}_5\text{H}_9\text{NO}_4 + \text{NH}_2\text{OH}$); Reaction 3 - Dismutation of hydroxylamine in the presence of H_2O_2 ($2\text{NH}_2\text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{NO}_2^- + \text{NH}_4^+ + 2\text{H}_2\text{O}$). PS – photosynthesis, TCA - tricarboxylic acid cycle, 2OG - 2-oxoglutarate

Summary and future directions

As our appreciation of the richness and dynamicity of microbial communities increases, so does the understanding that interactions between microorganisms are critical to the structure and function of these communities. Elucidating the roles of microbial interactions requires both well-characterized model organisms and a way to extend the insights from such models across the diversity of organisms and environmental conditions. Our results from the highly-simplified system of multiple *Prochlorococcus* and *Alteromonas* strains provide an important step towards this goal. Using the rich information on interaction phenotypes present in the growth and decline curves, we identify conserved and strain-specific facets of these interactions. Despite the genetic diversity across the *Alteromonas* strains studied, it was the identity of the *Prochlorococcus* strain that determined the interaction phenotype. This manifests in the growth and decline rates, in the shape of the curve (primarily the decline phase), and in the number of cells living in long-term, nitrogen-starved co-culture. Testing multiple mathematical models of culture decline suggests that cells in axenic cultures are accumulating damage and dying more rapidly over time, while cells in co-culture are acclimating, likely changing their physiology and also evolving to better deal with the emergent stress. Comparing the modelled and observed curves allows for the identification of consistent and reproducible shifts in physiology, for example the “second breath” of growth followed by rapid decline in NATL2A co-cultures (Fig 4b), that can then be studied in-depth.

Under our laboratory conditions, it is likely that nitrogen starvation is the main selection force driving the phenotypic outcome of long-term co-cultures, although other stressors such as the increase in osmolarity/salinity or the accumulation of waste products cannot be ruled out. We speculate that the genetic changes observed in the MIT9313 population are primarily indicative of processes occurring in the *Prochlorococcus* cells due to nitrogen starvation and/or osmotic stress, rather than representing a direct response to the interactions with *Alteromonas*. It is possible that a major effect of co-culture with *Alteromonas* is to slow down the deterioration in *Prochlorococcus* homeostasis, providing a “buffer” that enables *Prochlorococcus* to activate its own starvation survival responses, and subsequently to genetically evolve. *Alteromonas* may similarly allow *Prochlorococcus* to adapt to light starvation (Coe et al., 2016), yet the genetic mechanisms underlying this survival remain unclear (Coe et al., 2020). However, the buffering effect of *Alteromonas* cannot be taken for granted, as it also depends on culture conditions, for example CO_2 concentrations (Hennon et al., 2018).

Once mortality has been slowed down enough to allow acclimation and/or adaptation, the differences in the resulting shape of the co-culture curves are likely due to strain-specific

differences in nitrogen metabolism. For example, *Prochlorococcus* MIT9313 produces glycine betaine as a compatible solute (unlike MIT9312 and NATL2A (Klähn et al., 2010)), and it is possible that producing large amounts of this N-containing molecule constrains the metabolic responses of this strain to N starvation differently from other strains, resulting in this strain having a distinct phenotype. The reasons for the differences between the other *Prochlorococcus* strains are less clear, but may be related to differences in the regulatory networks (e.g. those affected by the ppGpp alarmone) or the capacity to utilize organic substances released by *Alteromonas* (Yelton et al., 2016). Some of the mutations were in hypothetical genes and in some cases in multiple hypothetical genes in the same putative operon (e.g. PMT1541 and PMT1542). Future work is needed to determine whether these represent “internal” *Prochlorococcus* processes, or whether they are part of the tools used by *Prochlorococcus* to interact with co-occurring bacteria.

Why is it the identity of the primary producer (*Prochlorococcus*) rather than the heterotrophic “recycler” (*Alteromonas*) that determines the outcome of the co-culture? *A-priori*, it was reasonable to assume that the co-culture phenotype would be affected by the differences between the *Alteromonas* strains in their ability to degrade and utilize polysaccharides and a variety of other organic molecule (Koch et al., 2019, 2020). We speculate that the growth of *Alteromonas* in the co-cultures is fueled primarily by the availability of major biomass components released by *Prochlorococcus* as they die, such as proteins, amino acids and nucleotides. Such common macromolecules do not require highly specialized metabolic processes to degrade and utilize, and hence could be utilized by all of the *Alteromonas* strains. It is possible that the differences between *Alteromonas* strains may manifest when more complex macromolecules are available, e.g. from plant material, or when all of the “easy to digest” (labile) organic matter has been utilized and only complex macromolecules remain (Teeling et al., 2012). These conditions may not have been met in our experiments. Regardless of the underlying mechanism, identifying to what extent the interaction phenotype is dependent on the identity of each of the interacting organisms requires an experimental setup spanning the diversity of both.

Our results identify patterns in the interactions between clades of abundant marine phototrophs and heterotrophs, including consistent shifts in physiology occurring after extended time in co-culture. The mechanistic basis of these shifts in physiology can now be studied in-depth. Given that these shifts occur under conditions where nutrients are scarce and their availability likely depends on recycling between phototrophs and heterotrophs, it is tempting to speculate that such mechanisms may be physiologically relevant in the oligotrophic ocean, much of which is N-stressed (Ustick et al., 2021). Nevertheless, the co-cultures did not reach a steady state, and did not represent a closed system. Thus, processes not represented in these simplified laboratory co-cultures, are necessary to explain the long-term stability over decades of *Prochlorococcus* in the oceans (Malmstrom et al., 2010). Such processes could include multi-organism interactions, as natural communities are much more complex than the laboratory co-cultures, as well as oceanographic processes such as nutrient injection through deep mixing. More generally, cell mortality is intimately linked with the amount and type of recycled organic matter, yet the rate of mortality in natural communities is highly unconstrained (Beckett et al., 2021). Hence, better representation of mortality in mathematical models (e.g. the use of appropriate mortality formulations) is likely important for understanding biogeochemical cycles (Beckett et al., 2021). Finally, this study is a reminder that bacterial growth curves represent an information-rich

representation of the life and death of cells, which can be mined to identify potential physiological changes of ecological relevance.

Materials and Methods

Strains and experiment set up

Axenic *Prochlorococcus* strains MED4, MIT9312, MIT0604, Natl2A and MIT9313 were maintained in Low N Pro99 media, based on natural sea water, with the addition of 100 μ M nitrogen, 50 μ M phosphorus and trace metal elements. Under these conditions, *Prochlorococcus* enter stationary stage due to the depletion of NH_4 from the media and nitrogen starvation (Grossowicz et al., 2017). *Alteromonas* strains HOT1A3, Black sea 11, ATCC27126, AltDE1 and AltDE were maintained in ProMM media. both under constant cold while light ($27 \mu\text{Em}^{-2} \text{s}^{-1}$) at 22°C in (L. R. Moore et al., 2007; Sher et al., 2011)). Prior to the experiment, the axenicity of the *Prochlorococcus* cultures was tested by inoculating 1 ml culture into 15 ml ProMM (L. R. Moore et al., 2007)). At the start of each co-culture experiment, *Alteromonas* cells from stationary-stage cultures (24-72 hour old) were centrifuged (15 minutes, room temperature, 15,000g), the growth media decanted, and the cells re-suspended in Pro99. The *Prochlorococcus* cultures (growing exponentially) and the re-suspended *Alteromonas* cells were then counted using BD FACSCanto™ II Flow Cytometry Analyzer Systems (BD Biosciences). For each co-culture, 1×10^6 *Prochlorococcus* cells mixed with 1×10^7 *Alteromonas* cells. For mono-cultures, 1×10^6 and 1×10^7 were added for *Prochlorococcus* and *Alteromonas*, respectively. The experiment was performed using triplicate 20ml cultures in borosilicate test tubes (2.5cm diameter, 15cm length).

Fluorescence and Flow cytometry

Bulk chlorophyll fluorescence (FL) (ex440; em680) was measured almost daily using a Fluorescence Spectrophotometer (Cary Eclipse, Varian). Samples for flow cytometry were taken for cell numbers after 60, 100 and 140 days of experiment 1 (Fig S1). Samples were fixed with glutaraldehyde (0.125% final concentration), incubated in the dark for 10 min and stored in -80 °C until analysis. Then, samples were thawed at room temperature and run on a BD FACSCanto™ II Flow Cytometry Analyzer Systems (BD Biosciences). To each sample, 2 μ m diameter fluorescent beads (Polysciences, Warminster, PA, USA) were added as an internal standard. At first, the natural fluorescence of the cells (chlorophyll pigments) was examined to identify *Prochlorococcus*, identified based on the PerCP-A and FSC-H. Samples were then stained with SYBR Green I (Molecular Probes/ ThermoFisher) to a final concentration according to the manufacturer's instructions and the *Alteromonas* was counted. Data were acquired and processed with FlowJo software. Flow rates were determined several times during each running session, and the average value for a specific run was used for calculating cells per ml.

PCA ordination

PCA ordination was run on the growth curves. The Cumulative sum of Fluorescence measurements were standardized via standard scalar by subtracting the mean and scaling to unit variance of each feature. Ordination was computed via principal component analysis (PCA). Data preprocessing was done by pandas (0.25.0). Scaling and PCA was done using sklearn (0.21.2).

Growth rate

Growth was computed - by solving the equation:

$$N_t = N_0 e^{\mu(t-L)}$$

Where N_t represents the number of cells at time t , N_0 is the initial number of cells, μ is the growth rate, and L is the growth lag. The LAN transformed equation was used to compute growth rate:

$$\ln(N_t) = \ln(N_0) + \mu(t - L)$$

Linear regression was run on the growth phase, predicting $\ln(N_t)$ based on time t with $R^2 > 0.9$. The growth rate μ is the regression coefficient.

Fit to Decline models

The following functions were used to fit against the measured fluorescence:

Exponential: $FL_t = FL_{max} e^{-a(t-t_{max})}$

Bi-exponential: $FL_t = FL_{max}(f e^{-a_1(t-t_{max})} + (1-f) e^{-a_2(t-t_{max})})$

Harmonic: $FL_t = FL_{max} \frac{1}{1+a t}$

Weibull: $FL_t = FL_{max} e^{-a t^n}$

Where FL_t is the Fluorescence measured at time t , FL_{max} is the maximum fluorescence measured, t_{max} is the time when the fluorescence was highest, and a, a_1, a_2, n, f are the model parameters estimated by the fitting function.

The decline function was fit against each growth curve via `curve_fit()` function from scipy package (1.3.0), using the parameters: `method='dogbox'`, `loss='soft_l1'`, `f_scale=0.1`. Each model was fit using random initial parameter values and the initial values of 0.5 per parameter, and the fit with the lowest RSME selected. Goodness of fit was measures using root mean square error (RMSE).

In the Weibull model the time needed to reduce the population by d factors of 10 (t_{d2}) was estimated as in (Van Boekel, 2002), using the formula:

$$t_d = a (-\ln(10^{-d}))^{\frac{1}{n}}$$

Random Forest Classification

Random Forest Classification was run on the growth curves. To detect difference in the curve pattern and not timing specific differences, the curves were aligned such that max growth point are at time zero, and time points from 10 days prior to max growth to 80 days after were selected. Since the specific measurement time points were different in different experiments and samples, rolling average was used to get mean fluorescence per day, and interpolation used to fill in missing measurements. The Fluorescence measurements were standardized via standard scalar by subtracting the mean and scaling to unit variance of each feature. Random Forest model was run in 10x cross validation to classify the curves by *Prochlorococcus* and by *Alteromonas* strains. To find the most significant days in *Prochlorococcus* classification, the model was built 30 times and the mean importance of all features (i.e., measurement days) calculated. Data preprocessing was done by pandas (0.25.0). Scaling and model fitting using sklearn (0.21.2).

Statistics

Statistics were computed using the statsmodels package in python. Multi test correction was done by `t_test_pairwise()` using Bonferroni correction. Permanova analysis by `adonis2` from R vegan package (R 3.6.1, vegan 2.5-7).

DNA extraction, genome resequencing and SNP analysis

Samples for genomic DNA sequencing was collected from parental strains, before experiment 1 and after 435 days from co-cultures (Fig S1, Fig 5a). Samples collected on a 0.22 µm filter (Supor, Pall Corporation), filled with 1 ml of storage buffer (40 mM EDTA, 50 mM Tris pH=8.3, 0.75 M Soucrose) and stored at -80°C. Before extraction samples were thawed on ice and storage buffer removed carefully. DNA extracted using DNeasy PowerWater Kit (Qiagen) according to the kit's protocol. DNA samples (minimum concentration of 1ng/µL) were submitted to the MIT BioMicroCenter in 96 well plate format. Samples were prepared for sequencing using Nextera flex preparation for intact DNA (high throughput) and a size selection of 300-600bp. DNA was sequenced using MiSeq flowcell (v3, 600nt). The samples have been processed using the BMC/BCC 1.7 pipeline updated on 01/11/2019. The NexteraPE adapter and the first 20 bases were trimmed by trimmomatic 0.39-1. And quality checked using fastqc and multiqc. All samples were mapped to the reference genome and variant called using snippy (based on bwa mem mapping, freebayes variant call, snpEff annotation). *Alteromonas* reads were removed from Co-culture samples by first mapping to *Alteromonas* HOT1A3 (NCBI accession: NZ_CP012202.1, NZ_CP012203.1), then running snippy a second time on reads that were not mapped to *Alteromonas*, mapping against *Prochlorococcus* MIT9313 (NCBI accession: NC_005071.1). The number of reads per sample was 550K-610K, 98-99% of the reads were mapped successfully to MIT9313 genome yielding mean coverage of 29-42X, 99-100% of the genes had >10X coverage. Mapping quality was assessed using qualimap. SNPs were called only if the read depth was at least 10 and the Genotype Quality, the Phred-scaled marginal (or unconditional) probability of the called genotype was at least 20. All raw reads can be downloaded from the NCBI Sequence Read Archive under accession numbers PRJNA771345.

535

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545 References

- 546 Aharonovich, D., & Sher, D. (2016). Transcriptional response of *Prochlorococcus* to co-culture
547 with a marine *Alteromonas*: Differences between strains and the involvement of putative
548 infochemicals. *ISME Journal*, 10(12), 2892–2906. <https://doi.org/10.1038/ismej.2016.70>
- 549 Amin, S. A., Hmelo, L. R., Van Tol, H. M., Durham, B. P., Carlson, L. T., Heal, K. R., Morales, R. L.,
550 Berthiaume, C. T., Parker, M. S., Djunaedi, B., Ingalls, A. E., Parsek, M. R., Moran, M. A., &
551 Armbrust, E. V. (2015). Interaction and signalling between a cosmopolitan phytoplankton
552 and associated bacteria. *Nature*, 522(7554), 98–101. <https://doi.org/10.1038/nature14488>
- 553 Antoniewicz, M. R. (2020). A guide to deciphering microbial interactions and metabolic fluxes in
554 microbiome communities. *Current Opinion in Biotechnology*, 64, 230–237.
555 <https://doi.org/10.1016/j.copbio.2020.07.001>
- 556 Bagchi, S. N., & Kleiner, D. (1990). Dismutation of hydroxylamine to ammonia and nitrite by a
557 novel, membrane-bound enzyme from the cyanobacterium *Phormidium uncinatum*.
558 *Biochimica et Biophysica Acta (BBA)/Protein Structure and Molecular*, 1041(1), 9–13.
559 [https://doi.org/10.1016/0167-4838\(90\)90115-V](https://doi.org/10.1016/0167-4838(90)90115-V)
- 560 Bagchi, S. N., & Kleiner, D. (1991). Interrelationship between hydrogen peroxide, ammonia,
561 glutamine, hydroxylamine and nitrite metabolism in the cyanobacterium *Phormidium*
562 *uncinatum*. *Archives of Microbiology*, 156(5), 367–369.
563 <https://doi.org/10.1007/BF00248712>
- 564 Becker, J. W., Hogle, S. L., Rosendo, K., & Chisholm, S. W. (2019). Co-culture and biogeography of
565 *Prochlorococcus* and SAR11. *ISME Journal*, 13(6), 1506–1519.
566 <https://doi.org/10.1038/s41396-019-0365-4>
- 567 Beckett, S. J., Demory, D., Coenen, A. R., Casey, J. R., Dugenne, M., Follett, C. L., Connell, P.,
568 Carlson, M. C. G., Hu, S. K., Wilson, S. T., Becker, K. W., Mende, D. R., Armbrust, E. V.,
569 White, A. E., Ribalet, F., & Weitz, J. S. (2021). *Diel population dynamics and mortality of*
570 *Prochlorococcus* in the North Pacific Subtropical Gyre. 1–20.
- 571 Biller, S. J., Berube, P. M., Lindell, D., & Chisholm, S. W. (2015). *Prochlorococcus*: The structure
572 and function of collective diversity. *Nature Reviews Microbiology*, 13(1), 13–27.
573 <https://doi.org/10.1038/nrmicro3378>

574 Biller, S. J., Coe, A., & Chisholm, S. W. (2016). Torn apart and reunited: Impact of a heterotroph
575 on the transcriptome of *Prochlorococcus*. *ISME Journal*, 10(12), 2831–2843.
576 <https://doi.org/10.1038/ismej.2016.82>

577 Brouwer, A. F., Eisenberg, M. C., Remais, J. V., Collender, P. A., Meza, R., & Eisenberg, J. N. S.
578 (2017). Modeling Biphasic Environmental Decay of Pathogens and Implications for Risk
579 Analysis. *Environmental Science and Technology*, 51(4), 2186–2196.
580 <https://doi.org/10.1021/acs.est.6b04030>

581 Christie-Oleza, J. A., Sousoni, D., Lloyd, M., Armengaud, J., & Scanlan, D. J. (2017). Nutrient
582 recycling facilitates long-term stability of marine microbial phototroph-heterotroph
583 interactions. *Nature Microbiology*, 2(June). <https://doi.org/10.1038/nmicrobiol.2017.100>

584 Cirri, E., & Pohnert, G. (2019). Algae–bacteria interactions that balance the planktonic
585 microbiome. *New Phytologist*, 223(1), 100–106. <https://doi.org/10.1111/nph.15765>

586 Coe, A., Biller, S. J., Thomas, E., Boulias, K., Bliem, C., Dooley, K., Rasmussen, A. N., Legault, K.,
587 Keefe, T. J. O., Eric, L., & Chisholm, S. W. (2020). *Coping with darkness: The adaptive*
588 *response of marine picocyanobacteria to repeated light energy deprivation*. 1–32.

589 Coe, A., Ghizzoni, J., LeGault, K., Biller, S., Roggensack, S. E., & Chisholm, S. W. (2016). Survival of
590 *Prochlorococcus* in extended darkness. *Limnology and Oceanography*, 61(4), 1375–1388.
591 <https://doi.org/10.1002/lno.10302>

592 Cole, J. J. (1982). *INTERACTIONS BETWEEN*. 291–314.

593 Cordero, O. X., Wildschutte, H., Kirkup, B., Proehl, S., Ngo, L., Hussain, F., Le Roux, F., Mincer, T.,
594 Polz, M. F., & Roux, F. Le. (2012). Ecological Populations of Bacteria Act as Socially Cohesive
595 Units of Antibiotic Production and Resistance. *Science (New York, N.Y.)*, 337(September),
596 1228–1231. <https://doi.org/10.1126/science.1219385>

597 Crane, S. R., & Moore, J. A. (1986). Modeling enteric bacterial die-off: A review. *Water, Air, &*
598 *Soil Pollution*, 27(3–4), 411–439. <https://doi.org/10.1007/BF00649422>

599 Cubillos-Ruiz, A., Berta-Thompson, J. W., Becker, J. W., Van Der Donk, W. A., & Chisholm, S. W.
600 (2017). Evolutionary radiation of lanthipeptides in marine cyanobacteria. *Proceedings of*
601 *the National Academy of Sciences of the United States of America*, 114(27), E5424–E5433.
602 <https://doi.org/10.1073/pnas.1700990114>

603 Diner, R. E., Schwenck, S. M., McCrow, J. P., Zheng, H., & Allen, A. E. (2016). Genetic
604 manipulation of competition for nitrate between heterotrophic bacteria and diatoms.
605 *Frontiers in Microbiology*, 7(JUN). <https://doi.org/10.3389/fmicb.2016.00880>

606 Dutkiewicz, S., Follows, M., Marshall, J., & Gregg, W. W. (2001). Interannual variability of
607 phytoplankton abundances in the North Atlantic. *Deep Sea Research Part II: Topical Studies*
608 *in Oceanography*, 48(10), 2323–2344.

609 Field, C. B., Behrenfeld, M. J., Randerson, J. T., & Falkowski, P. (1998). Primary production of the
610 biosphere: Integrating terrestrial and oceanic components. *Science*, 281(5374), 237–240.
611 <https://doi.org/10.1126/science.281.5374.237>

612 Finkel, S. E., & Kolter, R. (1999). Evolution of microbial diversity during prolonged starvation.
613 *Proceedings of the National Academy of Sciences of the United States of America*, 96(7),

614 4023–4027. <https://doi.org/10.1073/pnas.96.7.4023>

615 Foster, K. R., & Bell, T. (2012). Competition, not cooperation, dominates interactions among
616 culturable microbial species. *Current Biology*, 22(19), 1845–1850.
617 <https://doi.org/10.1016/j.cub.2012.08.005>

618 Fritts, R. K., McCully, A. L., & McKinlay, J. B. (2021). Extracellular Metabolism Sets the Table for
619 Microbial Cross-Feeding. *Microbiology and Molecular Biology Reviews*, 85(1).
620 <https://doi.org/10.1128/membr.00135-20>

621 Gau, A. E., Heindl, A., Nodop, A., Kahmann, U., & Pistorius, E. K. (2007). L-amino acid oxidases
622 with specificity for basic L-amino acids in cyanobacteria. *Zeitschrift Fur Naturforschung -*
623 *Section C Journal of Biosciences*, 62(3–4), 273–284. [https://doi.org/10.1515/znc-2007-3-](https://doi.org/10.1515/znc-2007-3-419)
624 419

625 Gralka, M., Szabo, R., Stocker, R., & Cordero, O. X. (2020). Trophic Interactions and the Drivers of
626 Microbial Community Assembly. *Current Biology*, 30(19), R1176–R1188.
627 <https://doi.org/10.1016/j.cub.2020.08.007>

628 Grossart, H. P. (1999). Interactions between marine bacteria and axenic diatoms (*Cylindrotheca*
629 *fusiformis*, *Nitzschia laevis*, and *Thalassiosira weissflogii*) incubated under various
630 conditions in the lab. *Aquatic Microbial Ecology*, 19(1), 1–11.
631 <https://doi.org/10.3354/ame019001>

632 Grossart, H. P., & Simon, M. (2007). Interactions of planktonic algae and bacteria: Effects on
633 algal growth and organic matter dynamics. *Aquatic Microbial Ecology*, 47(2), 163–176.
634 <https://doi.org/10.3354/ame047163>

635 Grossowicz, M., Roth-Rosenberg, D., Aharonovich, D., Silverman, J., Follows, M. J., & Sher, D.
636 (2017). Prochlorococcus in the lab and in silico: The importance of representing exudation.
637 *Limnology and Oceanography*, 62(2), 818–835. <https://doi.org/10.1002/lno.10463>

638 Hackl, T., Laurenceau, R., Ankenbrand, M. J., Bliem, C., Cariani, Z., Thomas, E., Dooley, K. D.,
639 Arellano, A. A., Hogle, S. L., Berube, P., Leventhal, G. E., Luo, E., Eppley, J., Zayed, A. A.,
640 Beaulaurier, J., Stepanauskas, R., Sullivan, M. B., DeLong, E. F., Biller, S. J., & Chisholm, S.
641 W. (2021). Novel Integrative Elements and Genomic Plasticity in Ocean Ecosystems. *SSRN*
642 *Electronic Journal*. <https://doi.org/10.2139/ssrn.3817805>

643 Harrington, V., Lau, L., Seddu, K., & Suez, J. (2021). *Ecology and Medicine Converge at the*
644 *Microbiome-Host*. 6(4), 4–8.

645 Hennon, G. M., Morris, J. J., Haley, S. T., Zinser, E. R., Durrant, A. R., Entwistle, E., Dokland, T., &
646 Dyhrman, S. T. (2018). The impact of elevated CO₂ on Prochlorococcus and microbial
647 interactions with a ‘helper’ bacterium *Alteromonas*. *ISME Journal*, 12(2), 520–531.
648 <https://doi.org/10.1038/ismej.2017.189>

649 Hogg, T., Mechold, U., Malke, H., Cashel, M., & Hilgenfeld, R. (2004). Conformational
650 antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates
651 (p)ppGpp metabolism during the stringent response. *Cell*, 117(1), 57–68.
652 [https://doi.org/10.1016/S0092-8674\(04\)00260-0](https://doi.org/10.1016/S0092-8674(04)00260-0)

653 Hou, S., López-Pérez, M., Pfreundt, U., Belkin, N., Stüber, K., Huettel, B., Reinhardt, R., Berman-
654 Frank, I., Rodriguez-Valera, F., & Hess, W. R. (2018). Benefit from decline: The primary

transcriptome of *Alteromonas macleodii* str. Te101 during *Trichodesmium* demise. *ISME Journal*, 12(4), 981–996. <https://doi.org/10.1038/s41396-017-0034-4>

Kettler, G. C., Martiny, A. C., Huang, K., Zucker, J., Coleman, M. L., Rodrigue, S., Chen, F., Lapidus, A., Ferreira, S., Johnson, J., Steglich, C., Church, G. M., Richardson, P., & Chisholm, S. W. (2007). Patterns and implications of gene gain and loss in the evolution of *Prochlorococcus*. *PLoS Genetics*, 3(12), 2515–2528. <https://doi.org/10.1371/journal.pgen.0030231>

Klähn, S., Steglich, C., Hess, W. R., & Hagemann, M. (2010). Glucosylglycerate: A secondary compatible solute common to marine cyanobacteria from nitrogen-poor environments. *Environmental Microbiology*, 12(1), 83–94. <https://doi.org/10.1111/j.1462-2920.2009.02045.x>

Koch, H., Dürwald, A., Schweder, T., Noriega-Ortega, B., Vidal-Melgosa, S., Hehemann, J. H., Dittmar, T., Freese, H. M., Becher, D., Simon, M., & Wietz, M. (2019). Biphasic cellular adaptations and ecological implications of *Alteromonas macleodii* degrading a mixture of algal polysaccharides. *ISME Journal*, 13(1), 92–103. <https://doi.org/10.1038/s41396-018-0252-4>

Koch, H., Germscheid, N., Freese, H. M., Noriega-Ortega, B., Lücking, D., Berger, M., Qiu, G., Marzinelli, E. M., Campbell, A. H., Steinberg, P. D., Overmann, J., Dittmar, T., Simon, M., & Wietz, M. (2020). Genomic, metabolic and phenotypic variability shapes ecological differentiation and intraspecies interactions of *Alteromonas macleodii*. *Scientific Reports*, 10(1), 1–14. <https://doi.org/10.1038/s41598-020-57526-5>

Latifi, A., Ruiz, M., & Zhang, C. C. (2009). Oxidative stress in cyanobacteria. *FEMS Microbiology Reviews*, 33(2), 258–278. <https://doi.org/10.1111/j.1574-6976.2008.00134.x>

Li, B., Sher, D., Kelly, L., Shi, Y., Huang, K., Knerr, P. J., Joewono, I., Rusch, D., Chisholm, S. W., & Van Der Donk, W. A. (2010). Catalytic promiscuity in the biosynthesis of cyclic peptide secondary metabolites in planktonic marine cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 107(23), 10430–10435. <https://doi.org/10.1073/pnas.0913677107>

Long, R. A., & Azam, F. (2001). Microscale patchiness of bacterioplankton assemblage richness in seawater. *Aquatic Microbial Ecology*, 26(2), 103–113. <https://doi.org/10.3354/ame026103>

López-Pérez, M., & Rodríguez-Valera, F. (2016). Pangenome evolution in the marine bacterium *alteromonas*. *Genome Biology and Evolution*, 8(5), 1556–1570. <https://doi.org/10.1093/gbe/evw098>

Malmstrom, R. R., Coe, A., Kettler, G. C., Martiny, A. C., Frias-Lopez, J., Zinser, E. R., & Chisholm, S. W. (2010). Temporal dynamics of *Prochlorococcus* ecotypes in the Atlantic and Pacific oceans. *ISME Journal*, 4(10), 1252–1264. <https://doi.org/10.1038/ismej.2010.60>

Mcdonagh, B., Domínguez-Martín, M. A., Gómez-Baena, G., López-Lozano, A., Díez, J., Bárcena, J. A., & García Fernández, J. M. (2012). Nitrogen starvation induces extensive changes in the redox proteome of *Prochlorococcus* sp. strain SS120. *Environmental Microbiology Reports*, 4(2), 257–267. <https://doi.org/10.1111/j.1758-2229.2012.00329.x>

Monod, J. (1949). A Certain Number. *Annual Reviews in M*, 3(XI), 371–394.

Moore, L. R., Coe, A., Zinser, E. R., Saito, M. A., Sullivan, M. B., Lindell, D., Frois-Moniz, K.,

696 Waterbury, J., & Chisholm, S. W. (2007). Culturing the marine cyanobacterium
697 Prochlorococcus. *Limnology and Oceanography: Methods*, 5(OCT), 353–362.
698 <https://doi.org/10.4319/lom.2007.5.353>

699 Moore, S. K., Baird, M. E., & Suthers, I. M. (2006). Relative effects of physical and biological
700 processes on nutrient and phytoplankton dynamics in a shallow estuary after a storm
701 event. *Estuaries and Coasts*, 29(1), 81–95. <https://doi.org/10.1007/BF02784701>

702 Morris, J. J., Johnson, Z. I., Szul, M. J., Keller, M., & Zinser, E. R. (2011). Dependence of the
703 cyanobacterium Prochlorococcus on hydrogen peroxide scavenging microbes for growth at
704 the ocean’s surface. *PLoS ONE*, 6(2). <https://doi.org/10.1371/journal.pone.0016805>

705 Morris, J. Jeffrey, Kirkegaard, R., Szul, M. J., Johnson, Z. I., & Zinser, E. R. (2008). Facilitation of
706 robust growth of Prochlorococcus colonies and dilute liquid cultures by “helper”
707 heterotrophic bacteria. *Applied and Environmental Microbiology*, 74(14), 4530–4534.
708 <https://doi.org/10.1128/AEM.02479-07>

709 Morris, J. Jeffrey, Lenski, R. E., & Zinser, E. R. (2012). The black queen hypothesis: Evolution of
710 dependencies through adaptive gene loss. *MBio*, 3(2), 1–7.
711 <https://doi.org/10.1128/mBio.00036-12>

712 Murray A.G. and Parslow J.S. (1999). The analysis of alternative formulations in a simple model
713 of a coastal ecosystem. *Ecological Modelling*, 119(2–3), 149–166.

714 Pacheco, A. R., Osborne, M. L., & Segrè, D. (2021). Non-additive microbial community responses
715 to environmental complexity. *Nature Communications*, 12(1), 1–11.
716 <https://doi.org/10.1038/s41467-021-22426-3>

717 Patra, P., & Klumpp, S. (2013). Population Dynamics of Bacterial Persistence. *PLoS ONE*, 8(5).
718 <https://doi.org/10.1371/journal.pone.0062814>

719 Pedler, B. E., Aluwihare, L. I., & Azam, F. (2014). Single bacterial strain capable of significant
720 contribution to carbon cycling in the surface ocean. *Proceedings of the National Academy
721 of Sciences of the United States of America*, 111(20), 7202–7207.
722 <https://doi.org/10.1073/pnas.1401887111>

723 Pruitt, K. M., & Kamau, D. N. (1993). Mathematical models of bacterial growth, inhibition and
724 death under combined stress conditions. *Journal of Industrial Microbiology*, 12(3–5), 221–
725 231. <https://doi.org/10.1007/BF01584194>

726 Ramanan, R., Kim, B. H., Cho, D. H., Oh, H. M., & Kim, H. S. (2016). Algae-bacteria interactions:
727 Evolution, ecology and emerging applications. *Biotechnology Advances*, 34(1), 14–29.
728 <https://doi.org/10.1016/j.biotechadv.2015.12.003>

729 Ratzke, C., & Gore, J. (2017). Modifying and reacting to the environmental pH drives bacterial
730 interactions. *Modifying and Reacting to the Environmental PH Can Drive Bacterial
731 Interactions*, 136838. <https://doi.org/10.1101/136838>

732 Rocap, G., Larimer, F. W., Lamerdin, J., Malfatti, S., Chain, P., Ahlgren, N. A., Arellano, A.,
733 Coleman, M., Hauser, L., Hess, W. R., Johnson, Z. I., Land, M., Lindell, D., Post, A. F., Regala,
734 W., Shah, M., Shaw, S. L., Steglich, C., Sullivan, M. B., ... Chisholm, S. W. (2003). Genome
735 divergence in two Prochlorococcus ecotypes reflects oceanic niche differentiation. *Nature*,
736 424(6952), 1042–1047. <https://doi.org/10.1038/nature01947>

737 Roth-Rosenberg, D., Aharonovich, D., Luzzatto-Knaan, T., Vogts, A., Zoccarato, L., Eigemann, F.,
738 Nago, N., Grossart, H. P., Voss, M., & Sher, D. (2020). Prochlorococcus cells rely on
739 microbial interactions rather than on chlorotic resting stages to survive long-term nutrient
740 starvation. *MBio*, 11(4), 1–13. <https://doi.org/10.1128/mBio.01846-20>

741 Saito, M. A., McIlvin, M. R., Moran, D. M., Goepfert, T. J., DiTullio, G. R., Post, A. F., & Lamborg,
742 C. H. (2014). Multiple nutrient stresses at intersecting Pacific Ocean biomes detected by
743 protein biomarkers. *Science*, 345(6201), 1173–1177.
744 <https://doi.org/10.1126/science.1256450>

745 Schriek, S., Rückert, C., Staiger, D., Pistorius, E. K., & Michel, K. P. (2007). Bioinformatic
746 evaluation of L-arginine catabolic pathways in 24 cyanobacteria and transcriptional analysis
747 of genes encoding enzymes of L-arginine catabolism in the cyanobacterium *Synechocystis*
748 sp. PCC 6803. *BMC Genomics*, 8, 1–28. <https://doi.org/10.1186/1471-2164-8-437>

749 Segev, E., Wyche, T. P., Kim, K. H., Petersen, J., Ellebrandt, C., Vlamakis, H., Barteneva, N.,
750 Paulson, J. N., Chai, L., Clardy, J., & Kolter, R. (2016). Dynamic metabolic exchange governs
751 a marine algal-bacterial interaction. *ELife*, 5(NOVEMBER2016), 1–28.
752 <https://doi.org/10.7554/eLife.17473>

753 Seyedsayamdost, M. R., Case, R. J., Kolter, R., & Clardy, J. (2011). The Jekyll-and-Hyde chemistry
754 of phaeobacter gallaeciensis. *Nature Chemistry*, 3(4), 331–335.
755 <https://doi.org/10.1038/nchem.1002>

756 Seymour, J. R., Amin, S. A., Raina, J. B., & Stocker, R. (2017). Zooming in on the phycosphere: The
757 ecological interface for phytoplankton-bacteria relationships. *Nature Microbiology*, 2(May).
758 <https://doi.org/10.1038/nmicrobiol.2017.65>

759 Sher, D., Thompson, J. W., Kashtan, N., Croal, L., & Chisholm, S. W. (2011). Response of
760 Prochlorococcus ecotypes to co-culture with diverse marine bacteria. *ISME Journal*, 5(7),
761 1125–1132. <https://doi.org/10.1038/ismej.2011.1>

762 Shoemaker, W. R., Jones, S. E., Muscarella, M. E., Behringer, M. G., Lehmkuhl, B. K., & Lennon, J.
763 T. (2021). Microbial population dynamics and evolutionary outcomes under extreme
764 energy limitation. *Proceedings of the National Academy of Sciences of the United States of*
765 *America*, 118(33), 1–8. <https://doi.org/10.1073/pnas.2101691118>

766 Steinchen, W., & Bange, G. (2016). The magic dance of the alarmones (p)ppGpp. *Molecular*
767 *Microbiology*, 101(4), 531–544. <https://doi.org/10.1111/mmi.13412>

768 Teeling, H., Fuchs, B. M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C. M., Kassabgy, M.,
769 Huang, S., Mann, A. J., Waldmann, J., Weber, M., Klindworth, A., Otto, A., Lange, J.,
770 Bernhardt, J., Reinsch, C., Hecker, M., Peplies, J., Bockelmann, F. D., ... Amann, R. (2012).
771 Substrate-controlled succession of marine bacterioplankton populations induced by a
772 phytoplankton bloom. *Science*, 336(6081), 608–611.
773 <https://doi.org/10.1126/science.1218344>

774 Ting, C. S., Hsieh, C., Sundararaman, S., Mannella, C., & Marko, M. (2007). Cryo-electron
775 tomography reveals the comparative three-dimensional architecture of Prochlorococcus, a
776 globally important marine cyanobacterium. *Journal of Bacteriology*, 189(12), 4485–4493.
777 <https://doi.org/10.1128/JB.01948-06>

778 Tolonen, A. C., Aach, J., Lindell, D., Johnson, Z. I., Rector, T., Steen, R., Church, G. M., & Chisholm,
779 S. W. (2006). Global gene expression of *Prochlorococcus* ecotypes in response to changes
780 in nitrogen availability. *Molecular Systems Biology*, 2.
781 <https://doi.org/10.1038/msb4100087>

782 Ustick, L. J., Larkin, A. A., Garcia, C. A., Garcia, N. S., Brock, M. L., Lee, J. A., Wiseman, N. A., Keith
783 Moore, J., & Martiny, A. C. (2021). Metagenomic analysis reveals global-scale patterns of
784 ocean nutrient limitation. *Science*, 372(6539), 287–291.
785 <https://doi.org/10.1126/science.abe6301>

786 Van Boekel, M. A. J. S. (2002). On the use of the Weibull model to describe thermal inactivation
787 of microbial vegetative cells. *International Journal of Food Microbiology*, 74(1–2), 139–159.
788 [https://doi.org/10.1016/S0168-1605\(01\)00742-5](https://doi.org/10.1016/S0168-1605(01)00742-5)

789 Vetsigian, K., Jajoo, R., & Kishony, R. (2011). Structure and evolution of streptomyces interaction
790 networks in soil and in silico. *PLoS Biology*, 9(10).
791 <https://doi.org/10.1371/journal.pbio.1001184>

792 Warringer, J., Anevski, D., Liu, B., & Blomberg, A. (2008). Chemogenetic fingerprinting by
793 analysis of cellular growth dynamics. *BMC Chemical Biology*, 8, 1–10.
794 <https://doi.org/10.1186/1472-6769-8-3>

795 Weisskopf, L., Schulz, S., & Garbeva, P. (2021). Microbial volatile organic compounds in intra-
796 kingdom and inter-kingdom interactions. *Nature Reviews Microbiology*, 19(6), 391–404.
797 <https://doi.org/10.1038/s41579-020-00508-1>

798 Yelton, A. P., Acinas, S. G., Sunagawa, S., Bork, P., Pedrós-Alió, C., & Chisholm, S. W. (2016).
799 Global genetic capacity for mixotrophy in marine picocyanobacteria. *ISME Journal*, 10(12),
800 2946–2957. <https://doi.org/10.1038/ismej.2016.64>

801 Zheng, Q., Wang, Y., Lu, J., Lin, W., Chen, F., & Jiao, N. (2020). Metagenomic and metaproteomic
802 insights into photoautotrophic and heterotrophic interactions in a *Synechococcus* culture.
803 *MBio*, 11(1). <https://doi.org/10.1128/mBio.03261-19>

804 Zoccarato, L., Sher, D., Miki, T., Segrè, D., & Grossart, H. P. (2020). Comparative whole-genome
805 approach to identify traits underlying microbial interactions. *BioRxiv*, 1–53.
806 <https://doi.org/10.1101/2020.06.30.179929>

807