Dynamic macromolecular composition and high exudation rates in *Prochlorococcus*

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Abstract

Every living cell is composed of macromolecules such as proteins, DNA, RNA and pigments or cofactors. The ratio between these macromolecular pools depends on the allocation of resources within the organism to different physiological requirements, and in turn determines the elemental composition of the organism and, potentially, how it may affect biogeochemical cycles of elements such as carbon, nitrogen and phosphorus. Here, we present detailed measurements of the macromolecular composition of *Prochlorococcus* MIT9312, a representative strain of a globally abundant marine primary producer, as it grows and declines due to N starvation in laboratory batch cultures. As cells reached stationary stage and declined, protein/cell decreased by ~30% and RNA/cell and pigments/cell decreased by an order of magnitude. The decline stage was associated with the appearance of chlorotic cells which had higher forward scatter (a proxy for cell size) but lower chlorophyll autofluorescence, as well as with changes in photosynthetic pigment composition. Specifically, during culture decline divinyl-chlorophyll-like pigments emerged, which were not observed during exponential growth. These

divinyl-chlorophyll-like pigments were also observed in natural samples from the Eastern Mediterranean. Around 80-85% of the carbon fixed by *Prochlorococcus* MIT9312 (but not of a different strain, NATL2A) was released into the growth media as dissolved organic carbon under these laboratory conditions. Broadly defined, the macromolecular composition of *Prochlorococcus* MIT9312 is more similar to eukaryotic phytoplankton than to marine heterotrophic bacteria, suggesting a different set of physiological constraints determines the macromolecular composition of these two broad classes of marine microorganisms.

Keywords: Prochlorococcus, Photosynthetic pigments, Macromolecular pools, exudation, ribosomes

Introduction

Like every cell on Earth, phytoplankton and bacterioplankton are composed of several classes of biological macromolecules that function together to maintain the cells structure and activity (Geider and La Roche 2002, Finkel et al. 2016). Proteins are the most abundant macromolecule (in terms of mass), responsible for most of the metabolic functionality of the cells. DNA stores hereditary information, whereas RNA (as mRNA or other regulatory RNAs) accesses this stored data and (as rRNA and tRNAs) uses it to produce protein. Lipids form the membrane(s) that separate the cell from its environment and, in the case of photosynthetic organisms, host the molecular machinery used to harvest light for energy. This molecular machinery includes, in addition to proteins, also photosynthetic pigments and other metabolites. Finally, various storage molecules (including some lipids, carbohydrates, polyphosphate and other storage molecules) allow cells to retain energy and elements for future use, and a host of other metabolites are involved in all forms of cellular function. The relative amount of each of these classes of macromolecules changes between different phytoplankton groups (Vargas et al. 1998, Geider and La Roche 2002, Finkel et al. 2016), and may also change within a single type of organism in response to environmental conditions (Vargas et al. 1998, Liefer et al. 2019). For example, the relative amount of chlorophyll-a per cell changes in response to light intensity (e.g. (Moore et al. 1995)) and may also change in response to nutrient starvation (Lourenço et al. 1998, Liefer et al. 2019). This affects the ability of photosynthetic cells to harvest light and fix carbon, impacting cell physiology and likely the interactions of the cell with other organisms, e.g. through the release of fixed organic carbon (Dubinsky and Berman-Frank 2001). Similarly, it has been suggested that the number of ribosomes per cell is a strong determinant of cellular growth rate, and is itself affected by temperature and nutrient (particularly phosphorus, P) availability (e.g. (Elser et al. 2003, Garcia et al. 2016, Martiny et al. 2016)). Thus, to some extent, the macromolecular composition of the cell is affected by, and may shed light on, the allocation of cellular resources to different functions.

The basic building blocks of biological macromolecules such as amino acids and nucleic acids have distinct elemental composition, and the macromolecular composition of the cell is thus intimately linked with its elemental composition (Geider and La Roche 2002, Finkel et al. 2016, Garcia et al. 2016, Liefer et al. 2019). In phytoplankton, proteins generally comprise the largest pool in terms of cell biomass (dry weight) as well as nitrogen and carbon (N and C) pools (Lourenço et al. 1998, Vargas et al. 1998, Geider and La Roche 2002, Finkel et al. 2016). Other macromolecules such as Nucleic acids and pigments contribute less to total cell biomass yet contribute significantly to the pools of N and phosphorus (P) (Geider and La Roche 2002, Finkel et al. 2016, Liefer et al. 2019). Thus, the macromolecular composition of the cell will determine its elemental composition, which in turn will determine the effect of the cell on biogeochemical cycles of elements. Over the last ~85 years (Redfield 1934) significant advancements have been made in our understanding of how the elemental ratios in marine organisms change over time and space, how they are affected by cell physiology, and how they are related to surrounding nutrient element concentrations (e.g. (Martiny et al. 2013, Gruber and Deutsch 2014)). Significantly less is known about the dynamics of the macromolecular composition of marine organisms, which underlie elemental composition. Similarly, most of the models of ocean communities and their effect on biogeochemistry are formulated using elements as the model currency rather than macromolecules (Omta et al. 2009, Goebel et al. 2010, Talmy et al. 2016). In order to obtain a better understanding of the dynamics of the macromolecular composition of organisms, and to link these to elemental ratios, experimental measurements are needed, particularly in abundant marine organisms.

Here, we measure the cellular pools of major macromolecules (protein, DNA, RNA and pigments) in laboratory batch cultures of *Prochlorococcus*, a highly abundant pico-cyanobacterium which is responsible for ~8.5% of the oceanic photosynthesis (Partensky and Garczarek 2010, Flombaum et al. 2013, Biller et al. 2014). *Prochlorococcus*, as a clade, comprise multiple genotypes, each different in

its physiology, genome structure and oceanic niche (Johnson et al. 2006, Biller et al. 2014). Many aspects of the physiology of the *Prochlorococcus* clade have bene intensively studied, ranging from its elemental composition (Bertilsson et al. 2003, Martiny et al. 2013) through its photophysiology (Moore et al. 1995, Moore and Chisholm 1999, Ting et al. 2002, Steglich et al. 2003, Komatsu et al. 2016) to its system-wide transcriptomic response to changes in environmental conditions (e.g. (Martiny et al. 2006, Tolonen et al. 2006, Thompson et al. 2011, Aharonovich and Sher 2016). Yet, detailed analyses of the macromolecular composition of the cells are lacking, even though changes in the elemental composition of *Prochlorococcus* that are observed in response to environmental conditions are in fact due to changes in the macromolecular composition of the cells (E.g. (Bertilsson et al. 2003, Van Mooy et al. 2006, Martiny et al. 2013, Martiny et al. 2016)). The strain we focus on, MIT9312, represents the high-light adapted HL-II clade which is the most abundant clade in the surface waters of large parts of the ocean (Bouman et al. 2006, Johnson et al. 2006). We chose to follow batch cultures of *Prochlorococcus* MIT9312 under conditions where entry into stationary stage occurs when the N source in the media is depleted for several reasons. First, N stress affects *Prochlorococcus* in large parts of the ocean (e.g.(Saito et al. 2014)), and significant information is available as to the physiological, transcriptomic and evolutionary responses of the cells to acute N starvation (Steglich et al. 2001, Moore et al. 2002, Tolonen et al. 2006, Gilbert and Fagan 2011, McDonagh et al. 2012, Read et al. 2017, Berube et al. 2019, Szul et al. 2019). Second, long-term analyses of batch cultures may identify cellular physiological processes not seen when cells are growing exponentially or exposed to sudden or short-term nutrient starvation (e.g. (Christie-Oleza et al. 2017, Roth-Rosenberg et al. 2019)). Indeed, it is an open question to what extent stationary-phase and culture decline in batch cultures represent conditions found in nature, yet we show that a pigment identified in our cultures during the culture decline stage is found in the oceans, raising the possibility that some cells in nature are indeed found in these physiological stages. Finally, we measured, in addition to the cellular macromolecular

pools, also the concentrations of TOC in the media, extracting from these measurements estimates of the release of DOC from the cells. This release may be due to passive exudation, active excretion and cell lysis (reviewed by (Thornton 2014)). Some of the released macromolecules are available for subsequent utilization by other organisms, whereas another part contributes to a huge pool of long-lived oceanic DOM, potentially impacting global biogeochemical cycles (Hansell et al. 2009).

Materials and Methods

Growth conditions and experimental procedure

To follow the growth and macromolecular composition of *Prochlorococcus* over the various stages of laboratory batch culture, axenic *Prochlorococcus* MIT9312 cultures were grown in 1 liter bottles of Pro99 media (Moore et al. 2007) where the NH_4 concentration was lowered to 100µM, leading to cessation of cell growth due to N starvation (Grossowicz et al. 2017). The experiment included 15 bottles, three of which were used for routine culture monitoring and the other 12 were used to collect samples at four time-points, corresponding to exponential growth (t=6 days), early stationary stage (t=10 days), late stationary stage (t=13 days) and culture decline (t=15 days). Samples were also collected from the starter culture to represent t=0. The number and timing of the samples collected for full analysis were selected in order to provide appropriate data for subsequent modeling (Omta et al. 2017). This experimental design was used to minimize the chance for contamination of the axenic cultures with heterotrophic bacteria. The cell numbers of the cultures used for macromolecular analysis were never statistically different from those of the cultures used for monitoring (Students t-test).

Prior to the beginning of the experiment, cells from a mid-exponential culture were counted by flow cytometry (FACSCantoII, BD), and diluted in the new media to an initial cell density of 10^6/ml. Cultures were maintained under constant light (22 µE), at 24.5 ±1ºC. 1mM $NaHCO_3$ was supplemented

at three different time points (T=0, T=7, T=11 days) to verify that the cultures are not carbon-limited (Moore et al. 2007, Grossowicz et al. 2017). Bulk culture fluorescence was measured in samples collected aseptically from the three monitoring bottles using a Carey Eclipse spectrofluorometer (Ex 440nm/Em 680nm). Samples for cell counting were fixed in 0.25% glutaraldehyde, kept in the dark for 10 minutes and transferred to a -80°C freezer for subsequent flow cytometry analysis.

Measurements of macromolecular pools and photosynthetic pigments.

At each of the five time-points selected, cells were collected by filtration. For protein measurements, cells were gently filtered on 0.22µm polycarbonate filters and kept in -80°C until extraction. For protein extraction, the filters were incubated in 500µl lysis buffer (50mM Tris pH6.8, 5mM EDTA, 2%SDS) on a lab rotator at 37°C for 20'. Following incubation, samples were sonicated in water bath for 10', centrifuged at 12,000g in a table-top micro-centrifuge for 5', and the supernatant liquid taken for measurement using the Bicinchoninic acid assay (BCA, Sigma). Nucleic acids were collected similarly on 0.22 µm Supor-200 Membrane Disc Filters (25 mm; Pall Corporation) and preserved in storage buffer (40 mM EDTA, 50 mM Tris pH 8.3, 0.75 M sucrose) and RNA Save (Biological Industries) for DNA and RNA measurements, respectively. Samples were kept at -80°C until extraction. DNA was extracted from the filters according (Massana et al. 1997). Total RNA was extracted using the mirVana miRNA kit (Ambion, Austin, TX, USA) as described in with lysozyme treatment for better lysis (Tolonen et al. 2006), follow by removal of contaminating DNA from total RNA by Turbo DNase (Ambion). Nucleic acids quantity measured using Qubit 2.0 (Invitrogen). For pigment analysis, samples were collected on Glass Fiber filters (25 mm GF/F, Whatman) and kept in -80C until extraction. Glass fiber filters were used, rather than polycarbonate filters, as the latter are not compatible with many extraction methods for pigments. Nevertheless, preliminary experiments using

a culture of *Prochlorococcus* MED4, which is similar in size to MIT9312, showed that with the vacuum pumps we used approximately 99.9% of the cells were retained on the GF/F membrane (Marmen et al. 2018), and thus the difference in pore size has minimal effect on the results. Cells were extracted in 100% methanol at 25°C for 2h. The cell extracts were clarified with Syringe filters (Acrodisc CR, 13 mm, 0.2 µm PTFE membrane, Pall Life Sciences), and kept at -20ºC until analysis by column chromatography on a C8 column (1.7 µm particle size, 2.1 mm internal diameter, 50 mm column length, ACQUITY UPLC BEH, 186002877) using an ACQUITY UPLC system equipped with a Photodiode Array detector (Waters). The chromatography protocol was based on LOV method (Hooker *et al.* 2005) modified for UPLC. A linear gradient was applied over 14 minutes, where solvent A was 70:30 methanol:0.5 M ammonium acetate, and solvent B was B. 100% methanol. The flow rate was 0.5 ml/min, and the column and injection heating were set to 50ºC and 30ºC, respectively. To identify and calculate pigments concentration, the following standards were used: Divinyl chlorophyll A (DVchlA), chlorophyll B (chlB), chlorophyll C (chlC), zeaxanthin (Zea), α- carotene (α-car), Divinyl Protochlorophyllide (MGDVP), Pheophorbide a, Pheophythin a and Chlorophyllide a. All standards were from DHI, Denmark.

Measurements of inorganic nutrients and TOC

The GF/F filtrates (see above) were kept in polypropylene at -20ºC until analysis. For measurements, samples were adjusted to 25ºC and diluted 4-fold in DDW to reduce the salt level. NH_4 (NH 3-N) was measured using an HI 96715 meter (Hanna instrument), and PO_4 was measured by HI 96713 meter (Hanna instrument). In both cases, low concentration kits were used. For Total Organic Carbon (TOC) analysis, samples were collected into cleaned, pre combusted, acid washed (10% HCL) 40ml vials and HCl was added to remove dissolved inorganic C before preservation at -20ºC. Before analysis, samples were combusted at 680°C using catalytic (platinum) oxidation method in oxygen-rich environment. TOC analysis was performed using TOC-L analyzer (Shimadzu ASI-L Autosampler, Columbia, MD).

For inorganic carbon (DIC), samples were collected in 14ml dark glass vials with screw caps and 75µl (0.05% v/v) saturated $HgCl_2$ solution (Dickson et al. 2007) was added to the samples. For alkalinity, 60 ml samples were collected in glass vials. All samples were kept in 4°C until measurement. CO_2 was extracted from the samples by acidification with phosphoric acid (H_3PO_4, 10%) using a custom, automated CO_2 extractor and delivery system (AERICA by MARIANDA) using high grade N_2 as a carrier gas, connected online with a LiCor 6252 IR CO_2 analyzer. Measurements were calibrated using seawater Certified Reference Materials (CRMs) from Dickson's lab. In order to check and correct for drift the CRM was run after every 4 samples.

Model of *Prochlorococcus* resolving key macromolecular pools

We formulated a mathematical model of the growth of a generalized axenic phytoplankton cell, which resolves the macromolecular pools measured in the experiments – protein, RNA, DNA and photosynthetic pigments, as well as N storage and dissolved organic carbon (Figure S3). The model is formulated in units of N in each macromolecular pool, with the C in each pool determined by the elemental ratios in each type of macromolecule (Geider and La Roche 2002). In this model, proteins act as enzymes in the synthesis of RNA, DNA and Chl, whereas RNA acts as an enzyme for the synthesis of proteins. The total organic carbon in the media (TOC, the sum of cellular C and DOC) is determined by the balance between photosynthesis and respiration. Since the cells are defined in terms of N, and the TOC is defined in units of C, the concentration of DOC is an emergent property of the model, and enables us to test under what conditions the model can reproduce high DOC production rates. We fit the model to the experimental data presented below, using the Metropolis-Hastings algorithm (Metropolis et al. 1953). A more detailed description of the model, including equations, can be found in the supplementary information, and the model itself (written in FORTRAN) is available on https://github.com/AWO-code/DalitDaniel_Model.

Results

Dynamics of cell numbers and macromolecular composition during growth and nitrogen starvation

To determine to what extent the macromolecular composition of *Prochlorococcus* changes between the different physiological states of a batch culture, we grew strain MIT9312 in laboratory batch cultures where the N:P ratio of the media was set to 2, thus leading to cessation of growth due to N starvation (Grossowicz et al. 2017). The bulk culture fluorescence, often used to monitor phytoplankton growth in a non-invasive way, increased exponentially until day 10, after which the culture fluorescence declined rapidly, with no clearly observable stationary stage (Fig 1a). The decline of the culture fluorescence coincided with the reduction of soluble NH_4 to below detection threshold (<10uM), while PO_4 and DIC levels remained high throughout growth and decline (Fig 1c, d). Cell counts by flow cytometry also showed an increase in the cell numbers until day 10, with a growth rate (μ) of 0.353 day^{-1} (±0.0239) and doubling time of 1.968 days (±0.129). However, unlike the bulk culture fluorescence that decline rapidly after this day, the cell numbers remained relatively stable for an additional 3-4 days, before starting to decline. During this period, a clear population of cells with low chlorophyll autofluorescence (low-fl) emerged, identified by flow cytometry (Fig 1b, 67% low-fl cells on day 13 and 88% on day 15, (Roth-Rosenberg et al. 2019)). These low-fl ("chlorotic") cells were also stained much more weakly than the high-fl ones with the dye Sybr Green, which binds nucleic acids (primarily dsDNA but also ssDNA and RNA, Fig S1b). At the same time, an increase in Sybr-Green staining was observed in the high-fl cells, potentially related to the arrest of the cells in G2 stage (Sup Fig. S1, (Zinser et al. 2009)). At the same time, the median forward scatter, a proxy for cell size, increased for both the high-fl and low fl cells. (Fig S1c).

Based on the observed fluorescence curves (Fig 1a), we sampled cells during exponential stage (day 6), early stationary stage (day 10), late stationary stage (after bulk culture fluorescence had started to

decline but while cell numbers were still stable, day 13) and culture decline (day 15) (Table S1). An additional estimate of the macromolecular pools from exponentially-growing cells was obtained from the starter cultures used to start the experiment (treated here as day 0). The per-cell content of proteins and RNA were relatively stable during exponential phase (0-10 days), but was reduced during the stationary and decline phases (days 13 and 15) (Fig 1e-f). The reduction in RNA/cell (a drop of approximately 75% between days 10 and 13) was much larger than that of protein/cell (10-30%). The total photosynthetic pigment concentration per cell started declining earlier than RNA or protein, with a drop of approximately 75% between days 6 and 10, as the cells moved from exponential growth to early stationary stage (Fig 1g).

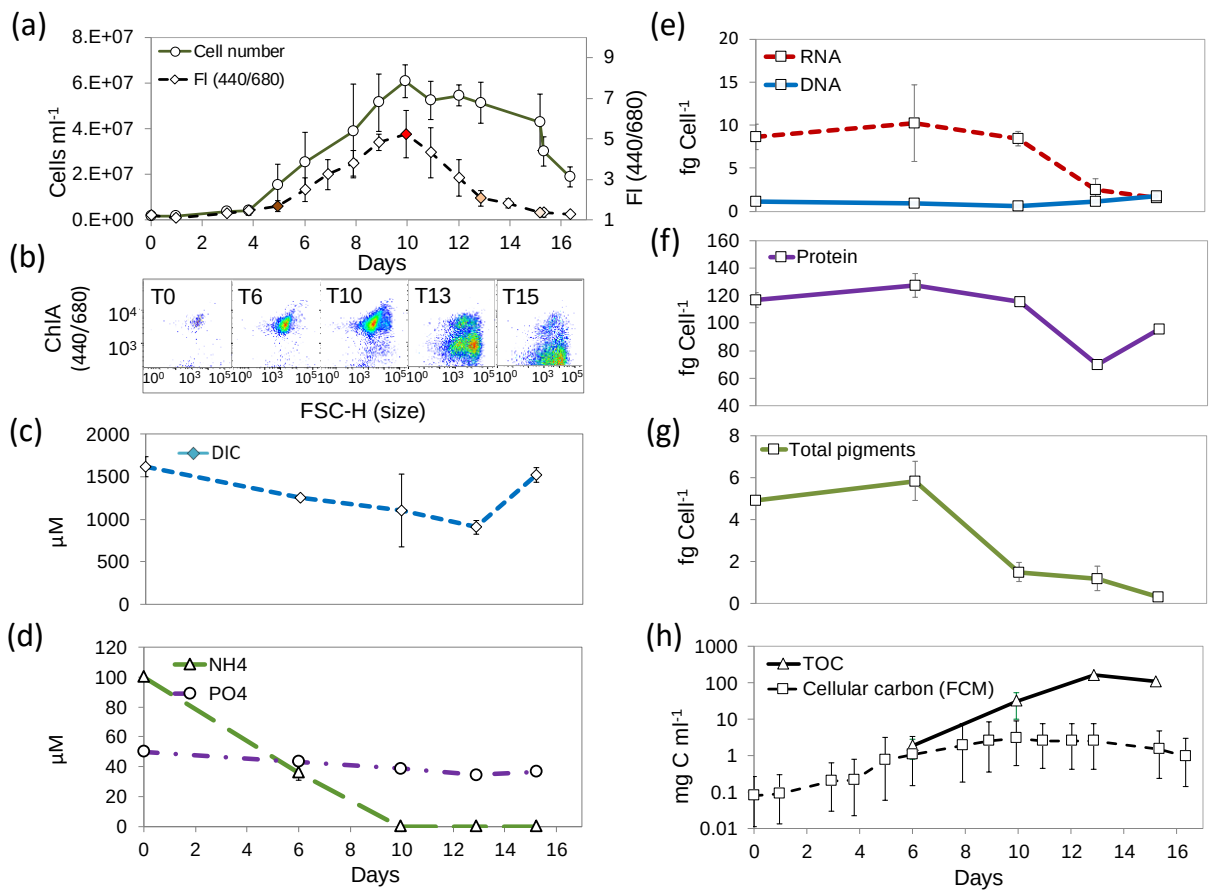

Figure 1: Dynamics of culture growth, plateau and death in batch *Prochlorococcus* MIT9312 cultures reaching stationary growth due to nitrogen starvation. (a) Cell numbers and

fluorescence curves of MIT9312 population shows a log-phase growth until day 10 than stationary and decline of the culture. (a) Flow cytometry scatter gram of specific measurements points. (c-d) Analysis of external inorganic nutrients shows that NH_4^+ drops below detection limit, while PO_4 and DIC are still available. Thus, the cultures enter stationary stage due to N starvation. Results are means and ranges of triplicate cultures. (e-g): Changes in the cell quotas of major macromolecules-DNA and RNA (e) proteins (f) and total pigments (g). While protein/cell is relatively stable, pigment and RNA pools drop as cells become starved and the culture declines. (h) Accumulation of large amounts of DOC. Triangles show measured Total Organic Carbon (TOC), squares show calculated C in cell biomass, based on flow cytometry counts and on per-cell C quotas. The error bars shown represents uncertainty due to the differences in estimates of per-cell C quotas between studies (Table 1), with the squares showing a value of 50 fg $cell^{-1}$.

Changes in photosynthetic pigment composition across the different growth stages

The reduction in chlorophyll autofluorescence per cell suggests changes to the photosynthetic machinery of the cells as they enter stationary stage and during culture decline. Indeed, as shown in Fig 2a, in addition to a reduction in the total pigment quota per cell (Fig 1g), the ratio of some accessory pigments to divinyl chlorophyll A (DVchlA) changed over time. Chlorophyll B and a chlorophyll-C like pigment were reduced compared to DVchlA after day 10, whereas the ratio of zeaxanthin (and also α-carotene) to DVchlA increased after this day (Fig 2b). In addition, on day 10 and later, UPLC several peaks were observed close to that of DVchlA that differed from it in their retention time. These peaks were not due to overloading of the UPLC column (were not seen when higher amounts of pigments were injected),and were reproducibly observed in other cultures from multiple

Prochlorococcus strains. One of these peaks (marked with an arrow in Fig 2c), which was especially prominent, had an absorption spectrum similar to that of DVchlA (Fig 2d) but was not one of the known degradation products (pheophytin-A eluted later, whereas phaeophorbide A, chlorophyllide A and divinyl protochlorophillide A all eluted much earlier, consistent with the loss of the phytol moiety). Assuming a similar molar absorption rate as DVchlA, this pigment (which may be a DVchlA', an epimerization product of DVchlA, (Komatsu et al. 2016)) comprised as much as 21% of the total DVchlA pigments on day 15, at the end of the decline stage. Importantly, pigments with the same elution time and absorbance were repeatedly observed in samples collected from the oligotrophic Eastern Mediterranean Sea, as demonstrated in Fig S2.

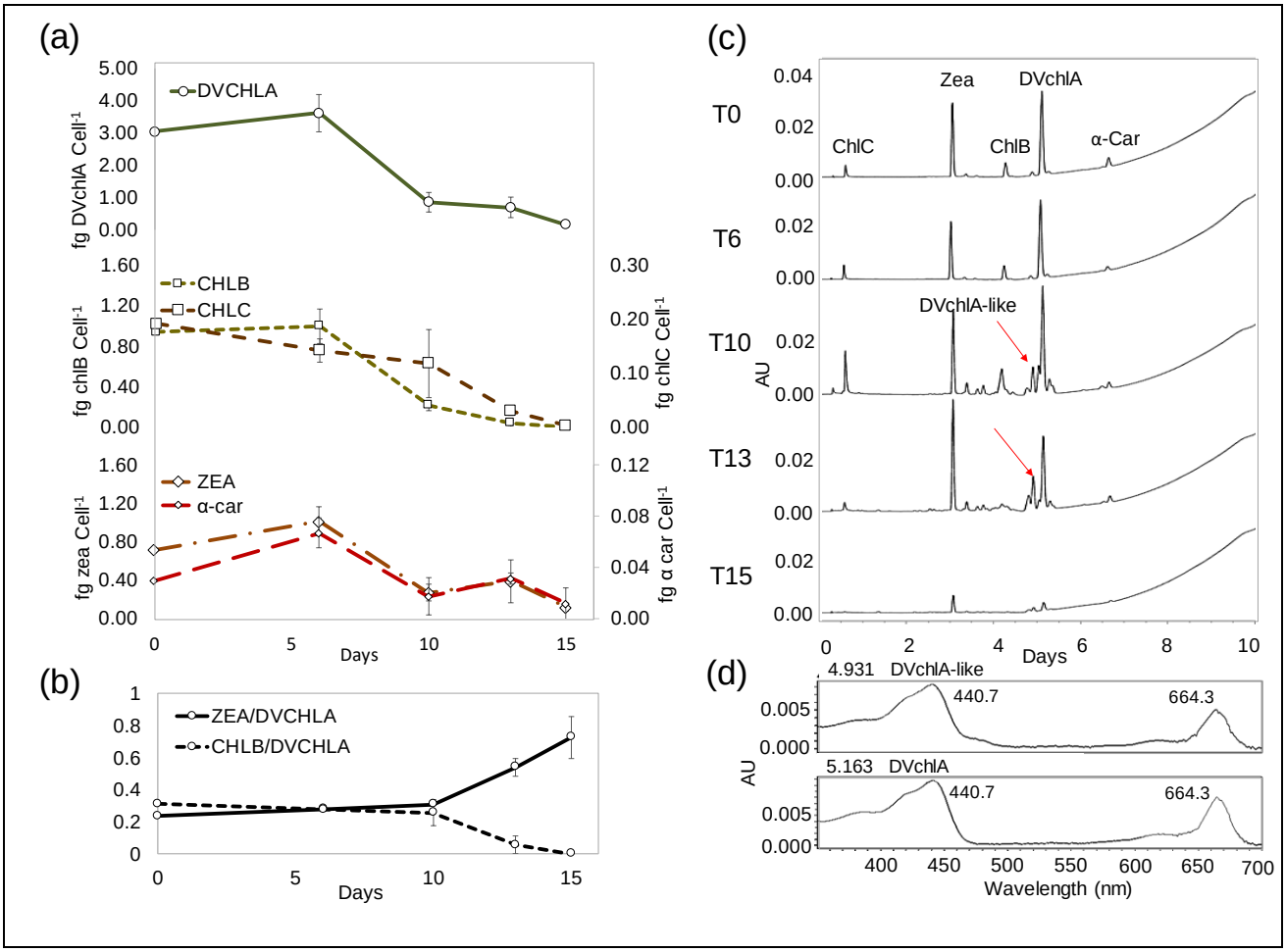

Figure 2. Pigments content and relative composition change during growth. (a) Changes in the major per-cell pigment quotas. Divinyl chlorophyll A - DVchlA, chlorophyll B - chlB, a chlorophyll C-like pigment - chlC , zeaxanthin - Zea, and α- carotene - α-car. (b) The relative abundance of zea to DVchlA increases, while that of chlB to DVchlA decreases. (c) UPLC chromatograms at different times show the appearance of additional pigments during stationary phase. The pigment marked with an arrow has an absorption spectrum similar to that of DVchlA (panel d), but differs from it in its retention time.

Reproducibly high rates of DOC exudation in *Prochlorococcus* MIT9312.

Part of the organic carbon fixed by phytoplankton is released from the cell due to exudation and cell mortality, and this organic carbon provides sustenance for co-occurring heterotrophic bacteria. To assess how much organic C is released by *Prochlorococcus*, we measured the total organic C in our cultures, and compared it to the amount of C expected to be in the particulate fraction based on the number of cells and the carbon quota of *Prochlorococcus* from previous studies (10-130 μg/cell, Table 1). During exponential stage (day 6), the measured TOC was within the range expected based on cell numbers and C quota, however, during the stationary and decline phases the amount of TOC was up to 23-fold higher than could be explained by cell biomass, suggesting a large amount of organic C was released into the growth media (Fig 1g). To check if the high release of organic carbon is common to different *Prochlorococcus* strain, we repeated this experiment using both strain MIT9312 and strain NATL2A which belongs to the Low Light I clade (Biller et al. 2014). High extracellular concentrations of organic C (up to 13-fold higher than the predicted cellular biomass) were observed again for

MIT9312, whereas strain NATL2A that grew under the same N starvation conditions showed TOC values much lower than MIT9312, within the expected range of cell quota (Fig 3).

Table 1: *Prochlorococcus* Carbon biomass estimated in different studies, expanded from (Bertilsson et al. 2003)

Strain	**fg C cell^{-1}**	**Analytical method**	**Reference**
Natural population, Atlantic Ocean	10-70	X ray microanalysis	(Grob et al. 2013)
PCC9511, EQPAC, SB, GP2, SARG, NATL1	17-34	X ray microanalysis	(Heldal et al. 2003)
PCC9511	17-38	POC	(Claustre et al. 2002)
MED4	17-40	POC	(Fu et al. 2007)
VOL7(MED4), VOL8(MIT9515), VOL29, VOL4 (MIT9312), VOL1(MIT9215), UH18301	18-55	POC	(Martiny et al. 2016)
MIT9301, MED4, NATL2A, MIT9313	30-33 (HL), 45 (LLI), 80 (LLIV)	Macromechanical mass sensor, assuming C is 50% of wet biomass	(Cermak et al. 2016)
MIT9301, MED4, MIT9313	44-51 (HL), ~180 (LLIV)	POC	(Becker et al. 2014)

MED4	46-61	POC	(Bertilsson et al. 2003)
CCMP1378(MED4)	49+/-9	POC	(Cailliau et al. 1996)
Generic (0.6 µm diameter cell)	53	Cell size and volume-carbon conversion (470 fg C/µm$^{3)}$	(Campbell et al. 1994)
Natural population, Atlantic Ocean	56	Cell size and volume-carbon conversion (325 fg C/mm3)	(DuRand et al. 2001)
Generic (0.8 µm diameter cell)	59	Cell size and volume-carbon conversion (220 fg C/mm3)	(Li et al. 1992)
MIT9211, MIT9215, MIT9312, MED4, SS120, MIT9302, MIT9303, MIT9313	61-94	Primary production, potential overestimation (does not account for respiration and exudation)	(Moore 1997)
MIT9312	67-123	Macromolecular pools	This study
MED4, MIT9401, SS120	78+/-27	Dry weight, assuming C is 50% of biomass	(Shaw et al. 2003)
Generic (0.8 µm diameter cell)	124	Cell size and volume-carbon conversion (294 fg C/cell)	(Veldhuis and Kraay 1990)

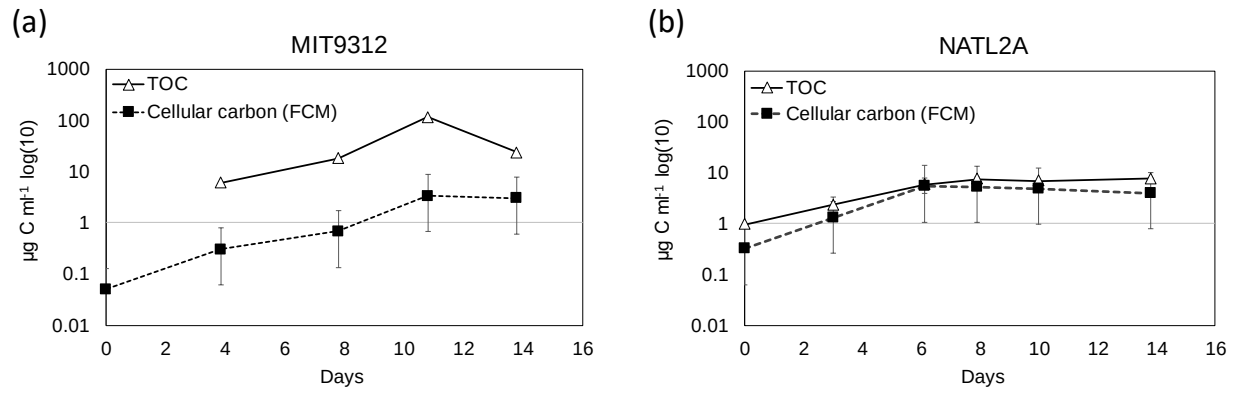

Figure 3: Differences in exudation rates between two axenic *Prochlorococcus* strains, MIT9312 (a) and NATL2A (b). The two strains were grown under identical conditions. Triangles show measured Total Organic Carbon (TOC), squares show calculated C in cell biomass, based on flow cytometry counts and on per-cell C quotas. The range shown represents uncertainty due to the differences in estimates of per-cell C quotas between studies (Table 1), with the square markers showing a value of 50 fg cell^{-1}. Panel a shows an independent experiment from that shown in Fig 1h, yet the high release of DOC is consistent.

Figure 3: Differences in exudation rates between *Prochlorococcus* strains.

Discussion

Quantitative measurements of cellular parameters, including macromolecular composition and elemental quotas, are fundamental to our understanding (and modeling) of microbial systems.

However, measurements of the per-cell C quota of *Prochlorococcus*, performed using different techniques, range over an order of magnitude (Table 1, (Bertilsson et al. 2003)). In this study, we directly measured the cell quotas of DNA, RNA and photosynthetic pigments at different physiological stages of laboratory batch culture in *Prochlorococcus* MIT9312. With some assumptions, these measurements can be used to constrain upper and lower bounds of the total cell quotas of C, N and P (Supplementary text, Supplementary Table 1). Measuring these macromolecular pools requires less biomass and can be performed at higher throughput compared to measurements of total particulate or dissolved C and N (Supplementary text). Assuming a lower bound on the C:N ratio of 6 (Bertilsson et al. 2003, Martiny et al. 2013, Martiny et al. 2016), the cellular C quota we estimate (~67-123 fg $cell^{-1}$, Supplementary Table 1) is at the upper range of measurements from other studies (Table 1), and is driven primarily by the high (and mostly stable) measurements of protein/cell. Like any other experimental result, these measurements are sensitive to the experimental design and the methodology used. For example, while the method we used for protein determination (BCA) is relatively insensitive to the effects of salts and detergents (if these are incorporated into the standard curve), the quantitative results can be affected by, for example, the protein standards used (e.g. Bovine Serum Albumin or Immunoglobulin). Similarly, our estimates of DNA/cell are about one-third those expected from cell number and the genome size of MIT9312 (1.7Mbp, (Kettler et al. 2007)). Experimental measurements of DNA/cell that are not consistent with genome size have been observed also in other studies (Zimmerman et al. 2014), and may be caused by differences in the DNA extraction efficiency across organisms or culture conditions. Finally, the *Prochlorococcus* population is not homogenous, containing at times two distinct sub-populations of cells, with different flow cytometry signatures (as discussed in detail below, Fig S1, see also (Coe et al. 2016, Roth-Rosenberg et al. 2019)**)**. We did not measure the amount of the macromolecular pools in each sub-population separately, due to the difficulty in obtaining sufficient biomass from non-fixed cells sorted by fluorescence-activated cell

sorting. With these uncertainties in mind, in the following sections we use the measurements of macromolecular pools at different growth stages, as well as the estimates of cellular C, N and P, to discuss the relationship between cell physiology, macromolecular pools and elemental budgets. Doing so, we note the need for an in-depth comparison of measurements between labs, growth stages and methods, perhaps along the lines of similar studies used to calibrate pigment measurements (e.g. (Claustre et al. 2004)).

Dynamic changes in the macromolecular composition of *Prochlorococcus* in batch culture – changes in the average cell or sub-populations?

In our laboratory batch cultures, *Prochlorococcus* grow exponentially until they run out of their nitrogen source (NH_4), at which stage they stop growing, enter a short stationary stage and finally die. During this time, the cultures lose their green color, the cell population became heterogeneous, and populations of low-fl cells emerge, forming the majority of the population as the cultures decline (Fig 1a, Fig S1a). The low-fl cells also stain weaker with the dye Sybr-green (Fig S1), which binds both DNA and RNA (albeit with a higher affinity for DNA, (Martens-Habbena and Sass 2006)). We propose that a significant part of the changes in the mean per-cell concentration of chlorophyll and RNA is caused by the presence of these low-fl cells, rather than by a gradual change in the concentration of these pools per cell. In the model cyanobacteria *Synechococcus elegantus* PCC7942 and *Synechocystis* PCC6803, the loss of culture chlorophyll (chlorosis) is part of a developmental program to generate resting stages, that can survive long-term starvation (e.g. (Sauer et al. 2001, Klotz et al. 2016)). In contrast, we have recently shown that *Prochlorococcus* cultures that become chlorotic cannot revive when nutrients are added, unless co-cultured with a heterotrophic bacterium (Roth-Rosenberg et al. 2019). Nevertheless, some of the chlorotic cells are still active (photosynthesize and take up NH_4,

(Roth-Rosenberg et al. 2019)). Thus, we interpret the changes observed in the macromolecular pools of the MIT9312 cultures, and primarily those that can be attributed to the low-fl (chlorotic) population, as physiological responses to N starvation that nevertheless are not sufficient to enable the cells to survive long-term nutrient stress.

In parallel to the observed loss of culture fluorescence and the appearance (and dominance) of low-fl cells, changes were also in the per-cell concentration and composition of the photosynthetic pigments (Fig 1h, Fig 2). Specifically, the mean relative amount of zeaxanthin increases compared to DVchlA, whereas the relative amount of chlorophyll B decreases (Fig 2b). Similar changes were observed in nutrient-replete batch cultures of other *Prochlorococcus* (strains MED4 and SS120) in response to increasing light intensities (Moore et al. 1995). One possible interpretation, which we deem less likely, is that the changes in the pigment ratio as the cells enter stationary phase and start declining are due to increased light levels as many of the cells become chlorotic and the cultures become clear. An alternative interpretation is that the increase in relative abundance of zeaxanthin is a general stress response, as these pigments may act as antioxidants in cyanobacteria (Zhu et al. 2010). Indeed, an increase in the zeaxanthin-DVchlA ratio was observed upon nitrogen starvation also in an additional *Prochlorococcus* strain, SS120 (Steglich et al. 2001). The decrease in chlorophyll B can also be interpreted independently from a potential change in light levels. The reduction in DVchlB could simply be related to the deactivation of the photosystems, in agreement with previous studies that showed a decrease in photosynthetic activity (e.g. Fv/Fm) and psbA protein levels upon nitrogen starvation (Steglich et al. 2001, Tolonen et al. 2006). Alternatively, the reduction in DVchlB may be part of a cellular mechanism to reduce the use of N-containing pigments under N starvation, as chlorophylls contain nitrogen, whereas xanthophylls do not. This is consistent with other mechanisms whereby *Prochlorococcus* conserve N, including changes in the transcriptional profiles (Tolonen et al.

2006), use of shorter transcripts (Read et al. 2017) and, potentially, "thrifty" N use in proteins expressed in response to N starvation (Gilbert and Fagan 2011).

The emergence of the DVchlA-like pigment is currently unexplained, although this pigment may be an epimerization product of DVchlA (DVchlA'). DVchlA' has been previously observed in several *Prochlororoccus* strains (Komatsu et al. 2016), and in our study the DVchlA-like pigment is associated with culture decline. Generally speaking, harvesting cells from late exponential stage or early stationary stage is a common practice when high biomass is required, yet as discussed above the physiology of the cells can change during stationary phase in comparison with exponentially-growing cells. Additionally, it is currently unclear to what extent processes observed in late stages of laboratory batch cultures, such as nutrient starvation and the subsequent changes in cell physiology, occur in nature. Nevertheless, the observation that a pigment with the same retention time and absorption spectrum as the DVchlA-like pigment observed at-sea suggests that at least some of the cells in nature may be undergoing processes similar to those we observe in the laboratory batch cultures.

The high-fl cells also exhibit an increase in mean forward scatter, a proxy for cell size. This occurs despite the overall reduction in RNA, pigments and (to a lesser extent) protein (Fig 1e, f), which are the major N-containing macromolecular pools. Assuming the increase in forward scatter represents an increase in cell biomass, this suggests that the cells are accumulating primarily C-rich macromolecules such as storage carbohydrates and lipids, leading to an increased C:N ratio. A similar response to nitrogen starvation has been predicted by mathematical models of N-starved cells (Grossowicz et al. 2017) and observed in other phytoplankton species (Hu et al. 2008, Breuer et al. 2012, Liefer et al. 2019). If these processes occur in nature, they could impact the C:N ratio of DOM released from dead *Prochlorococcus* cells (e.g. (Agusti and Sanchez 2002, Llabrés et al. 2011, Ribalet et al. 2015)) as well as POM exported by these cells (Richardson and Jackson 2007, Lomas and Moran 2012, Zhao et al. 2017).

The macromolecular composition of *Prochlorococcus* MIT9312 is more akin to eukaryotic phytoplankton than to heterotrophic bacteria

Phytoplaknton and heterotorphic bacteria are inherently different in their life histories, and this likely determines and constrains the relative allocation of energy and elements to different molecular functions. Phytoplankton need to allocate resources to the photosynthetic and carbon fixation machineries and are likely less constrained by the availability of carbon (which can be derived from photosynthesis) compared to other elements such as nitrogen. Eukaryotic phytoplankton are also often larger than bacteria, and thus are less constrained by the internal volume of the cell. Heterotrophic bacteria, in contrast, do not need to allocate resources to photosynthesis but are potentially limited by C and by cell size. As shown in Fig 4, the limited number of measurements of macromolecular pools in marine microorganisms available in the literature supports the idea phytoplankton and bacteria allocate resources differently.

Generally speaking, phytoplankton have a lower RNA/protein ratio than heterotrophic bacteria with the same RNA/DNA ratio. Assuming that the ribosomes of phytoplankton and bacteria have similar maximal rates of protein production (chain elongation), this suggests that phytoplankton ribosomes are working at a higher relative capacity. In phytoplankton, a significant amount of protein production needs to be invested in maintaining the photosynthetic apparatus, for example in the replacement of the core proteins (Zavřel et al. 2019). An alternative (but non-exclusive) explanation is that heterotrophic bacteria (primarily copiotrophic ones) maintain more ribosomes than needed, using the "spare" production capacity to allow rapid response to changes in growth conditions. Such a strategy has been demonstrated experimentally for *E. coli* (e.g. (Li et al. 2018). Notably, some bacteria, such as *Psychrobacter Mor119*, *Halomonas Hal146*, *Vibrio Vib2d* and *Ruegeria Oce241*, are more similar in

the RNA/protein ration to phytoplankton (Fig 4). The reason for this similarity, which may represent a lower ability to allocate ribosome resources to rapid changes in gene expression, is unclear, and may depend on the specific experimental conditions employed in their study (Zimmerman et al. 2014). While *Prochlorococcus* is a bacterium (prokaryote), its RNA/protein and RNA/DNA ratios at different stages of the growth curve all fall within the range of those from eukaryotic phytoplankton. The single measurement form *Synechococcus* is also closer to other phytoplankton (Fig 4). This could be the result of both the need to invest in maintaining the photosystem and, potentially, that *Prochlorococcus* may have a lower ability compared to fast-growing heterotrophs to "ramp up" their translation in order to rapidly respond to changes in environmental conditions.

Nevertheless, *Prochlorococcus* may still maintain some unused ribosome capacity. In our study, protein/cell remained relatively stable, with a decline of no more than ~30% as the culture declined. RNA/cell, in contrast, dropped by more than 80% over the same period (Fig 1). The decline in RNA/cell also started earlier. This suggests either that processes resulting in the loss of protein (e.g. degradation, exudation or excretion) were strongly reduced in stationary and decline phase cells, or that the actual rate of protein production per ribosome increased in declining cultures compared to exponentially-growing ones. These two explanations - decrease in loss processes or increase in ribosome efficiency - are not mutually exclusive, but evidence is lacking for either of them in most organisms, including *Prochlorococcus*.

Figure 4. RNA/DNA and RNA/protein compared to those of other phytoplankton and bacteria.

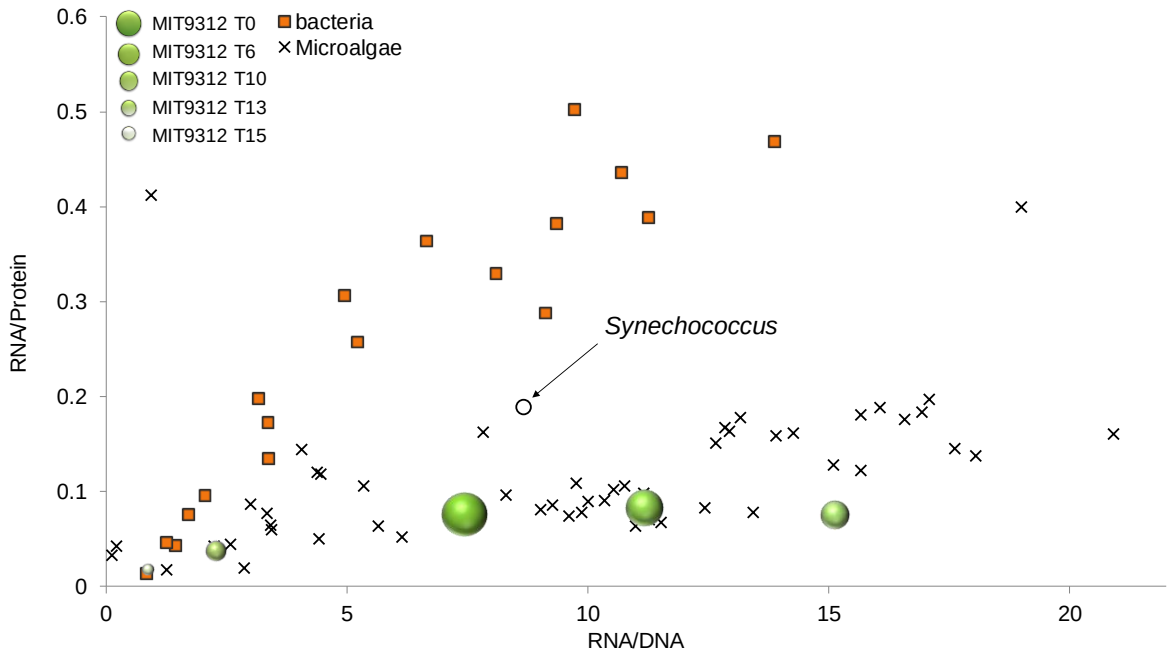

Figure 4. RNA/DNA and RNA/protein compared to those of other phytoplankton and bacteria. The ratios in phytoplankton (from (Finkel et al. 2016)), presented in cross markers , show a high diversity of RNA/DNA, while relatively low diversity of RNA/Proteins compositions. Heterotrophs bacteria however (squares) are highly diverse in both RNA/Proteins and RNA/DNA composition (Churchward et al. 1982, Neidhardt et al. 1990, Zimmerman et al. 2014). *Prochlorococcus* MIT9312 at different growth phases (spheres of different sizes) are in fact similar to microalgae rather to bacterial macromolecule composition. The most phylogenetically related cyanobacteria, Synechococcus WH7803, marked in circle, shows similarity to MIT9312 in the RNA/DNA but not in the RNA/Protein ratios.

DOC accumulation and its potential mechanism

One of the surprising observations of this study was the high rate of accumulation of DOC during the exponential growth of MIT9312. By the time the cultures reached stationary stage (day 13 in Fig 1h

and day 11 in Fig 3a) the particulate organic carbon (i.e. cell biomass) was only 3-7% of the total organic carbon (considering the two experiments shown in Fig 1h and 3h, and assuming a cell C quota of 120 fg cell^{-1}). This suggests that 80-85% of the organic carbon fixed by *Prochlorococcus* in two separate experiments was released as dissolved organic carbon. Pervious experimental studies where DOC release was measured using ^{14}C as a tracer suggested that 2-24% of the primary productivity is released (Bertilsson et al. 2005, Lopez-Sandoval et al. 2013). However, indirect assessments suggest that a somewhat higher fraction of the fixed carbon is lost from the cells. First, a comparison of carbon uptake through photosynthesis and growth rate in *Prochlorococcus* MED4 over a diel cycle suggested that ~30% of the fixed carbon is released or respired (Zinser et al. 2009). Second, the theoretical growth rate of MIT9312 based on the expected yield of photosynthesis (taking into account published values for illumination, cross section, efficiency and chlorophyll/cell, (Moore and Chisholm 1999)) is almost two-fold higher than measured (expected μ=0.64 day^{-1}, compared to 0.35±0.02 day^{-1} measured during exponential stage, see Supplementary Information for more details). Third, we used a mathematical model of a generalized phytoplankton, resolving the macromolecular pools measured in this experiment, to test whether the exudation rates we observed are feasible. The model was able to reproduce quite well some of the experimental results, including the accumulation of DOC (observed as an increase in TOC, Figs S3-S5, Supplementary Table S2). This model reproduced well the cell density, the uptake of inorganic N and the reduction after stationary stage in the cellular quotas of RNA and chl-a, although the timing in the decline of the protein quota per cell was predicted to be earlier than observed experimentally. Importantly, while the values of C fixation per unit chl-a in most of the model simulations were within the range of the maximum photosynthesis measured for *Prochlorococcus* (up to 10-20mol C[g divinyl Chl-a]$^{-1}$day^{-1}, (Moore and Chisholm 1999, Bruyant et al. 2005, Felcmanová et al. 2017)), this value had to vary over the dynamics of batch culture for the model to reproduce well the accumulation of TOC, increasing transiently as the cells entered the

decline phase (Fig S5). This phenomenon – an increase in photosynthetic efficiency in cells entering chlorosis (potentially caused by a reduction in shelf-shading, (Felcmanová et al. 2017)) - has not, to the best of our knowledge, been described previously. An alternative explanation is that the respiration rates decrease in relation to growth rate as the cells enter chlorosis (Fang et al. 2019). The current model formulation cannot distinguish between these two possibilities, and thus experimental measurements of photosynthesis and respiration across batch culture are required to test these two alternative model predictions. Taken together, these considerations show that while the rates of DOC release recorded here are 2-3 fold higher than those demonstrated experimentally (e.g. using ^{14}C release assays), they are within the range of growth rates possible based on theoretical considerations.

The differences between our results, based on accumulation of DOC over time, and estimates of exudation based on ^{14}C partitioning between the particulate and dissolved phases, may also be due to loss of volatile substances during the acidification and subsequent venting of inorganic ^{14}C during radioactive incorporation experiments. Indeed, *Prochloroccoccus* produce several volatile substances (e.g. organic acids, organohalogens, methane and isoprene, (Shaw et al. 2003, Bertilsson et al. 2005, Hughes et al. 2011, Bižić-Ionescu et al. 2019)), although to what extent these substances would be lost from the media during the protocol for measuring ^{14}C incorporation is unclear. Thus, our results showing high release of DOC are consistent with theoretical calculations and (some) previous studies, but also highlight the need for additional studies, specifically addressing DOC release using different analytical methods.

The DOM released by *Prochlorococcus* can include, in addition to C, also other elements such as N and P. While we did not measure this experimentally, a rough estimate of the loss processes N and P can be obtained by comparing the total amount of N and P in the original media and the estimated

amount of N and P in the cells at their maximum density. Since *Prochlorococcus* do not fix dinitrogen or perform denitrification, the N "missing" from the cell biomass when reach stationary stage under N-starved conditions should represents organic N that were lost from the cells in a format that is not available for re-uptake (i.e. refractory to *Prochlorococcus*). At the peak of the *Prochlorococcus* growth in our experiment, on day 10, the cellular N accounted for 79% of the total N in the media. Given that, on that day, NH_4 in the media was depleted, this suggests that ~20% of the N had already been released from the cells in a form that is not available for re-utilization. Thus, while C rich, the DOM released by *Prochlorococcus* MIT9312 contains significant amounts of other elements, likely (at least in part) in the form of proteins, amino acids, DNA, RNA and nucleotides. This is consistent with studies in *Synechococcus* (Christie-Oleza et al. 2015, Christie-Oleza et al. 2017, Zhao et al. 2017) and with the effect of *Prochlorococcus*-derived DOM on gene expression in natural communities (Sharma et al. 2014). With regard to P, a similar calculation shows that only ~3.2% of the total P in the media was found in cellular biomass, while the PO_4 concentration in the media dropped from 50μM to 34-36μM, a reduction of ~30%. Thus, either we have significantly underestimated total cellular P (i.e. there is an additional, unmeasured pool besides RNA, DNA and phospholipids), or a significant amount of P has been converted from PO_4 to extracellular DOP.

While our understanding of biogeochemistry is framed in terms of elements, cells actually release the same macromolecules from which they are built, e.g. the proteins, DNA and RNA measured in this study. Previous studies have suggested that *Prochlorococcus* release, in addition to the volatile compounds described above, also TEP (Iuculano et al. 2017), and that at least part of the produced DOM (the hydrophobic fraction that can be analyzed following solid-phase extraction) differs from strain to strain, and even between replicate cultures (Becker et al. 2014). *Prochlorococcus* have also been shown to release vesicles containing proteins and nucleic acids (Biller et al. 2014). Furthermore, DOC loss from cultures can be due not only to passive leakage from the cells (exudation) or active

release (excretion) but also to lysis of dead cells (reviewed by (Thornton 2014)), with the latter process likely releasing most or all of the cellular macromolecular content. Several studies have indicated that up to ~20% of the cells in exponentially-growing *Prochlorococcus* cultures may be dead (Agusti and Sanchez 2002, Hughes et al. 2011). Therefore, it is likely that all of these processes (exudation, excretion, vesicle production and mortality) contribute to DOM formation.

Several studies have suggested that high rates of DOC release occur in oligotrophic regions of the oceans, and that DOC, rather than POC, contributes significantly to carbon export from the photic zone in these areas (Guyennon et al. 2015, Roshan and DeVries 2017). It is tempting to speculate that *Prochlorococcus* may be at least partially responsible for this DOC accumulation, based on the high rates of DOC production observed here by *Prochlorococcus* MIT9312, a member of the HL-II clade that numerically dominates large areas of the oligotrophic ocean (Bouman et al. 2006, Johnson et al. 2006). In support of this speculation, DOM released by *Prochlorococcus* in the surface waters of the Pacific Ocean (which was dominated by the HL-II clade) has been suggested to provide as much as 75% of the daily organic carbon produced (Ribalet et al. 2015), although it is unclear to what extent mortality compared to exudation feeds this organic pulse. It has also been suggested that DOM derived from *Prochlorococcus* contributes a significant amount to deep sea DOM (Zhao et al. 2017). We note, however, that DOC production rates may differ based on the physiological states of the cells and the surrounding nutrient conditions. For example, similar to our study, Bertilsson and co-workers also noted differences between two strains of *Prochlorococcus*, MIT9312 and MED4, with the former releasing approximately twice as much DOC (Bertilsson et al. 2005), and Becker and co-workers documented differences in the composition of released DOM (Becker et al. 2014). Future work is required to determine the to what extent DOM production changes between strains and across culture conditions, what are the mechanisms of this process, and to what extent assessments of DOM release under lab conditions can be extended to the ocean.

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Author contributions

DR, DA, AWO, MJF and DS conceived the study and designed the experiments. DR and DA performed the experiments and analyzed the samples. AWO performed the modelling. DR and DS wrote the manuscript with contributions from all authors.

Competing interests

The authors declare no competing interests

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803