Nutrient recycling facilitates long-term stability of marine microbial phototroph–heterotroph interactions

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Biological interactions underpin the functioning of marine ecosystems, be it via competition, predation, mutualism or symbiosis processes. Microbial phototroph–heterotroph interactions propel the engine that results in the biogeochemical cycling of individual elements, and they are critical for understanding and modelling global ocean processes. Unfortunately, studies thus far have focused on exponentially growing cultures in nutrient-rich media, meaning knowledge of such interactions under in situ conditions is rudimentary at best. Here, we have performed long-term phototroph–heterotroph co-culture experiments under nutrient-amended and natural seawater conditions, and show that it is not the concentration of nutrients but rather their circulation that maintains a stable interaction and a dynamic system. Using the Synechococcus–Roseobacter interaction as a model phototroph–heterotroph case study, we show that although Synechococcus is highly specialized for carrying out photosynthesis and carbon fixation, it relies on the heterotroph to remineralize the inevitably leaked organic matter, making nutrients circulate in a mutualistic system. In this sense we challenge the general belief that marine phototrophs and heterotrophs compete for the same scarce nutrients and niche space, and instead suggest that these organisms more probably benefit from each other because of their different levels of specialization and complementarity within long-term stable-state systems.

Marine primary production is mainly driven by microscopic phytoplankton, because phototrophic picocyanobacteria and picoeukaryotes contribute to almost all photosynthesis that takes place in the vast photic zones of the oligotrophic open ocean1. Numerically, picocyanobacteria (that is, Prochlorococcus and Synechococcus) are the most abundant primary producers on Earth2, and their abundance is predicted to increase due to climate change3. Marine planktonic microorganisms generally show stable cell numbers, with growth and loss largely balanced4,5. Ultimately, all primary production will be converted into particulate or dissolved organic matter (DOM), which becomes the main source of carbon and energy for the complex marine food web6,7. DOM is thought to be generated by cell death, viral lysis and inefficient grazing, but living organisms are also known to be, per se, inevitably or ‘intentionally’ leaky, for example, through the production of extracellular vesicles8, active efflux processes or, simply, permeable membrane leakage. In this sense, phytoplankton drive bacterial community dynamics because they are the main suppliers of organic matter9. Interestingly, despite reports showing how marine picocyanobacteria acquire simple organic compounds such as amino acids or glucose10,11, these organisms generally cannot use complex DOM as they lack the necessary pool of secreted enzymes12,13, potentially creating a dependence on remineralized nutrients released by the heterotrophic community.

Here, we set out to understand the long-standing anecdotal observation that cultures of phototrophic organisms are more robust and have a much longer lifespan when indigenous heterotrophic bacterial ‘contaminants’ are present, in both natural and nutrient-amended seawater. In phototroph–heterotroph systems, heterotrophs clearly benefit through the acquisition of organic matter as a source of carbon and energy14. From the phototroph perspective, previous studies concluded that the interaction is based on the heterotrophic scavenging of oxidative stress15–18, supply of vitamins19–24 or the exchange of growth factors25. These are clearly important physiological dependency events that have occurred in strong mutualistic interactions during the evolution of streamlined genomes in stable environments26. However, these may only be species-specific co-evolution events and do not explain the general aspects that underpin phototroph–heterotroph interactions. For example, unlike Prochlorococcus, which is deficient in the catalase–peroxidase mechanisms involved in oxygen radical protection and detoxification17, and picoeukaryotes, which are usually deficient in vitamin production27, marine Synechococcus are capable of both physiological functions, but still require heterotrophic microbes for long-lasting growth, as shown in this study.

Previous reports analysing marine picocyanobacteria–heterotroph interactions have focused on exponential phase cultures in nutrient-rich media18,28–31, but we focus here, for the first time, on the long-term stable-state growth phase, both in rich media and natural oligotrophic seawater. We provide robust evidence to suggest that mutualistic phototroph–heterotroph interactions are based on nutrient cycling. The phototroph inherently produces and leaks organic matter in the form of photosynthate, which is deoxygenated to unlock inorganic contaminants17, and picoeukaryotes, which are usually deficient in vitamin production27, marine Synechococcus are capable of both physiological functions, but still require heterotrophic microbes for long-lasting growth, as shown in this study.

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Results
Physiology of long-term Synechococcus-heterotroph co-cultures. We assessed the growth of axenic Synechococcus sp. WH7803 in artificial seawater (ASW) medium with different Roseobacter strains as well as various heterotrophic bacterial ‘contaminant’ strains obtained from other non-axenic Synechococcus cultures (Fig. 1a,b). In the presence of various heterotrophs, Synechococcus sp. WH7803 cultures could persist for over 10 months, whereas axenic cultures died after 4–6 weeks. No significant difference in growth rate or maximum cell densities was observed for Synechococcus sp. WH7803 when grown in the presence/absence of the heterotroph during the exponential phase (38.2 and 38.7 h doubling times, respectively) (Fig. 1c). Some of the heterotrophs tested seemed less efficient in stably sustaining Synechococcus in long-term culture, but after an initial death phase of the phototroph, the co-culture re-established high cell density (Fig. 1a; and Tropicibacter sp. in Fig. 1c). Ruegeria pomeroyi DSS-3 also sustained growth of other axenic Synechococcus strains (WH8102 and WH7805) (Supplementary Fig. 1 and Supplementary Note 1).

The heterotrophs in co-culture with Synechococcus reached high cell densities, despite the absence of any exogenous addition of an organic source of carbon and energy, vitamins or nutrient requirements (Fig. 1d and Supplementary Note 2). Hence, there is a clear benefit from this mutualistic interaction through the acquisition of photosynthate. This beneficial effect is unsurprisingly higher if Synechococcus is alive and continuously producing and releasing organic matter (Supplementary Fig. 2).

The death of axenic Synechococcus cultures in ASW medium is due to the accumulation of organic matter. The death of Synechococcus cultures was not due to the accumulation of reactive oxygen species, variation in pH, or to the lack of auxotrophic supplements (for example, vitamins) or nutrients (Supplementary Note 3 and Supplementary Fig. 3), but rather was due to the accumulation of organic matter. The accumulation of carbohydrate and protein was monitored over time in axenic Synechococcus cultures, with up to 200 and 400 µg ml\(^{-1}\) of carbohydrate and protein, respectively, being produced after 35 days (Supplementary Fig. 4a). The large quantity of protein compared to carbohydrates indicates that Synechococcus produces N-rich DOM. This build-up of DOM (0.06% wt/vol when considering only protein and carbohydrates) is potentially toxic to Synechococcus. Indeed, via the addition of known concentrations of exogenous organic matter (that is, peptone and yeast extract, Supplementary Fig. 4b), we observed that concentrations of DOM between 0.01 and 0.1% wt/vol accelerated the death of axenic Synechococcus sp. WH7803 cultures, whereas the presence of R. pomeroyi DSS-3 rescued the culture, except when the concentration of organic carbon was too high (1% wt/vol).

Molecular interactions in ASW phototroph–heterotroph co-cultures assessed by proteomics. Comparative proteomics analysis (Supplementary Tables 1 and 2) suggests the following:

(1) Synechococcus is a specialized biological system for carrying out photosynthesis and CO\(_2\) fixation. Almost 45% of Synechococcus sp. WH7803 protein abundance has a direct role in photosynthesis or CO\(_2\) fixation (Table 1). This percentage increases when other processes directly linked to photosynthesis and CO\(_2\) fixation are included (for example, ATP synthase complex, oxidative phosphorylation or biosynthetic pathways of tetrapyrrole ring systems).

(2) Comparative analysis of Synechococcus when grown in axenic culture versus in co-culture showed a small number of differentially expressed proteins (14 and 27 proteins, respectively,

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**Figure 1** | Synechococcus sp. WH7803 grown in axenic (ax) culture and in co-culture with different heterotrophs. a, Synechococcus sp. WH7803 grown for 24 weeks with heterotrophs isolated from other non-axenic Synechococcus strains and identified by partial 16S rDNA sequencing. C1 (Tropicibacter sp.) and C2 (Stappia sp.) were isolated from Synechococcus sp. CC9311 and B1 (Paracoccus sp.), and B2 (Marinobacter sp.) and B3 (Muricauda sp.) were isolated from Synechococcus sp. BL107. b, Synechococcus sp. WH7803 grown for 40 weeks with different Roseobacter strains: R1 (R. pomeroyi DSS-3), R2 (Ruegeria lascaeurilensis ITI1157), R3 (Dinoroseobacter shibae DFL12) and R4 (Roseobacter denitrificans OCh114). The highly pigmented cultures observed in week 1 are typical of a healthy culture, whereas white (bleached) cultures indicate dead Synechococcus. Representative cultures of three biological replicates are shown in a and b. c, Growth curves of Synechococcus sp. WH7803 in ASW in axenic culture and in the presence of heterotrophs R. pomeroyi DSS-3 or Tropicibacter sp. The arrow indicates the time point at which cells were collected for proteomic analysis (35 days). d, Growth curves of heterotrophs R. pomeroyi DSS-3 and Tropicibacter sp. in the presence of Synechococcus sp. WH7803. Synechococcus cell counts were determined by flow cytometry and heterotrophic growth was monitored by c.f.u. on marine agar plates. Three culture replicates (n = 3) of each condition are presented in c and d.
increased or decreased when using stringent cutoff values: fold change $>2$ and $P$ value $<0.01$ (Table 2). Interestingly, those proteins with the highest increase in co-culture were five proteins of unknown function. Curiously, the two most abundantly increased proteins, P1017 and P1706 (38.7- and 6.3-fold increase in co-culture, respectively), both contain COG5361 with DUF1254 and DUF1214 conserved regions (Supplementary Fig. 5a). The architecture of COG5361 is conserved and encoded in almost 20% of genomes through all domains of life (Supplementary Fig. 5b and Supplementary Table 3). The fact that most of the highest differentially expressed proteins are of unknown function mirrors observations in short-term phototroph–heterotroph co-culture experiments and highlights our current lack of knowledge of specific microbial interaction processes. We also detected an increase in proteins involved in pyruvate and oxaloacetate biosynthesis from phosphoenolpyruvate (that is, pyruvate kinase and phosphoenolpyruvate carboxylase), favouring the citric acid cycle, when \textit{R. pomeroyi} was present. Other proteins with an increased abundance in the presence of \textit{R. pomeroyi} included those involved in twitching motility, amino-acid transport, a glucose-methanol-choline-like oxidoreductase and a multi-copper oxidase, among others (Table 2).

(3) \textit{Synechococcus} sp. WH7803 appears to use ammonium when grown in co-culture with \textit{R. pomeroyi}, despite ASW containing nitrate, a feature tentatively concluded from previous \textit{Synechococcus–Vibrio} co-culture work. This is suggested by the increase in proteins involved in ammonium assimilation via glutamate synthase and ammonium ligase, the shutdown of alternative pathways for acquiring nitrogen (for example, nitrate or cyanate) and the reduction of proteins involved in the transamination/deamination of amino acids (Supplementary Table 2). This is highly suggestive of the heterotroph remineralising the organic matter produced by the phototroph and making ammonium available (see section ‘Nutrient analysis of seawater cultures’).

(4) At the functional level, a larger proportion of the \textit{Synechococcus} proteome was allocated to the production of photosynthesis and membrane transport when the heterotroph was present (7 and 61%, respectively; Fig. 2a). Such an obvious increase in membrane transport protein capacity is largely due to the increase in abundance of two periplasmic binding proteins specific for amino acids and iron, both of which are highly detected proteins (0.5 and 0.9% of all \textit{Synechococcus} proteins detected, respectively). Conversely, a decrease in mechanisms dealing with oxidative stress (12%, Fig. 2a), vitamin/cofactor and nucleic acid metabolism was observed in co-culture (22 and 16%, respectively, Fig. 2b). The 3.6-fold increase in abundance of a \textit{Synechococcus} ABC-type amino-acid transporter and the decrease in proteins related to central metabolic pathways (for example, biosynthesis of nucleic acids and cofactors) in the presence of the heterotroph are potentially indicative of the hydrolysed organic matter becoming available and re-asimilated by the phototroph, as suggested previously during \textit{Prochlorococcus–Alteromonas} interactions.

(5) Only the 37 most abundant proteins of \textit{R. pomeroyi} DSS-3 were detected, as this organism represented only 6% of the cells within the co-culture at the time the samples were taken (Supplementary Table 2). Interestingly, ten of these proteins were components of active transport systems, mainly for amino acids or peptides, but the sn-glycerol-3-phosphate ABC transporter (YP\textsubscript{165509.1}) was also highly represented (over 2% normalized spectral count abundance factor, NSAF). Altogether, active nutrient transport systems represented 20% of the total detected proteins, without taking into account the major outer membrane porin (YP\textsubscript{168626.1}, 16.2% NSAF).

\textbf{Phototroph–heterotroph interactions in natural seawater.} Given that nutrient availability is a key variable limiting ocean productivity and it is unlikely that organic matter will build up to toxic levels in natural open ocean environments, we co-cultured \textit{Synechococcus} and \textit{R. pomeroyi} in filter-sterilized autoclaved natural seawater to assess phototroph–heterotroph interactions under natural (low nutrient) conditions. Despite reaching relatively low cell densities (~10\textsuperscript{5} cells ml\textsuperscript{-1}), the enhanced survival of \textit{Synechococcus} was again only observed when the heterotroph was present (Fig. 3a). Most interestingly, the phototroph reached a stable 1:10 cell density equilibrium with the heterotroph, which was independent of the starting inoculum (Fig. 3b), a density akin to that observed in natural surface seawater\textsuperscript{3}. Increasing the nutrient load of the natural seawater (by adding ASW medium) showed that culture cell yield was proportional to the concentration of nutrients present in the system, and only at high nutrient concentrations were cell yields limited by light (Supplementary Fig. 3c).

\textbf{The phototroph–heterotroph mutualistic interaction is based on nutrient exchange.} Using nitrogen as a case study, we grew \textit{Synechococcus} and \textit{R. pomeroyi} in ASW with no nitrogen, as well as in ASW containing either 1 mM nitrate or 0.01% (wt/vol) peptone (≥1 mM nitrogen) as the only nitrogen source. As expected, monocultures of \textit{Synechococcus} or \textit{R. pomeroyi} could not use peptone or nitrate, respectively (Fig. 3c,d) and behaved similarly to those cultures grown in ASW lacking nitrogen. Monocultures of \textit{Synechococcus} and \textit{R. pomeroyi} showed robust growth when grown with an accessible source of nitrogen (NO\textsubscript{3} or peptone, respectively), but initiated a cell density decline during the stationary phase (Fig. 3c,d). In contrast, when grown in co-culture, both strains reached similar cell yields but with cell numbers being maintained across the 60-day timescale of the experiment. Most interestingly, each strain reached high growth yields, even in those cultures in which the source of nitrogen was not accessible to them, highlighting a cross-feeding between strains. Hence, regardless of the nitrogen source used in co-culture, even if inaccessible to one partner, for example, peptone for \textit{Synechococcus} or nitrate for \textit{R. pomeroyi}, an ultimate exchange of accessible nitrogen is achieved.

Further confirmation that the continuous supply of remineralized nutrients is the driving force of this mutualistic interaction was seen in the extended survival of \textit{Synechococcus} in natural seawater through periodic addition of small amounts of nutrients (1:1,000 diluted ASW every three to four days, Fig. 3e), mimicking the role of the heterotroph through a constant release of small amounts of nutrients.

We then determined which of the essential nutrients (nitrogen, phosphorus or trace metals) played a key role in the mutualistic
interaction. *Synechococcus* sp. WH7803 was again grown in axenic culture in natural seawater, but this time small amounts of ASW (1:1,000 diluted), each lacking one of the different nutrients, were added (Fig. 3f). All nutrients proved essential to sustain the survival of *Synechococcus* sp. WH7803 alone, R. *pomeroyi* alone and a co-culture of both. Cultures were then incubated for 7 days under continuous-light conditions to maintain phototrophic activity, followed by 7 days under continuous darkness. *Synechococcus* did not survive the dark period. Dissolved phosphorus showed the strongest detrimental effect (similar to those where no nutrients were added, Fig. 3f), despite the known phosphatase activity of *Synechococcus* sp. WH7803 proteins increased or decreased in abundance during co-culture with *R. pomeroyi* DSS-3 in nutrient-rich ASW medium compared to an axenic grown control culture (>2-fold increase/decrease and P ≤ 0.01).

| NCBI reference | Annotated function | Fold change | P value |
|----------------|--------------------|-------------|---------|
| YP_001224740.1 | Hypothetical protein SynWH7803_1017 | 38.7 | <0.001 |
| YP_001225429.1 | Hypothetical protein SynWH7803_1706 | 6.3 | <0.001 |
| YP_001225426.1 | Hypothetical protein SynWH7803_1703 | 5.5 | 0.10 |
| YP_001226079.1 | Hypothetical protein SynWH7803_2356 | 4.7 | 0.003 |
| YP_001226036.1 | Hypothetical protein SynWH7803_2312 | 3.0 | 0.009 |
| YP_001224941.1 | Pyruvate kinase | 5.5 | 0.007 |
| YP_001224177.1 | Phosphoenolpyruvate carboxylase | 2.4 | 0.001 |
| YP_001224671.1 | Putative multicopper oxidase | 5.4 | <0.001 |
| YP_001225564.1 | Twitching motility protein | 5.1 | <0.001 |
| YP_001225795.1 | ABC-type amino acid transport system | 3.6 | <0.001 |
| YP_001224436.1 | Flavoprotein related to choline dehydrogenase | 3.1 | 0.007 |
| YP_001224753.1 | N-acetylglucosamine-1-P uridylyltransferase | 2.6 | 0.002 |
| YP_001224095.1 | NAD(P)H-quinone oxidoreductase subunit H | 2.6 | 0.007 |
| YP_001225323.1 | Prototrophin IX Mg-chelatase subunit Chl | 2.1 | 0.005 |
| YP_001224303.1 | Long-chain acyl-CoA synthetase | −7.9 | <0.001 |
| YP_001225887.1 | Phosphoglucomutase | −6.4 | 0.001 |
| YP_001223965.1 | UDP-glucose 4-epimerase | −2.0 | 0.003 |
| YP_001224133.1 | 50S ribosomal protein L17 | −4.3 | 0.001 |
| YP_001224092.1 | ATP-dependent Clp protease adaptor protein | −3.7 | 0.004 |
| YP_001224152.1 | 50S ribosomal protein L22 | −3.0 | 0.009 |
| YP_001224147.1 | 50S ribosomal protein L14 | −2.8 | <0.001 |
| YP_001224142.1 | 50S ribosomal protein L18 | −2.8 | <0.001 |
| YP_001225172.1 | Two-component system response regulator | −3.9 | 0.004 |
| YP_001224856.1 | Uracil phosphoryltransferase | −3.0 | <0.001 |
| YP_001223749.1 | Hypothetical protein SynWH7803_0026 | −2.5 | 0.006 |
| YP_001224362.1 | Uridylate kinase | −2.4 | 0.008 |
| YP_001225094.1 | Pyridoxal phosphate biosynthetic protein PdxJ | −3.8 | 0.001 |
| YP_001223792.1 | Hypothetical protein SynWH7803_0069 | −2.1 | 0.004 |
| YP_001224106.1 | Hypothetical protein SynWH7803_0383 | −2.0 | 0.007 |
| YP_001223992.1 | Imidazoleglycerol-phosphate dehydratase | −3.7 | 0.003 |
| YP_001221562.1 | Dihydrodipicolinate reductase | −2.3 | <0.001 |
| YP_001226037.1 | NAD(P)H-quinone oxidoreductase subunit H | −3.0 | <0.001 |
| YP_001225969.1 | Photosystem I assembly protein Ycf3 | −2.1 | 0.008 |
| YP_001226044.1 | Glutathione synthetase | −3.7 | 0.005 |
| YP_001223895.1 | Rod shape-determining protein MreB | −2.7 | 0.007 |
| YP_001223979.1 | ABC-type transport system, ATP comp. | −2.4 | 0.007 |
| YP_001225809.1 | Hypothetical protein SynWH7803_2086 | −7.4 | 0.001 |
| YP_001224473.1 | Hypothetical protein SynWH7803_0750 | −3.8 | 0.001 |
| YP_001225333.1 | Dienelactone hydrolase/uncharacterized domain | −3.3 | 0.007 |
| YP_001224023.1 | Hypothetical protein SynWH7803_0300 | −2.2 | 0.003 |
| YP_001225620.1 | Hypothetical protein SynWH7803_1897 | −2.2 | 0.001 |
| YP_001224075.1 | Hypothetical protein SynWH7803_0352 | −2.1 | 0.008 |
| YP_001224526.1 | Hypothetical protein SynWH7803_0803 | −2.1 | 0.003 |
| YP_001225623.1 | Hypothetical protein SynWH7803_1900 | −2.1 | 0.001 |
| YP_001225824.1 | Hypothetical protein SynWH7803_2101 | −2.0 | 0.005 |

**Nutrient analysis of seawater cultures.** Sterile seawater was inoculated with *Synechococcus* sp. WH7803 alone, *R. pomeroyi* alone and a co-culture of both. Cultures were then incubated for 7 days under continuous-light conditions to maintain phototrophic activity, followed by 7 days under continuous darkness. *Synechococcus* did not survive the dark period. Dissolved phosphorus showed the strongest detrimental effect (similar to those where no nutrients were added, Fig. 3f), despite the known phosphatase activity of *Synechococcus* sp. WH7803.
Figure 2 | Comparative proteomic analysis of *Synechococcus* sp. WH7803 proteins detected in the absence (axenic) and presence of *R. pomeroyi* DSS-3 (co-culture) (n = 3). a, b. All categories (a) and subcategories (b) from central metabolism are shown. Asterisks represent significant differences (t-test, P < 0.05). Bars on the left indicate percentage increase in functional groups in axenic culture. Bars on the right indicate percentage increase in functional groups in co-culture. TCA, tricarboxylic acid cycle.

Proteomic assessment of seawater cultures. The proteome of *Synechococcus* sp. WH7803 incubated in seawater (Supplementary Table 4) showed a similar proteomic profile to that observed in nutrient-rich ASW medium (Table 1), with 43.5% of the cellular proteins dedicated to photosynthesis and CO₂ fixation. Membrane transport was the only protein category that showed a large variation, mainly due to the detection of high amounts of potential phosphorus stress-induced porins (YP_001225958.1 and YP_001225959.1, 5.3% and 1.0%, respectively) and a periplasmic binding protein component of an ABC transporter system for phosphate (0.9%; Supplementary Table 5). Interestingly, while *R. pomeroyi* was only able to regenerate it to its original concentration of its proteome to active membrane transport (over 21%), mainly focused on the uptake of amino acids and amine compounds) and an outer membrane porin (over 11%; Supplementary Table 5).

As previously observed in ASW, *R. pomeroyi* devoted a large fraction of its proteome to active membrane transport (over 21%, mainly focused on the uptake of amino acids and amine compounds) and an outer membrane porin (over 11%; Supplementary Table 5). Most interestingly, while *R. pomeroyi* DSS-3 devotes most of its membrane transporters to the uptake of organic compounds (59% versus 2.7% for inorganic compounds), *Synechococcus* sp. WH7803 shows a larger investment in acquiring inorganic compounds (85.2% versus 2.8% for organic compounds), highlighting how both organisms potentially target different substrates (Fig. 5).

Finally, we carried out a comparative proteomics analysis of *R. pomeroyi* incubated in natural seawater in the presence and absence of *Synechococcus* (Supplementary Table 6). Despite having a clear nutrient-starved pattern in both conditions, the heterotroph showed a remarkable switch from phosphorus starvation and a generalist-scavenging metabolism when alone, to nitrogen starvation and DON-targeted metabolism in the presence of the phototroph (Supplementary Table 7 and Fig. 5). When alone, *R. pomeroyi* showed increased production of the PhoB regulon (24×), alkaline phosphatases (PhoD, 31×; PhoX, 2×), phosphate ABC transporter (3–7×) and phosphonate transport and metabolism (up to 57×). It also increased its metabolism involved in scavenging energy from a nutrient-poor environment, for

organic carbon (DOC), dissolved organic nitrogen (DON), oxidized nitrogen (nitrate and nitrite) and ammonium were measured at the end of each period (Fig. 4). DOC only showed a decrease in the presence of the heterotroph (H, from ~70 to 50 µM), while the concentration of DON was maintained slightly higher in the presence of the phototroph (57 µM). Interestingly, *Synechococcus* depleted ammonium from the medium after the light incubation, whereas *R. pomeroyi* was only able to regenerate it to its original level after the dark period (1.4 µM). Hence, the organic nitrogen contained within *Synechococcus* photosynthate is consumed by *R. pomeroyi* regenerating ammonium, which in turn is rapidly used by *Synechococcus*. *R. pomeroyi* cannot use or regenerate oxidized nitrogen and *Synechococcus* does not seem to deplete it completely from the medium. Phototroph monocultures enriched the DOM with DON (2.3 µM), decreasing the C:N ratio of marine DOM in seawater from 56 to 33, whereas the heterotroph increased the C:N ratio of DOM to 86–104, mainly due to the consumption of DON (Fig. 4).
Figure 3 | Growth curves under nutrient-limiting conditions. **a**, Growth of Synechococcus sp. WH7803 in seawater in the presence or absence of *R. pomeroyi* DSS-3. **b**, Growth curves of Synechococcus sp. WH7803-*R. pomeroyi* DSS-3 co-cultures in seawater where Synechococcus was inoculated at three different concentrations (~10^3, 10^4 and 10^5 cells ml^{-1}). Three culture replicates (*n* = 3) of each condition are presented in **a** and **b**. **c,d**, Growth curves of Synechococcus sp. WH7803 (**c**) and *R. pomeroyi* DSS-3 (**d**) grown in axenic culture and in co-culture in ASW with no nitrogen, and with nitrate and peptone as the only source of nitrogen. **e**, Growth curves of Synechococcus sp. WH7803 in seawater with (+nut) and without (−nut) periodic addition of small amounts of nutrients (1:1,000 diluted ASW every 3–4 days) in the presence or absence of *R. pomeroyi* DSS-3. The arrow indicates the time point at which the addition of nutrients was stopped or *R. pomeroyi* was added. **f**, Growth curves of axenic Synechococcus sp. WH7803 cultures in seawater with periodic addition of small amounts of nutrients as shown in **e**, but the added ASW was nitrogen, phosphorus or trace metals depleted. No nutrients were added in ‘Control’ cultures. The average value of triplicate cultures (*n* = 3) is shown in **c–f** (error bars show standard deviation).

Figure 4 | Nutrient analysis. **a,b**, Carbon (a) and nitrogen (b) nutrient analysis of seawater (C), seawater containing the heterotroph *R. pomeroyi* DSS-3 (H), seawater containing the phototroph Synechococcus sp. WH7803 (Ph) and seawater containing both microorganisms (H+Ph). DOC, ammonium, nitrate/nitrite and total nitrogen measurements were performed after seven days under optimum light conditions (yellow bars) and on the same cultures after a further seven days in the dark (black bars). DON was calculated after subtracting the inorganic nitrogen from the total nitrogen. C:N ratios in each condition are indicated above the DON bars. The average value of triplicate cultures (*n* = 3) is shown (error bars show standard deviation).
example, C1 metabolism (formate dehydrogenase, up to 337× and carbon monoxide dehydrogenase, 3×), transporters for sulfonates (3×) and carbohydrates (2–83×), or benzoate catabolism (3–8×).

On the other hand, Synechococcus seemed to be providing a constant source of amino acids and glycine betaine (increased detection of membrane transporters for these compounds, up to 16×) and vitamins, as the anabolic pathways for thiamine and biotin were strongly decreased (up to 59× and 4×, respectively) (Supplementary Table 7).

**Discussion**

Living organisms are never alone. The general belief that microbes compete for the same resources clashes with a basic concept in ecology whereby nutrients are recycled between phototrophic and heterotrophic organisms and, hence, how evolution favours specialization and collaborative behaviour in coexisting populations.

Here, we demonstrate experimentally how marine phototrophic and heterotrophic organisms represent key examples of this collaborative specialization, as highlighted by a clear functional partitioning of roles and the nutrient resources they target (Fig. 5). Ultimately, this complementarity of functions implies that one group of organisms cannot survive without the other, which favours a mutualistic interaction based on nutrient recycling. We believe this long-term mutualistic interaction is driven passively by the availability and balance of nutrients, although further research is required to determine if the active production of specific signalling molecules is also involved, as was recently observed in marine diatom–bacterial interactions.

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**Figure 5** | Schematic representation of the nutrient circulation process taking place in marine phototroph (Synechococcus sp. WH7803)–heterotroph (R. pomeroyi DSS-3) co-cultures in both ASW and seawater conditions. Abundantly detected processes/pathways/transporters either higher (red) or lower (blue) in co-culture are represented in the phototrophic cell. Proteins with higher abundance in natural seawater conditions are represented in green. The relative abundance of different groups of membrane transporters is also shown, summing 100% for each cell type. The abundance of other processes is relative to the total cellular proteome. PEP, phosphoenolpyruvate; Pyr, pyruvate; OAA, oxaloacetate; DOP, dissolved organic phosphorous; TCA, tricarboxylic acid cycle; Gln, glutamine; Glu, glutamate; GlnA, glutamine synthase; GdhA, glutamate dehydrogenase.
Nutrient circulation is needed for a functional system (Fig. 5). Inorganic macro- and micronutrients are constantly being made available in marine environments, despite their rapid assimilation and incorporation into organic matter by microbes via the recycling of nutrients within the microbial loop. This connects phytoplankton and heterotrophic bacterial niche space, because the former is limited by inorganic nutrients (for example, nitrogen, phosphorus and iron), whereas the latter is normally carbon- and energy-limited. Based on these ‘niche’ requirements, both groups of organisms reach a stable balance in their respective habitats and associated cell numbers are maintained. Interestingly, our natural seawater experiment shows that the simple two-strain system we present here is a good surrogate of the general heterotroph–phototroph cell ratio in the oceans (10:1), where cell yields are also limited by overall nutrient availability. Nevertheless, this Synechococcus–R. pomeroyi system lacks the presence of viruses or grazers, which clearly play an important role in keeping the ecosystem ‘young’ and dynamic by speeding up the recirculation of nutrients. In any case, the data we present here clearly demonstrate for the first time that the foundations of long-term self-sustained heterotroph–phototroph mutualistic interactions are based on nutrient recycling, which we posit is a basic concept in ecology.

Methods

**Bacterial growth and culture conditions.** Axenic marine Synechococcus strains WH7803, WH8102 and WH7805, and non-axenic strains CC9311 and BL107, were routinely grown in 100 ml ASW medium contained in 250 ml Erlenmeyer flasks and incubated at 22 °C at a light intensity of 10 µmol photons m−2 s−1 with shaking (140 r.p.m.). The ASW contained no vitamin supplements, and nitrogen and phosphorus were supplied as 8.8 mM nitrate and 0.18 mM phosphate, unless indicated differently in the text. Natural seawater experiments were performed with water collected from the Gulf Stream in the Gulf of Mexico, supplied by Sigma (Fig. 4 gives the DOC and nitrogen measurements from this seawater), autoclaved seawater inoculated with marine heterotrophs to eliminate nutrients before inoculating co-cultures. Synechococcus cell abundance was monitored by flow cytometry (BD FACScan), and viable heterotrophs were counted by colony forming units (c.f.u.) on marine agar (Difco). A detailed comparison of c.f.u. and flow cytometry cell counts is provided in Supplementary Note 4 and Supplementary Fig. 6. All experiments in this study were performed with independent biological triplicates.

**Preparation of cellular proteomes for nanoLC–MS/MS and data analysis.** Shotgun proteomic analysis of cellular extracts of Synechococcus sp. WH7803 grown in ASW and seawater medium in the presence and absence of R. pomeroyi DSS-3 was carried out to elucidate the molecular mechanisms occurring during the interaction process. We also analysed the proteome of R. pomeroyi DSS-3 in the presence and absence of the phototroph in seawater conditions. Cell pellets were collected by centrifugation (3,000g for 15 min at 4 °C) using 10 and 400 ml of ASW and seawater cultures, respectively. At the time point of collection (35 days for ASW and 10 days for seawater), the heterotroph represented 6 and 69% of the cells within the heterotroph–phototroph cell ratio in the oceans. Isolates were identified by sequencing the near full-length 16S rRNA gene using the universal primers F27 and R1492 (ref. 48). All heterotrophs were initially grown in marine broth (Difco). Cells were washed twice with autoclaved seawater to eliminate nutrients before inoculating co-cultures. Synechococcus cell abundance was monitored by flow cytometry (BD FACScan), and viable heterotrophs were counted by colony forming units (c.f.u.) on marine agar (Difco). A detailed comparison of c.f.u. and flow cytometry cell counts is provided in Supplementary Note 4 and Supplementary Fig. 6. All experiments in this study were performed with independent biological triplicates.
protein identifications were validated using Scaffold (v. 4.3.4, Proteome Software) in all cases using the described standard parameters. Proteins were only validated with two or more different peptides. Protein quantification of ASW samples was performed by NSAF whereas for the seawater samples Progenesis QI was used (v. 2.0.5, Nonlinear Dynamics, Waters). Protein categories were based on KEGG annotations, with manual curation assessed by the Conserved Domain search tool from NCBI.

Oxidative stress and nutrient analyses. An Amplex Red Hydrogen Peroxide/ Peroxidase Assay Kit (ThermoFisher) was used to measure oxidative stress in the cultures. Protein and carbohydrate analyses were performed with supernatants obtained from Synechococcus cultures grown in ASW. Cells were centrifuged (13,000 g for 1 min) and supernatants were further filtered through a 0.2-µm-diameter pore filter (Sartorius Minisart). Protein quantification was performed with the Quant-iT BCA assay kit (Sigma-Aldrich). Carbohydrate quantification was performed using a phenol sulfuric acid assay. Nutrient analyses were performed using a FluoroSELECT ammonia kit (Sigma-Aldrich) with a small modification of the indicator reaction. Nitrate and nitrite were measured with an ion analyser with the TNM-L accessory module (Shimadzu). NH₃ was measured using the Peroxidase Assay Kit (ThermoFisher) was used to measure oxidative stress in the cultures. Oxidative stress and nutrient analyses.

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Data availability. The data that support the findings of this study are available in the Supplementary Information.
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Author contributions

J.A.C.-O. and D.J.S. conceived the study. J.A.C.-O. designed the experiments. J.A.C.-O., D.S. and M.L. performed the experiments. J.A. carried out the proteomic analyses. J.A.C.-O. analysed the data. J.A.C.-O. and D.J.S. wrote the paper with contributions from J.A.

Additional information

Supplementary information is available for this paper.
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Competing interests

The authors declare no competing financial interests.