Regulatory and metabolic adaptations in the nitrogen assimilation of marine picocyanobacteria

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Abstract

Prochlorococcus and *Synechococcus* are the two most abundant photosynthetic organisms on Earth, with a strong influence on the biogeochemical carbon and nitrogen cycles. Early reports demonstrated the streamlining of regulatory mechanisms in nitrogen metabolism and the removal of genes not strictly essential. The availability of a large series of genomes, and the utilization of latest generation molecular techniques have allowed elucidating the main mechanisms developed by marine picocyanobacteria to adapt to the environments where they thrive, with a particular interest in the strains inhabiting oligotrophic oceans. Given that nitrogen is often limited in those environments, a series of studies have explored the strategies utilized by *Prochlorococcus* and *Synechococcus* to exploit the low concentrations of nitrogen-containing molecules available in large areas of the oceans. These strategies include the reduction in the GC and the cellular protein contents; the utilization of truncated proteins; a reduced average amount of N in the proteome; the development of metabolic mechanisms to perceive and utilize nanomolar nitrate concentrations; and the reduced responsiveness of key molecular regulatory systems such as NtcA to 2-oxoglutarate. These findings are in sharp contrast with the large body of knowledge obtained in freshwater cyanobacteria. We will outline the main discoveries, stressing their relevance to the ecological success of these important microorganisms.

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Introduction

Two genera of marine picocyanobacteria, namely *Prochlorococcus* and 55 *Synechococcus*, are the most abundant photosynthetic organisms in most oceans (Scanlan *et al.*, 2009). Together with the rest of the cyanobacteria they play a crucial role in the nitrogen and carbon cycles of the Earth (Partensky *et al.*, 1999, Mella-Flores *et al.*, 2012). This fact gives them a significant ecological position in two of the main challenges faced by present and future generations: food supply and global warming. *Prochlorococcus* and 60 *Synechococcus* have been estimated to contribute about 25% of ocean primary production (Partensky *et al.*, 1999, Flombaum *et al.*, 2013, Larkin *et al.*, 2019) although in some oligotrophic areas it could be as high as 80% (Rii *et al.*, 2016). Collectively *Prochlorococcus* fixes about four gigatons of C per year (Flombaum *et al.*, 2013), which approximately represents the same production as that of global croplands (Huston & 65 Wolverton, 2009). These picocyanobacteria show a very wide distribution in the ocean based on light, temperature, and nutrient limitations. Both cyanobacteria are quite abundant but have their own preferences, e.g. *Prochlorococcus* is most abundant in warm oligotrophic waters, and it is much less common when the water temperature drops below 15°C (Larkin *et al.*, 2016). Furthermore, although *Prochlorococcus* is more abundant near 70 the sunlit surface, it can thrive in deep waters (even more than 100 m below the sea surface). By contrast, *Synechococcus* thrives both in coastal and the open ocean (Partensky *et al.*, 1999), in a wider temperature range than *Prochlorococcus*, even in polar waters, although it is less abundant at warm waters where *Prochlorococcus* grows best (Larkin *et al.*, 2019). Moreover, *Synechococcus* populations grow down to 100 m depth, 75 being undetectable in deeper waters (Partensky *et al.*, 1999).

The importance of N availability to maintain biodiversity in marine ecosystems is well known. Oceans are the largest reservoir of fixed N on Earth, containing about 5 times more than the land biosphere; therefore the importance of N metabolism in the marine environments is outstanding (Gruber, 2008). This is reinforced by taking into account that 80 the primary production has similar values in both the ocean and the terrestrial environments (Gruber, 2008). Nitrogen restrains primary productivity in many parts of the oceans, particularly in the low-latitude oceans (Moore *et al.*, 2013, Glibert *et al.*, 2016, Bristow *et al.*, 2017), thus playing a critical role in the uptake of atmospheric CO_2. This biologically driven biogeochemical loop is essential for the regulation of the planet's

85 climate, as it is one of the processes that control the concentration of CO_2 in the atmosphere (Gruber, 2008). Thus N cycle can have a big impact on Earth's climate (Gruber & Galloway, 2008) although the exact nature and the direction of the possible change it can produce is still unclear (Gruber, 2008, Basu & Mackey, 2018, Naafs *et al.*, 2019). Different aspects of the marine microbial N cycle, including the impacts of human

90 activities, have been recently reviewed (Pajares & Ramos, 2019).

N-limited areas are remarkably occupied by the cyanobacteria *Synechococcus* and *Prochlorococcus* (Scanlan & Post, 2008). The abundance of these picocyanobacteria in these areas supports their important role in the N cycle and the primary production in oligotrophic areas. Marine phytoplankton, including cyanobacteria, reduce about 2,000

95 Tg of nitrate per year (Duce *et al.*, 2008). Nitrogen can be found in five relatively stable oxidation states in marine environments: N_2, NO_3^-, NO_2^-, NH_4^+ and N_2O. Certainly, N_2 constitutes the largest amount of N in the oceans (about 94%) (Gruber, 2008) but this is an inaccessible form for most microorganisms. Marine phytoplankton assimilation of nitrate or ammonium into organic N is the process that quantitatively dominates the

100 marine N cycle (Gruber, 2008). The oxidation state of those N forms has important consequences on the energy required for its assimilation; therefore, NH_4^+ is the preferred one for phytoplankton, since its assimilation requires less energy than the assimilation of the other forms (Zehr & Ward, 2002, García-Fernández *et al.*, 2004). Thus, ammonium is the common denominator in inorganic N assimilation, so most forms of N are first

105 reduced to NH_4^+ before their incorporation into cellular material (Berges & Mulholland, 2008). Furthermore, ammonium assimilation metabolically links the C and N cycles since C backbones are required for amino acid synthesis. The central N assimilatory pathway, common to most photosynthetic microorganisms, is composed of the enzymes nitrate reductase, nitrite reductase, glutamine synthetase (GS), and glutamate synthase

110 (GOGAT), the last two constituting the GS/GOGAT cycle.

The role of marine picocyanobacteria on N cycling can be predicted from the stratification and the nutrient concentrations along the water column (Scanlan & Post, 2008). All phytoplankton was expected to grow on ammonium as the only N source despite ammonium being much less abundant than nitrate. However, that lesser

115 abundance can be due to ammonium being uptaken at a greater rate than other forms of N (Lewis *et al.*, 1986). Since nitrate is abundant in the ocean, most phytoplankton species have the enzymes needed to ensure its assimilation no matter if it is energetically more expensive, requiring the transfer of 8 electrons per NO_3^- reduced to NH_4^+. In early studies

on marine picocyanobacteria, *Prochlorococcus* and some *Synechococcus* strains were considered important exceptions to that rule (López-Lozano *et al.*, 2002, Moore *et al.*, 2002). It was later demonstrated that nitrate assimilation genes are missing in basal lineages of *Prochlorococcus*, but occurring within recently emerged clades (Martiny *et al.*, 2009, Berube *et al.*, 2015, Berube *et al.*, 2019).

In this manuscript, we will review the state of the art regarding the regulatory and metabolic adaptations unveiled in the N metabolism from the marine picocyanobacteria *Prochlorococcus* and *Synechococcus*, with the goal of understanding how these adaptations helped them to become the most abundant photosynthetic organisms on Earth. Readers can find general information about nitrogen metabolism in freshwater cyanobacteria or about some specific aspects of the relationship between the regulation of carbon and nitrogen metabolisms in a few excellent reviews recently published (Esteves-Ferreira *et al.*, 2018, Zhang *et al.*, 2018, Herrero & Flores, 2019, Forchhammer & Selim, 2020, Muro-Pastor & Hess, 2020, Forchhammer *et al.*, 2022).

1. Differential utilization of nitrogen sources

1.1. General aspects

All *Synechococcus* and *Prochlorococcus* strains require N as an essential nutrient, although they differ in some critical aspects related to N metabolism, echoing specific features of the environmental niches they occupy in the ocean. N is a limiting factor for picocyanobacteria and the concentration and type of nitrogen sources greatly influence the oceanic distribution of different strains. In coastal waters, and areas of deep mixing and upwellings, the concentrations of inorganic N molecules are generally low compared to those found in most freshwaters, reaching only the micromolar range. Concentration typically found in the surface layers of the oligotrophic oceans are even lower, often below the detection limit of 5 to 50 nM, depending on the N species and the method of determination (Scanlan *et al.*, 2009).

Cyanobacteria have been considered as a group of prokaryotes able to use all forms of N (Flores & Herrero, 1994), from the most oxidized sources (including molecular N or nitrate) to the most reduced ones (such as ammonium or urea), thus providing them remarkable metabolic flexibility to cope with a variety of environmental changes. Although it was traditionally accepted that reduced forms of N are preferentially used over the oxidized ones based on the energetic costs of their assimilative processes, it has been recognized that both types of N sources can be simultaneously used (Jenkins & Zehr, 2008).

It is unclear whether marine cyanobacteria have a hierarchy that allows them to determine the presence of different N sources in the environment and to establish the best order for the acquisition of these compounds. The only exception is a clear preference for ammonium utilization over all other N sources (Scanlan & Post, 2008, Casey *et al.*, 2022). Based on energy requirements calculations, it would be reasonable to utilize amino acids, ammonium, and urea preferentially, followed by nitrite, nitrate, and finally the fixation of molecular N, provided that these sources were all available and the organism possessed the required enzymatic machinery. A deeply mixed water column may contain several N forms, and marine cyanobacteria thrive in such environments with the capability of rapidly adapting to the differential utilization of combined N sources (Lindell & Post, 1995, Lindell & Post, 2001). Once N has been acquired, its assimilation proceeds via the GS/GOGAT pathway in all studied marine cyanobacteria. GS is required for ammonium assimilation irrespective of the primary source of N. Besides, an increasing importance

of organic nitrogen sources such as urea, cyanate, or amino acids as N forms has been demonstrated for marine picocyanobacteria (Mulholland *et al.*, 1999, Mulholland & Capone, 2000, Moore *et al.*, 2002, Kamennaya *et al.*, 2008, Mary *et al.*, 2008, Zubkov *et al.*, 2008, Zinser *et al.*, 2009, Berthelot *et al.*, 2019).

To the best of our knowledge, none of the marine *Synechococcus* or *Prochlorococcus* strains studied thus far has shown physiological or genomic hints of N fixation (although early reports were suggesting the occurrence of this trait in some *Synechococcus* strains (Spiller & Shanmugam, 1987)). This is a particularly striking absence since there are other marine cyanobacteria, either free-living groups (*Trichodesmium*, *Crocosphaera*) (Capone *et al.*, 1997, Zehr, 2011) or symbiotic ones (UCYN-A) (Zehr *et al.*, 2016) which have been reported to be important N fixers in the ocean. The very high energetic cost of N fixation is probably why this process is absent in both *Synechococcus* and *Prochlorococcus* (García-Fernández *et al.*, 2004). Interestingly, a recent study has shown that even *Trichodesmium*, a genus defined as a N fixer for decades, includes some strains unable to fix N (Delmont, 2021). This fact suggests that fundamental physiological traits, such as this one, may have been subjected to evolutive selection, leading to their disappearance in some specific cases where they might not be worth keeping for ecological reasons.

Nitrate and ammonium were thought to be the prevailing N species influencing total phytoplankton production in the ocean. While nitrate was considered the primary source of N promoting new production, ammonium would be the main regenerated N source (Mulholand & Lomas, 2008). This distinction between 'new' and 'regenerated' primary productivity (Dugdale & Goering, 1967) means that new production supports net growth (which could be exported) while regenerated production maintains populations (Dugdale & Goering, 1967, Eppley & Peterson, 1979). However, the discovery of new organisms and new places (e.g., the surface ocean) where N cycling is occurring blurred the distinction between 'new' and 'regenerated' production (Zehr & Kudela, 2011), as it is the case for nitrate formed by oxidation within the euphotic zone and recycled to phytoplankton (Ward *et al.*, 1989, Dore & Karl, 1996). Furthermore, in addition to nitrate and ammonium, it is now recognized that organic N compounds can be abundant in the ocean (Voss *et al.*, 2013) and substantially contribute to the primary productivity (Mulholand & Lomas, 2008). Reduced N compounds resultant from N fixation can be considered 'new production', while nitrate produced from nitrification is not 'new production' because it was generated from ammonia already existing in the environment.

Consequently, the difference between 'new' and 'regenerated' production is a complex concept, and it should not be based on the type of N compound: it should consider how that compound has been produced, and where it comes from.

205 All *Prochlorococcus* strains can use ammonium, while none can fix molecular N, however, they differ in their capability to assimilate other forms of N, including nitrate, nitrite, urea, cyanate, and amino acids (figure 1) (Palinska *et al.*, 2000, Rippka *et al.*, 2000, Moore *et al.*, 2002, Fuhrman, 2003, Zubkov *et al.*, 2003, García-Fernández *et al.*, 2004, Mulholand & Lomas, 2008, Martiny *et al.*, 2009, Kamennaya & Post, 2011). Unlike *Prochlorococcus*, most *Synecchococcus* strains can utilize those N sources (Garczarek *et*

210 *al.*, 2021). Genomic data indicate that N-stress responses may greatly differ in cyanobacteria since they can rely on different gene pools to acquire alternative N sources, some of which have not been considered usual N sources in the marine environment (tables 1 and 2). However, the significance of these alternative N sources is supported by the high degree of similarity to amino acid, cyanate, nitrite, oligopeptide transporters, and

215 the enzymes required to metabolize them, observed in genes from marine picocyanobacteria (Scanlan & Post, 2008, Herrero *et al.*, 2019, Larkin *et al.*, 2019). Recent metagenomic studies have proposed the loss of nitrate and nitrite assimilation genes in *Synechococcus* as an adaptation to severe iron limitation in high nitrogen, low chlorophyll regions where ammonium availability is higher (Sharpe *et al.*, 2022).

220 In order to study a possible correlation between the availability of N sources and the presence of genes involved in N assimilation in marine picocyanobacteria, we used the TARA metagenomic dataset (Sunagawa *et al.*, 2015, Villar *et al.*, 2018, Vernette *et al.*, 2022). A Pearson correlation analysis was carried out between each gene abundance and nutrient concentration available in each TARA Station. Overall, there was a negative

225 correlation between the abundance of N assimilation genes and N concentration (supplementary table 1 and supplementary figure 1), suggesting that marine picocyanobacterial populations are adjusting their genomic repertoire to adapt to the availability and diversity of key nutrients such as N-containing molecules. For example, in ammonium-limited regions, we found a higher abundance of genes involved in the

230 assimilation of other N sources such as *cynS, glnA, nirA, nrtP,* and *urtA*.

The low-light (LL) adapted *Prochlorococcus* strains MIT9313 and NATL2A retain the genes for nitrite utilization and effectively they can grow on nitrite as the sole N source (Moore *et al.*, 2002, Rocap *et al.*, 2003). This capability is coherent with the depth distribution of LL adapted *Prochlorococcus* strains which concentrate at depths where

235 nitrite is particularly abundant. Recent studies have shown that some nitrate-utilizing *Prochlorococcus* strains can release nitrite, which can in turn be used by other *Prochlorococcus* strains possessing nitrite reductase but not nitrate reductase (Berube *et al.*, 2022). Low-light adapted *Prochlorococcus* strains may also contribute to new primary

production, however it is estimated to be slightly significant, since these *Prochlorococcus*
populations are less abundant than those of high-light (HL) adapted *Prochlorococcus*
strains, and light may become a limiting factor considering these populations undergo a
much lower irradiance, around 5% of surface intensities (Scanlan & Post, 2008).

1.2. Nitrate assimilation

Differences in the ability to use several N sources among cyanobacterial groups
(figure 1) can be due to the presence or absence of a specific gene (tables 1 and 2),
generally related to the presence and concentration of a particular N compound in the
environment where a given cyanobacterium thrives. *Prochlorococcus* strains were
thought not to grow on nitrate as a sole N source (Rippka *et al.*, 2000, López-Lozano *et
al.*, 2002, Moore *et al.*, 2002), until it was reported that some *Prochlorococcus*
populations, flow cytometrically sorted from the Sargasso Sea, were capable to assimilate
significant amounts of nitrate (Casey *et al.*, 2007). Later, genomic studies identified *narB*,
coding for assimilatory nitrate reductase, in the genome of some *Prochlorococcus* strains
(Martiny *et al.*, 2009, Berube *et al.*, 2015). Genes necessary for nitrate assimilation
associated with *Prochlorococcus* were identified in the global ocean sampling
metagenomic database (Martiny *et al.*, 2009) of flow-cytometry-sorted *Prochlorococcus*
populations (Batmalle *et al.*, 2014) and a metagenomic analysis of anoxic zones of the
Eastern Tropical South Pacific (Astorga-Elo *et al.*, 2015), but only for a few strains.
Currently, six *Prochlorococcus* strains are known to possess *narB* in their genomes, all of
them also including the *nirA* gene (coding for nitrite reductase) and the *nrtP* gene (coding
for a nitrate/nitrite transporter) (table 2) (Garczarek *et al.*, 2021). Furthermore, several
Prochlorococcus strains have been reported to grow on nitrate (Berube *et al.*, 2015). The
situation is quite different for the *Synechococcus* genus, since most of its strains include
narB and *nrtP* in their genomes, while all of them possess *nirA* (table 1), and consequently
show the ability to grow on nitrate. The analysis of the genomes from different strains of
Prochlorococcus not only revealed that the arrangement of genes related to nitrate
assimilation differs among strains, but also showed evidence of acquisition, loss, and
horizontal transfer of N assimilation-related genes for some HL strains, and of retention
of those genes in some LL ecotypes during the evolutionary divergence from their shared
ancestor with *Synechococcus* (Berube *et al.*, 2015, Berube *et al.*, 2019).

The high energetic costs of the assimilation of nitrate (and to a lower extent, of
nitrite) have been proposed to be the main evolutive reason to explain why many

Prochlorococcus strains can not utilize this N source. But there might be other reasons related to ecological competition for N: the maximum abundance of marine picoeukaryotes can be found in the nitracline (layer of the water column where nitrate concentration changes abruptly with depth) of the eastern subtropical North Atlantic Ocean. Picoeukaryotes living there are taking up nitrate at high rates (nitrate uptake in the nitracline is 10-fold higher than in the nitrate-poor waters of upper layers) (Painter *et al.*, 2014). This might have pushed the populations of both *Prochlorococcus* and *Synechococcus* to higher layers of the water column, and in turn, in the long evolutive run, might have driven the loss of the capability to assimilate nitrate in some *Prochlorococcus* strains. However, this would not be the only selective pressure for genes involved in nitrate assimilation. As nitrification occurs throughout the euphotic layer, *Prochlorococcus* strains thriving in this zone could have faced intense competition for ammonium; therefore, the ability to assimilate nitrate may act as a selective advantage as they can use the end products of both ammonium and nitrite oxidations (Berube *et al.*, 2016). More than the availability of a given N compound, the relative abundance of the main N sources could drive the loss of these genes for strains located in the higher layers of the water column.

During steady-state growth under N-sufficient conditions, the level of N assimilation in cyanobacteria represents about 20% of CO_2 assimilation, which means that around 30% of the reducing equivalent generated by photosynthesis is utilized for nitrate assimilation in the freshwater cyanobacterium *Anacystis nidulans* (Flores *et al.*, 1983). Since the proportion of the reducing power used for N assimilation can be diminished to 10% of the total by assimilating ammonium, it is reasonable to anticipate that cyanobacteria would show a strong preference for ammonium over nitrate. Similar calculations and results have been reported for marine cyanobacteria (García-Fernández *et al.*, 2004). Moreover, three *Prochlorococcus* strains recently reported to be capable of growth on nitrate have been analyzed regarding their physiology in relation to the N source available. It has been shown that the growth of HL adapted strains on nitrate is ≃17% slower than their growth on ammonium (Berube *et al.*, 2015).

1.3. Urea assimilation

Urea is the most abundant form of dissolved organic nitrogen (DON) in most aquatic ecosystems and many organisms can use it as an N source by taking it up and hydrolyzing it into CO_2 and two ammonium molecules which then will be assimilated

(figure 1). Intracellular hydrolysis of urea occurs through one of the two enzymatic pathways: either catalyzed by urease (urea amidohydrolase, EC 3.5.1.5) or ATP:urea amidolyase (UALase, EC 6.3.4.6), although the later has not been found in cyanobacteria (Solomon *et al.*, 2010). Seven polypeptides are needed for urease activity. UreA, UreB and UreC are needed for the activity, and all are highly conserved. In addition, four accessory proteins, UreD, UreE, UreF, and UreG, are needed for the enzyme assembly (Berges & Mulholland, 2008). Most *Prochlorococcus* and *Synechococcus* strains possess the genes required for urea utilization (tables 1 and 2), and several strains have been reported to grow with this organic compound as the sole N source (Rippka *et al.*, 2000, Moore *et al.*, 2002, Mulholand & Lomas, 2008). *Synechococcus* sp. strain WH7803, known for its inability to grow on urea, lacks the genes for urea utilization (Moore *et al.*, 2002, Berube *et al.*, 2015). Urea uptake may be much more important in the open ocean than previously thought (Mulholand & Lomas, 2008). Besides autotrophic microorganisms, heterotrophic bacteria also have ureases, indicating that they can use urea, although the available data show that autotrophic phytoplankton has lower K_m values for urea than bacteria (Berges & Mulholland, 2008). It has long been realized that urea uptake can support a substantial amount of regenerated production in a broad variety of environments (Bronk, 2002, Sipler & Bronk, 2015). If urea uptake were excluded from the estimation of regenerated production, the results could overestimate the role of ammonium in this process. It was further recognized that the large DON pool might be more labile and available to phytoplankton than it was formerly thought (Mulholand & Lomas, 2008).

In general, urea concentrations in aquatic ecosystems are lower than those of nitrate and ammonium, but urea availability may exceed the concentrations of those inorganic N forms occasionally, and for short periods (Glibert & Burkholder, 2011). Furthermore, as the production and consumption of all these N sources are tightly coupled (Widner *et al.*, 2018), the relevance of urea contribution to N assimilation in marine cyanobacteria could be even more underestimated. Urea can be formed naturally in the water column as a by-product of cellular metabolism including regeneration by heterotrophic bacteria, excretion by zooplankton, or release by phytoplankton (Solomon *et al.*, 2010). The contribution of urea to the total N taken up by planktonic communities widely varies, from nearly zero to over 50%, being urea generally preferred over nitrate (Collier *et al.*, 2009). Under specific conditions, rates of urea uptake can meet most of the phytoplankton demand for N. A study on the abundance of proteins in the North Pacific Subtropical

Gyre showed that the most abundant one was the *Prochlorococcus* urea transporter (Saito *et al.*, 2014), underlying the important role of urea as a nutritional source for that picocyanobacterium. It is important to mention that, as proposed by Scanlan and coworkers (Scanlan *et al.*, 2009), differences in potential N utilization based on gene

345 content do not define ecotypes, since some strains within a given ecotype encode the genes needed for urea utilization, while others do not. There seems to be some species-specific differences in the degree of constitutive expression versus upregulation of urease activity, independently of the nutrient cyanobacteria previously used (Berges & Mulholland, 2008). Cyanobacteria possess constitutive and ammonium-repressible

350 ureases. However, expression of high-affinity urea transporter is subjected to N control (Herrero *et al.*, 2001, Valladares *et al.*, 2002). Surprisingly, the values of urease activity detected in *Synechococcus* sp. WH7805 cultures were higher growing on nitrate than on either ammonium or urea (Collier *et al.*, 1999). Urease-coding genes in marine *Synechococcus* strains (WH7805, WH8102) are also part of the NtcA regulon, so that

355 they are N regulated (Berges & Mulholland, 2008).

1.4.   Cyanate assimilation

Cyanate was first identified as a N source in surface waters from the Red Sea for some cyanobacteria populations, namely *Prochlorococcus* sp. MED4 and *Synechococcus*

360 sp. WH8102 (Kamennaya *et al.*, 2008). The most likely origin of cyanate is the decomposition of ambient urea that can be produced by excretion from zooplankton and lysis of cells. Both strains have the whole set of genes for the uptake and metabolization of cyanate (Kamennaya *et al.*, 2008), thus sustaining the hypothesis that the growth of both strains could be supported by cyanate as the sole N source (García-Fernández *et al.*,

365 2004). This role of cyanate was also reported in other oceanic areas, such as North Atlantic Ocean (Widner *et al.*, 2016). Cyanase (EC 4.2.1.104) catalyzes the decomposition of cyanate (NCO^-) into CO_2 and ammonium (Johnson & Anderson, 1987) and it has been reported to be present in the genomes of both freshwater and marine cyanobacteria such as *Anabaena* sp. PCC 7120, *Synechocystis* sp. PCC 6803,

370 *Prochlorococcus* sp. MED4, *Synechococcus elongatus,* and *Synechococcus* sp. WH8102 (Su *et al.*, 2005). In the genome of the last three organisms, the cyanase coding gene *cynS* is clustered with the *cynABD* genes, encoding the ABC-type cyanate transporter CynABC/D, probably forming an operon in strains PCC 6801 and MED4. Thus, those three cyanobacterial strains are likely to use cyanate as an N source. However, no cyanate

375   transporters are known to be encoded by any genes in the *Anabaena* sp. PCC 7120 and
       Synechocystis sp. PCC 6803 genomes (Su *et al.*, 2005).

              Although it has been proposed that cyanate might serve as a significant N source
       for *Prochlorococcus* populations but less so for *Synechococcus* (Kamennaya & Post,
       2013), our analysis of the Cyanorak data showed a much higher number of
380   *Synechococcus* strains than *Prochlorococcus* ones possessing the genes needed for
       cyanate assimilation (tables 1 and 2). The only *Synechococcus* strains lacking *cynS* are
       WH5701, CC9616, KORDI-100, and NOUM97013, a representative of estuarine
       Synechococcus, and three from open ocean (Garczarek *et al.*, 2021). However, only 8 out
       of the 47 listed in table 1 also have the genes needed for the transport of cyanate, all of
385   them belonging to the subclade IIIa except for A15-28, from IIIb. Furthermore, the strains
       possessing the set of transport genes are much more homogeneous, including their GC
       content, that the ones where these genes are absent (table 1). On the other side, among 43
       Prochlorococcus genomes, only 8 carried a *cynS* ortholog, and only 4 of them have the
       genes encoding for the cyanate transporter (table 2).

390   The genomic situation of *cynS* differs in marine cyanobacterial strains. In
       Prochlorococcus, it seems to depend on the set of cyanate assimilation genes that a
       particular strain has. For instance, in strains NATL1A and NATL2A, lacking genes for
       cyanate transport, *cynS* is located among conserved hypothetical genes. However, in
       Prochlorococcus sp. MED4, *cynS* is positioned immediately downstream of *cynABD*,
395   and it is probably transcribed as part of a polycistronic messenger RNA (Kamennaya &
       Post, 2011). *Synechococcus* sp. WH7803 and WH7805 carry an ORF near *cynS* named
       cynH, identified as a cyanate hydratase (i.e., cyanase) in the automated annotation. The
       predicted amino acid sequence for that cyanase appears unique and orthologs are found
       in seven marine *Synechococcus* genomes (Kamennaya & Post, 2011). Furthermore, most
400   of the *Synechococcus* strains collected in the Cyanorak database include *cynH* in their
       genomes although, as is the case for *cynS*, they do not possess the genes for cyanate
       transport (table 1). It is worth noting that not a single *Prochlorococcus* strain sequenced
       so far has *cynH*, which is curious since it is a smaller protein and so it could save N, while
       exerting the same function as *cynS*. Sequence comparison revealed no significant
405   similarity between known CynS sequences and the short protein encoded by *cynH*
       (Kamennaya & Post, 2011). The physiological and ecological roles of cyanase in marine
       cyanobacteria have not clearly been elucidated yet. Cyanase may play a role in cyanate
       assimilation provided a specific transporter exists, but the majority of marine

cyanobacteria that contain *cynS* lack the genes related to cyanate acquisition (tables 1 and 2), suggesting for cyanase a possible role in the detoxification of internally generated cyanate which accumulates as a by-product of the urea cycle or via the degradation of carbamoyl phosphate (Kamennaya & Post, 2013).

1.5.   Ammonium incorporation by glutamate dehydrogenase

The enzyme glutamate dehydrogenase (GDH) provides an alternative pathway to incorporate ammonium through the reductive amination of 2-oxoglutarate (2-OG) and it has been characterized in different cyanobacteria (Florencio *et al.*, 1987, Martínez-Bilbao *et al.*, 1988) (figure 1). However, due to its high K_m value for ammonium (millimolar range), GDH is thought to play a minor role in N assimilation in photosynthetic microorganisms, including those from marine environments (Muro-Pastor *et al.*, 2005). Unlike the main pathway of ammonium incorporation by the GS-GOGAT cycle, the assimilation through GDH does not require ATP (Chávez *et al.*, 1999). Experiments with a *Synechocystis gdhA* mutant showed that the presence of NADP-GDH can provide a selective advantage to *Synechocystis* cells in the late stages of growth, when the energy supply may be limited. (Berges & Mulholland, 2008). Genes encoding GDH are not present in most marine cyanobacteria, although they have been found in the genomes of five *Prochlorococcus* strains belonging to the clade LLIV (table 2) and in *Trichodesmium erythraeum* (García-Fernández *et al.*, 2004, Muro-Pastor *et al.*, 2005). Although the precise role of GDH in these cyanobacteria is not currently supported by experimental data, it has been reported that it could be involved in the assimilation of amino acids to take advantage of glutamate released in aged cultures (Rangel *et al.*, 2009). Recent reports suggest this enzyme might also perform the aminating reaction to incorporate ammonium into 2-OG in LL, high N environments in *Prochlorococcus* (Casey *et al.*, 2022). GDH aminating function does not seem to play any relevant role in marine microorganisms considering the low ammonium concentration in those environments and the high K_m of this enzyme for ammonium (Rees *et al.*, 1999).

2.   Affinity and uptake rates of nitrogen transporters

The extremely low concentrations of N forms available in large areas of the ocean suggest that high-affinity, active transporters (Herrero *et al.*, 2001, Berges & Mulholland, 2008) are required for organisms living in those areas to successfully scavenge N. This

strategy has already been reported for glucose uptake in *Prochlorococcus* and marine *Synechococcus* strains (Muñoz-Marín *et al.*, 2013, Muñoz-Marín *et al.*, 2017). The genetic capacity to utilize different N sources along the water column has been linked to speciation (Kettler et al. 2007). Strain-level differences have been observed in the peripheral substrate-binding protein, which determine the specificity of nutrient uptake in ABC transporters, showing that individual strains have access to different portions of the nutrient pool across distinct environments (Ford et al., 2021). Therefore, nutrient transporters are an essential link between cells and their environment. It could be predicted that the transporters for different N forms were maximally expressed near the time of greatest N demand by the cell (Zinser *et al.*, 2009). The number of transport proteins per cell seems to be a function of the cellular physiological state, although it is not known either the mechanism underlying the variation in the number of transporters or the signals stimulating their expression. It has been proposed that the number of transporters per cell increases under N limitation (Harke & Gobler, 2015) although other data contradict this idea (Jenkins & Zehr, 2008). Many of the most abundant proteins in the Sargasso Sea are high-affinity ABC transporters (Sowell *et al.*, 2009, Ford *et al.*, 2021), highlighting their importance for species living in extremely nutrient-depleted environments.

Two types of nitrate transporters (NRT) have been identified in cyanobacteria: the ABC-type NRT comprised of the four proteins NrtA, NrtB, NrtC, and NrtD (Omata *et al.*, 1993) and the major facilitator superfamily (MFS) transporter encoded by the *nrtP* gene (Sakamoto *et al.*, 1999, Wang *et al.*, 2000, Aichi *et al.*, 2006). Both kinds of NRT are differentially scattered among the cyanobacterial strains having the capacity for nitrate assimilation. The ABC-type NRT can be found in almost all freshwater cyanobacteria (Omata, 1995), while marine strains capable of nitrate assimilation, usually have NrtP as a unique NRT system (Sakamoto *et al.*, 1999, Aichi *et al.*, 2006, Ohashi *et al.*, 2011). ABC-NRT transporter needs a high amount of substrate-binding protein in the plasma membrane, which is energetically very expensive. This could explain why marine strains, that live mainly in oligotrophic environments, have chosen the NrtP permease (Ohashi *et al.*, 2011). Besides, the source of energy required in each case is different: in the ABC-type nitrate transporter, hydrolysis of ATP is presumed to provide the energy for the active uptake of nitrate (Omata, 1995) while in the NrtP type it could be provided by a gradient of H^+ or Na^+ (Sakamoto *et al.*, 1999, Scanlan *et al.*, 2009). Furthermore, recent studies suggest the occurrence of specific mechanisms to detect (and possibly

uptake) nanomolar concentrations of nitrate in the ocean in *Synechococcus* sp. strain WH7803 (Domínguez-Martín *et al.*, 2022), in good agreement with previous studies showing the occurrence of *Synechococcus* blooms in the ocean after nanomolar changes in the nitrate concentration (Glover *et al.*, 1988). NrtP transports nitrite as well as nitrate, being its affinity substantially lower for nitrite (Aichi *et al.*, 2006). Thus, cells expressing NrtP as the only nitrate/nitrite transporter are virtually unable to take up nitrite in the presence of nitrate (Aichi *et al.*, 2006). This explains the presence of NitM (also designated as FocA), a putative nitrite-specific transporter, together with NrtP to assimilate nitrite in most marine *Synechococcus* and *Prochlorococcus* strains. This fact has relevant physiological importance since it allows the cells to take up nitrite, even in the presence of nitrate (Ohashi *et al.*, 2011), which can be released by coexisting cyanobacterial strains upon nitrate reduction (Berube *et al.*, 2022).

In marine cyanobacteria, N control consists of the repression of some N assimilation pathways when more easily assimilated or preferred N sources are available as it occurs in freshwater cyanobacteria (Herrero *et al.*, 2001). The N control gene *ntcA,* which is present in all studied cyanobacteria (Frías *et al.*, 1994, García-Fernández & Diez, 2004), regulates genes associated with nitrate (Lindell *et al.*, 1998, Herrero *et al.*, 2001) and urea uptake (Collier *et al.*, 1999, Rocap *et al.*, 2003). The regulation of transporters mediated by NtcA is a crucial part of the response of *Prochlorococcus* cells to N-scarcity (Lindell *et al.*, 2002, Tolonen *et al.*, 2006). Besides, the expression of *nrtP* and *narB* was higher in *Synechococcus* sp. strain WH8103 in response to N starvation and nitrate addition but was strongly repressed by ammonium under low irradiation (Bird & Wyman, 2003). There may be important exceptions to that general rule of the regulatory system. For example, *amt1*, encoding a high-affinity ammonium transporter, is expressed at high levels both in the presence of ammonium and during different stages of N-deprivation in *Prochlorococcus* sp. PCC 9511 (Lindell *et al.*, 2002). This regulation differs from the increased expression of *amt1* observed in the absence of ammonium in freshwater *Synechococcus* strains (Vázquez-Bermúdez *et al.*, 2002). The constitutive *amt1* expression in *Prochlorococcus* sp. strain PCC 9511, together with the absence of a typical *ntcA* binding site upstream of *amt1*, suggests that the expression of *amt1* is not regulated by *ntcA* in this *Prochlorococcus* strain (Lindell *et al.*, 2002) even though it appeared to be regulated by N availability in other strains of *Prochlorococcus* (Rocap *et al.*, 2003). These results reinforce the differences in the regulatory systems between marine and freshwater cyanobacteria (Scanlan & Post, 2008). Remarkably, marine eukaryotic

phytoplankton, which has divergent types of *amt* genes, showed a similar pattern of expression as in *Prochlorococcus* sp PCC 9511, with a high expression both under standard growth conditions and under N depletion (McDonald *et al.*, 2010). The most rapidly and highly upregulated genes in *Prochlorococcus* MED4 and MIT9313, are N

515   transport-related genes such as *urtA*, *cynA*, and *nitM* (Tolonen *et al.*, 2006).

Other different features have been observed among the distinct ecotypes of *Synechococcus* (Glibert & Ray, 1990) and *Prochlorococcus* (Tolonen et al., 2006). Differential ammonium and nitrate uptake features were observed in coastal vs open ocean *Synechococcus* strains (Glibert & Ray, 1990). During N limitation many genes,

520   among them the ammonium (*amt1*) and urea (*urt*) transporters, were activated both in HL and LL *Prochlorococcus* ecotypes (Tolonen *et al.*, 2006). However, transporters for urea and nitrite or urea and cyanate are regulated differently in both ecotypes depending on the available N source. The velocity and maintenance in the time of the transcriptional response to N limitation were also different in both ecotypes, apparently reflecting the

525   different conditions of the environments where they thrive (Tolonen *et al.*, 2006). In this scenario, *Prochlorococcus* may sense limitation of N sources as a reduction in the rate of N assimilation, probably via 2-OG (Forchhammer, 1999, Tandeau de Marsac *et al.*, 2001), and replies by activating the transport of all N sources at the same time (Tolonen *et al.*, 2006).

530   Expression of the ammonium transporter gene (*amt1*) reaches a peak in the evening, near sunset but displays a high expression level over the diel cycle. This suggests that there is a need for constant high production of Amt1 to ensure efficient uptake of any available ammonium from the oligotrophic waters where *Prochlorococcus* thrives (Zinser *et al.*, 2009). The expression of genes related to ammonium assimilation exhibits a similar

535   periodicity as the transporter, showing the maximum level of expression in the evening. 2-OG is produced by isocitrate dehydrogenase, encoded by the *icd* gene, which showed also maximum expression in the evening (Zinser *et al.*, 2009).

The urea and cyanate transporters (*urtAB* and *cynA*) showed maximum expression soon after sunrise and a secondary peak at night, although cyanate and urea were not

540   present in the media (Zinser *et al.*, 2009). Natural populations of *Prochlorococcus* can also obtain significant amounts of organic N from amino acids, particularly methionine and leucine (Zubkov *et al.*, 2003, Zubkov & Tarran, 2005, Mary *et al.*, 2008, Zubkov & Tarran, 2008) and their accumulation are substantially higher at dusk than at dawn (Mary *et al.*, 2008). The authors proposed that there is clear competition for amino acids between

545 natural populations of *Prochlorococcus* and heterotrophs in oligotrophic areas of de Arabian Sea and some areas of the Southern Atlantic gyre (Zubkov *et al.*, 2004, Zubkov & Tarran, 2005). By contrast, the amino acids were a very minor N source for *Synechococcus* (Zubkov & Tarran, 2005). Moreover, genomic and metagenomic studies suggest that many strains of *Prochlorococcus* and *Synechococcus* can take up amino acids 550 and could also degrade them to obtain ammonium for biosynthesis (Yelton *et al.*, 2016, Garczarek *et al.*, 2021). However, the physiological function of genes annotated as amino acid transporters have not been experimentally tested yet, and growth of these organisms on amino acids as N source has not been published. The distribution of these genes in selected *Synechococcus* and *Prochlorococcus* strains is shown in supplementary tables 2 555 and 3, respectively. An inducible ABC-type permease, encoded by the gene cluster *urtABCDE*, is required for urea uptake at low concentrations (<1 µM) in cyanobacteria, even though the internal urease is constitutively expressed (Valladares *et al.*, 2002).

The utilization of other forms of dissolved organic C has not been studied in detail in marine picocyanobacteria. However, some studies suggest that both *Prochlorococcus* 560 and *Synechococcus* release fluorescent organic matter, which might be degradation products of phycobilin pigments (Zhao *et al.*, 2017). The importance of these compounds as N sources in the oceans remains to be investigated in the field, but initial estimations suggest it might be significant (Zhao *et al.*, 2017).

565 **3. Adaptation in nitrogen regulatory mechanisms**

It has been proposed that the tight regulation of N metabolism by ammonium could be related to the high N content in cyanobacteria (Ohashi *et al.*, 2011). In addition, several assumptions seem to be common in the interaction between ammonium and nitrate. For 570 instance, environmental concentrations of ammonium can reduce, but not completely avoid nitrate uptake, and there are significant differences among phytoplankton species concerning the ammonium concentration threshold at which nitrate uptake is reduced (Mulholand & Lomas, 2008). Moreover, the repression of nitrate uptake by ammonium is not direct but mediated by organic metabolites products of its assimilation, likely 575 glutamine or glutamine/2-OG ratio, meaning that regulation of nitrate uptake is also related to the overall N status of the cell (Ohashi *et al.*, 2011).

A crucial point for the regulation of ammonium assimilation is to detect its presence or absence in the environment or the cell. The N regulatory networks of cyanobacteria

involve the transcription factor NtcA, which regulates the transcription of co-regulated genes, the signal transduction protein P_{II}, and the regulatory protein PipX. The three regulatory proteins coordinate N and C metabolism in cyanobacteria (figure 2) (Herrero *et al.*, 2001, Tandeau de Marsac *et al.*, 2001, García-Fernández *et al.*, 2004, Berges & Mulholland, 2008). *Prochlorococcus* and marine *Synechococcus* possess the corresponding regulatory genes, *ntcA* (NtcA)*, glnB* (P_{II}), and *pipX* (PipX) (Ohashi *et al.*, 2011). Although N control in marine cyanobacteria is less known, some important differences with respect to freshwater strains have been shown (El Alaoui *et al.*, 2001, Lindell *et al.*, 2002, Wyman & Bird, 2007, López-Lozano *et al.*, 2009, Domínguez-Martín *et al.*, 2016).

3.1. Regulatory adaptation of key enzymes in nitrogen metabolism in marine cyanobacteria

The enzymes implicated in N assimilation (figure 1) are regulated in response to environmental variables such as N sources and light availability, and to the intracellular concentration of various metabolites such as 2-OG or glutamine (García-Fernández *et al.*, 2004, Scanlan *et al.*, 2009).

GS is a key regulatory point in the N metabolism of cyanobacteria, subjected to control by the transcriptional regulator NtcA and the P_{II} protein (Herrero *et al.*, 2001). Besides, *Prochlorococcus* also possesses another regulatory system based on metal-catalyzed oxidative modification (MCO) shown to regulate GS degradation under nutrient starvation, including N (Gómez-Baena *et al.*, 2001, Gómez-Baena *et al.*, 2006, McDonagh *et al.*, 2012, Gómez-Baena *et al.*, 2015). Two different types of GS have been found in cyanobacteria. Although most strains have only one GS type I (GSI), encoded by *glnA*, some cyanobacterial strains have in addition GS type III (GSIII) encoded by *glnN*. Both are differently regulated (Muro-Pastor *et al.*, 2005, Berges & Mulholland, 2008).

Synechococcus sp. WH7803 possesses the two GSs, and their regulation has been demonstrated to be different (Domínguez-Martín *et al.*, 2016). GSI is not regulated by light while GSIII lost the responsiveness to N availability in sharp contrast with the enzymes studied in freshwater cyanobacterial strains (Reyes *et al.*, 1997, Domínguez-Martín *et al.*, 2016). GSI is up-regulated in ammonium-grown cells compared to those subjected to N starvation (El Alaoui *et al.*, 2001, Bird & Wyman, 2003, El Alaoui *et al.*, 2003). However, in freshwater cyanobacteria, GSI is usually up-regulated when cells are subjected to N starvation (Reyes & Florencio, 1995, Reyes *et al.*, 1997). This unusual

615 response to N limitation in marine cyanobacteria could be due to the characteristics of the oligotrophic environment of the oceans where these species thrive (Bird & Wyman, 2003, García-Fernández *et al.*, 2004).

In marine cyanobacteria, nitrate reductase regulation appears to be less important in controlling N assimilation than in their freshwater counterparts. On one hand, many 620 *Prochlorococcus* strains lack this enzyme (Palinska *et al.*, 2000, López-Lozano *et al.*, 2002, Moore *et al.*, 2002, Berube *et al.*, 2019). On the other hand, although it is present and regulated by the availability of N in most *Synechococcus* ecotypes (Bird & Wyman, 2003), its role is not that crucial since nitrate concentration in marine environments is significantly more constant than in freshwater cyanobacteria (García-Fernández *et al.*, 625 2004). Intriguingly, neither *nirA* nor *ntcA* are tightly regulated by ammonium in marine *Synechococcus* (Bird & Wyman, 2003). The arrangement of genes involved in nitrate assimilation also differs between freshwater and marine cyanobacteria (Domínguez-Martín *et al.*, 2022).

Enzymes such as urease and cyanase also have an important role in the N 630 metabolism in marine cyanobacteria (figure 1). In natural phytoplankton communities, urease activity seems to be inversely correlated with ammonium and nitrate concentrations, although there are some exceptions to this general rule. For instance, *Synechococcus* sp. WH8112 showed no difference in urease activity on ammonium versus urea, having higher urease activity when the cells were grown on nitrate (Solomon 635 *et al.*, 2010). Cyanate-related genes including those involved in its transport form part of the NtcA regulon, and in *Prochlorococcus* sp. MED4 have elevated transcript levels in N-deprived cells. However, *cynS* was not differentially expressed (Tolonen *et al.*, 2006). In fact, in *Synechococcus* sp. WH8102 a putative NtcA binding site was detected upstream of *cynA* but not of *cynS* indicating that transcription of *cynS* might be disconnected from 640 the response to N stress in that strain (Kamennaya & Post, 2011).

3.2. Adaptative responses of regulatory proteins

In order to analyze whether the regulatory genes *ntcA, glnB,* and *pipX* are also 645 widely spread in environmental samples, we determined their presence and abundance in the TARA metagenomic dataset (supplementary figure 2). Gene abundance was estimated, and the geographical distribution of the homologs was visualized for each gene in each station, using the *psbO* gene (encoding a photosynthetic protein) as a control to compare the abundances of regulatory genes. In general, we found similar patterns for the 650 presence of the *Synechococcales* genes in all TARA stations. We found *ntcA*, *pipX,* and

psbO sequences homologs only in cyanobacteria, but *glnB* was identified in many organisms (15% cyanobacteria). The abundances measured for *ntcA* and *psbO* genes were very similar, showing the importance of this key gene in the ocean.

The role of NtcA in *Prochlorococcus* and *Synechococcus* seem to differ from that in freshwater cyanobacteria (Lindell *et al.*, 2002, García-Fernández *et al.*, 2004). Furthermore, some freshwater strains can carry other regulatory elements as a secondary transcriptional activators-like (*ntcB*), encoding a LysR-type transcriptional activator necessary for optimal utilization of nitrate (Aichi & Omata, 1997, Aichi *et al.*, 2001); *gifA* and *gifB*, codifying inhibitory factors for GS activity (García-Domínguez *et al.*, 2000), and *nblA* required for the degradation of the phycobilisome under N deprivation (Collier & Grossman, 1994, Schwarz & Grossman, 1998, Luque *et al.*, 2001). All of them seem to be missing in marine strains. NtcA induces the expression of key genes required to utilize of several N sources, such as nitrate, nitrite, cyanate, or urea. These genes are characterized by the presence of a NtcA binding site, with the sequence $GTAN_8TAC$, in the promoter region (figure 2) (Picossi *et al.*, 2014, Domínguez-Martín *et al.*, 2017). In addition, a downstream -10 σ^{70}-like box was also required for the action of NtcA (Herrero *et al.*, 2001). The situation is not so clear for genes required for the utilization of other N sources, like amino acids, oligopeptides, etc. Their open reading frames often lack putative NtcA binding sites and thus their function in the N stress response is unclear (Scanlan & Post, 2008). However, a certain variability in these binding sites cannot be discarded, which might hide the responsiveness of those genes to NtcA. Further physiological studies are required to clarify this topic.

A new method for the prediction of cis-regulatory binding sites has allowed to predict new genes that can be controlled by NtcA in cyanobacterial genomes. It is worth noting that NtcA promoters were found for many genes involved in different stages of photosynthesis and C fixation (figure 2) (Su *et al.*, 2005). This work postulated for the first time that NtcA works as a regulatory protein coordinating two critical processes for cyanobacteria as photosynthesis and N assimilation (Su *et al.*, 2005), a hypothesis supported by other studies (Szul *et al.*, 2019) and validated by experimental procedures (Giner-Lamia *et al.*, 2017). It has been reported the presence of NtcA promoters in the chlorophyll a/b-binding light-harvesting genes (*pcb*) from low-light adapted *Prochlorococcus* strains SS120 and MIT9313, but not for *pcb* genes in high-light MED4 (Su *et al.*, 2005). On the other hand, NtcA promoters have been found for the photosystem II *psb* genes in MED4, WH8102, and PCC 7120, but not for their orthologues in SS120,

685 MIT9313, and PCC 6301 (Su *et al.*, 2005). These differences in the NtcA-regulated genes among ecotypes might be the result of acclimation to their ecological niches (Su *et al.*, 2005).

Interaction among the three regulatory proteins is well-studied in freshwater cyanobacteria (figure 2). P_{II} is a sensor-transducer protein that conveys high or low
690 carbon, energy, and nitrogen signals, translating them into changes in the activities of enzymes, channels, or gene expression. A small protein, PipX, interacts with P_{II} or NtcA; the 2-OG level regulates this interaction. In the presence of low 2-OG (high N within the cell), PipX is sequestered by P_{II}. When the N status is scarce, the level of 2-OG increases and PipX swaps partner by binding to the 2-OG activated transcriptional regulator NtcA,
695 co-activating the expression of the genes with NtcA-promoters. Nonetheless, in this scenario, NtcA can be self-activated (figure 2) (Espinosa *et al.*, 2006, Llácer *et al.*, 2010, Forcada-Nadal *et al.*, 2018). However, in marine cyanobacteria, little is known currently about this network.

Studies on the regulation of P_{II} in *Prochlorococcus marinus* PCC 9511 showed that
700 it was not phosphorylated when growing with different N sources, despite its highly conserved amino acid sequence including the Ser49, the phosphorylated residue of this protein in freshwater cyanobacteria (Palinska *et al.*, 2002). Also, the motif that binds DNA in NtcA from *Prochlorococcus* MIT9313 differs from other cyanobacteria in a substitution of serine by alanine and this change could affect the DNA binding specificity
705 of NtcA in that particular strain (Su *et al.*, 2005). The only in vitro study of the interaction NtcA-DNA in *Prochlorococcus* demonstrated that the NtcA response to 2-OG differs in the studied strains, underlying the diversity of C/N balance regulation in this genera (Domínguez-Martín *et al.*, 2018).

Expression studies highlighted differences of regulatory genes in *Synechococcus*
710 strains WH7803 and WH8103, and *Prochlorococcus* strains MED4 and MIT9313. When cells are deprived of ammonium, *ntcA* transcription is upregulated in *Synechococcus* sp. WH7803, but not in WH8103 (Lindell *et al.*, 1998, Lindell & Post, 2001, Wyman & Bird, 2007). On the other hand, *Prochlorococcus* MED4 responded to N starvation by upregulating more genes than MIT9313 (Tolonen *et al.*, 2006), although the *ntcA*
715 expression increased in both strains. A clear difference in the response of *ntcA* and *glnB* to N deprivation also occurs in *Synechococcus* sp. WH8102, where *ntcA* was upregulated whereas *glnB* was repressed (Su *et al.*, 2006), thus indicating that different responses of these two N regulators could be a common element in marine cyanobacteria (Tolonen *et*

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al., 2006). Moreover, *glnB* regulation under N stress in *Prochlorococcus* indicates that P$_{II}$ function may be independent of N utilization (Tolonen *et al.*, 2006). *Synechococcus* sp. strain WH7803 harbors a copy of *ntcA*, but unlike other marine picocyanobacteria, this strain is able of assimilating nitrate when grown in the presence of ammonium, as shown also in a recent study (Domínguez-Martín *et al.*, 2022). The expression of *nrtP, narB*, and *amt1* seems to be NtcA dependent in this marine cyanobacterium, but this is not the case for *nirA*. It should be highlighted that *ntcA* expression, in *Synechococcus* sp. WH7803 is regulated by ammonium concentration at the levels found in the oceanic environment (Lindell & Post, 2001). Ecologically, *ntcA* expression may be able to differentiate between regenerated and new primary production by cyanobacterial phytoplankton. It would appear then that *ntcA* expression by *Synechococcus* in nature responds to the ammonium flux rather than to ammonium concentration, the latter being the case for cultures (Post, 2005, Scanlan & Post, 2008).

3.3. Adaptations through transcriptional regulation of N metabolism: role of sigma factors and small RNAs

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In *Prochlorococcus*, like in other cyanobacteria, some specific sigma factors are induced in response to N starvation (Caslake *et al.*, 1997). It has been reported that *Prochlorococcus* cells have only a few regulatory proteins, a much lower number than other cyanobacteria (Dufresne *et al.*, 2003, Mary & Vaulot, 2003, Rocap *et al.*, 2003, Steglich *et al.*, 2008). This could be due to their relatively stable environment and contributes to the compaction of their genomes (García-Fernández *et al.*, 2004). However, this scarce baggage of regulatory proteins could be compensated by the relatively high number of regulatory non-coding RNA (ncRNAs). *Prochlorococcus*, like other streamlined microorganisms even so different as *Helicobacter pylori*, has a remarkably complex transcriptome for such a small genome, including regulating RNA, other nc RNAs, and mRNAs with short half-lives among others (Steglich *et al.*, 2010, Voigt *et al.*, 2014). The mRNA half-life of *Prochlorococcus* is 2.4 minutes, the shortest reported for any organism (Steglich *et al.*, 2010). The authors have proposed that a rapid mRNA turnover strategy might be a great advantage to recycle nucleotides for novel mRNA synthesis, thus allowing a rapid response to changing environmental conditions. Furthermore, it has been proposed that some of these ncRNAs can be among the transcripts regulated by NtcA (Muro-Pastor & Hess, 2020). These particular regulatory

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RNAs could be replacing in these organisms the complex protein-based regulatory network typical of other cyanobacteria (Steglich *et al.*, 2008, Steglich *et al.*, 2010).

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4. Interactions between nitrogen and carbon metabolism

Cyanobacteria are essential constituents of oceanic microbial communities contributing up to two-thirds of fixed CO_2 in the oceans. Nitrogen plays a crucial role in the uptake of CO_2 (Falkowski, 1997) and is a key component in how the ocean responds to global environmental changes (Pajares & Ramos, 2019). During photosynthesis, C and N metabolisms are coordinated to produce nitrogenated molecules such as amino acids and nucleotides. Thus, the balance between C and N metabolisms is critical for the growth and welfare of all photosynthetic organisms. Since there is a close connection between the marine C and N cycles, it was suggested that changes in the marine N cycle may have had a role in past alterations of the global C cycle and, particularly, in the atmospheric CO_2 concentration (Falkowski *et al.*, 1998, Falkowski *et al.*, 2008).

It has been estimated that the ratio of the maximal rates of C to N assimilation is roughly 2 to 2.5 times lower in cyanobacteria than in marine algae under optimized assay conditions (Sakamoto *et al.*, 1999). This suggests that, although the C and N assimilation ratio can vary, there is an intense competition for electrons generated by photosynthetic water oxidation, mainly when cells grow on nitrate (Flores *et al.*, 1983), therefore both processes should be precisely coordinated. Nitrate reduction in terrestrial environments is estimated to be six times smaller (ca. 333 Tg of nitrate per year) than in marine environments (2,000 Tg of nitrate per year (Duce *et al.*, 2008)) due to the lower N requirement of land plants, requiring about one molecule of N per 40 molecules of fixed C, while marine algae need one molecule of N per 6.6 fixed C molecules (Kuypers *et al.*, 2018). C and N metabolisms are among the most complex biochemical routes in terms of the number of enzymes and the extent of regulatory processes involved. However, these pathways are far more straightforward in marine picocyanobacteria compared to other photosynthetic organisms (García-Fernández *et al.*, 2004).

It has been demonstrated that the signal molecule 2-OG (Vazquez-Bermudez *et al.*, 2002, Flores & Herrero, 2005) is used as the indicator of C/N balance in cyanobacterial cells (Herrero *et al.*, 2001, Muro-Pastor *et al.*, 2001, Vazquez-Bermudez *et al.*, 2002). Since cyanobacteria do not have the enzyme 2-OG dehydrogenase (tricarboxylic acid cycle component), for a long time it was considered that the only fate for 2-OG was the

conversion to glutamate/glutamine through the GS/GOGAT pathway (Herrero *et al.*, 2001). Consequently, 2-OG accumulates under N limitation, acting as a signal for a high C/N ratio in the cell (Muro-Pastor *et al.*, 2001). Although this statement holds for most cyanobacteria, including marine strains, it must be considered that non-canonical ways of closing the TCA cycle have been described in some cyanobacteria (Zhang & Bryant, 2011, Steinhauser *et al.*, 2012). In *Prochlorococcus*, 2-OG accumulates under N limitation (Szul *et al.*, 2019), thus activating NtcA, which consequently binds 2-OG and PipX. The resulting complex acts then in the upregulation of the *ntcA* gene, N assimilation transporters and enzymes, and C fixation (Forchhammer & Selim, 2020). However, in *Prochlorococcus* the interaction of 2-OG with NtcA is of a lower strength than in freshwater cyanobacteria (figure 3) (Domínguez-Martín *et al.*, 2018). This suggests that a higher threshold 2-OG concentration is required to trigger the NtcA response; in other words, the NtcA regulon seems less responsive to small changes in 2-OG than in freshwater cyanobacteria; this might be another evidence of the streamlining of regulatory pathways in marine picocyanobacteria.

The signal transducer P_{II} has been shown to coordinate cellular C and N relationships in many cyanobacteria (Ninfa & Atkinson, 2000); it has been recently proposed that it can play an essential role in the control of other metabolic processes (Forchhammer *et al.*, 2022). As NtcA, P_{II} responds to 2-OG levels in freshwater cyanobacteria (Forchhammer, 1999, Tandeau de Marsac *et al.*, 2001). The increase in 2-OG levels intensifies P_{II} phosphorylation (Forchhammer & Hedler, 1997). This protein controls the activity of transporters for nitrite/nitrate and bicarbonate (Forchhammer, 2004). The *glnB* gene, encoding the P_{II} protein, is transcriptionally activated by NtcA (Tolonen *et al.*, 2006). Furthermore, complete activation of NtcA-regulated genes under N stress needs P_{II} (Paz-Yepes *et al.*, 2003), indicating that P_{II} and NtcA are functionally interdependent. The *Prochlorococcus* P_{II} shows a typical amino acid sequence for a cyanobacteria (Palinska *et al.*, 2002) but remarkably, it forms a separate subclade with other oceanic strains within the *glnB* cyanobacterial radiation (García-Fernández *et al.*, 2004). Moreover, proteomic studies on *Prochlorococcus* SS120 subjected to N stress indicate that NtcA increases while PipX and P_{II} decrease (Domínguez-Martín *et al.*, 2017). Further studies are needed to decipher the interplay between these molecules.

In most marine picocyanobacteria analyzed so far, inducible high-affinity C transport systems have not been found, in contrast to most freshwater cyanobacteria (Scanlan *et al.*, 2009). This implies that most picoplanktonic marine *Synechococcus* and *Prochlorococcus* strains lack the capacity for active CO_2 uptake unless they have developed some novel uptake system (Badger *et al.*, 2002, Badger & Price, 2003). From this, it can be inferred that inorganic C is available in sufficient quantities for growth and

other nutrients could represent the main limiting factors for these microorganisms (Scanlan *et al.*, 2009, Zinser *et al.*, 2009, Hopkinson *et al.*, 2014).

How N limitation can affect C fixation in *Prochlorococcus* has also been studied. As expected, it was found that under N limitation, the whole amount of photosynthesis performed was much lower than that observed in well-supplied cultures (Szul *et al.*, 2019). Furthermore, another interesting aspect related to N and C metabolism relationship is that phytoplankton excretes organic C as a way to reduce the excess of reducing power under a nutrient limitation or extreme light (Fogg, 1983). Thus, a study comparing the metabolic flux in N repleted vs N limited cells of *Prochlorococcus* reported that a large amount of fixed C in N limited cultures is released into the environment (Szul *et al.*, 2019). This fascinating behavior might be connected to an evolutionary strategy proposed in *Prochlorococcus* to increase the bioavailability of material and, hence, oceanic biomass (Braakman *et al.*, 2017).

Besides, *Prochlorococcus* sp. VOL29 stores a more significant amount of polysaccharides, although C fixation is lower, under N limited than in N replete conditions (Szul *et al.*, 2019). These results are similar to those reported for freshwater cyanobacteria (Osanai *et al.*, 2007, Joseph *et al.*, 2014). The above-described results are consistent with the overflow hypothesis for C metabolism (Cano *et al.*, 2018): when C fixation exceeds metabolic demand due to low N content, C is used to synthesize glycogen. The excess of C could then be liberated to the environment. This scenario reinforces the relationships between the limitation of N and C metabolism. These results also show that N limitation in *Prochlorococcus* has a significant effect on C fixation, which could have important consequences for the impact of this cyanobacterium on CO_2 fixation by the phytoplankton population.

However, a previous study comparing expression patterns of C metabolism genes in *Prochlorococcus* sp. MED4 (representative of HL strains) and *Prochlorococcus* sp. SS120 (representative of LL strains) showed that the two strains integrate N and C metabolisms differently (Tolonen *et al.*, 2006). Besides, the regulation of the metabolism of glycogen during N stress differs in these two strains of *Prochlorococcus* compared to freshwater cyanobacteria. Freshwater cyanobacteria accumulate glycogen during N starvation, while MED4 and MIT9313 strains increased transcription of the glycogen phosphorylase, and MED4 also repressed genes for glycogen synthesis. It has been proposed that *Prochlorococcus*, which lives in a relatively homogenous environment, responds to N stress by expending C reserves (Tolonen *et al.*, 2006); this might be related

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to the evolutionary trend of *Prochlorococcus* to increase the excretion of organic C (Braakman *et al.*, 2017). However, freshwater cyanobacteria could respond to N

860 starvation by storing C in preparation for a future change of N supply. Thus, as it happens with the N assimilation regulatory systems the C metabolism regulation behaves somehow differently in *Prochlorococcus* strains from other cyanobacteria.

Cyanobacteria lack a complete glycolytic pathway, and it was also thought they lack the Entner–Doudorhoff (ED) pathway. So, they should use the oxidative pentose

865 phosphate pathway (PPP) to originate pyruvate and, eventually, 2-OG for N assimilation. Two crucial genes involved in the PPP are upregulated during N limitation. The *zwf* gene, codifying for the enzyme that catalyzes the first step in the PPP, was upregulated in MED4. The *tal* gene, encoding the transaldolase that reorganizes the C skeletons in the PPP, was upregulated in MED4 and MIT9313 strains (Tolonen *et al.*, 2006). However, it

870 has been later described that the ED pathway can be operative in cyanobacteria such as *Synechocystis* (Chen *et al.*, 2016) and *Prochlorococcus* SS120 (Muñoz-Marín *et al.*, 2017). On the other hand, the *icd* gene (encoding isocitrate dehydrogenase) is upregulated in MED4 and downregulated in MIT9313 under N starvation. As mentioned above, it has been suggested that MED4 and MIT9313 respond to N starvation by degrading glycogen.

875 The C liberated could then be channeled through the PPP towards synthesizing 2-OG to efficiently assimilate intracellular N (Tolonen *et al.*, 2006). In *Prochlorococcus* SS120, similar results to those of MIT9313 (both LL ecotypes) have been described (López-Lozano *et al.*, 2009). Analogous results to those of MED4 have been reported for PCC 9511 (also HL ecotype) in response to the addition of azaserine that, by inhibiting

880 glutamate synthase, produces N stress in the cells (Domínguez-Martín *et al.*, 2014).

Metatranscriptomic studies showed that urea and ammonium treatments resulted in significant increases in Chl *a* concentration, primary productivity, and cell densities of *Prochlorococcus* (Shilova *et al.*, 2020), but cell densities did not significantly increase in *Synechococcus* under those conditions (Shilova *et al.*, 2017). The addition of urea or

885 ammonium seems to relieve signs of N stress, as shown not only by diminishing transcription of N-related genes but also by the increase in photosynthesis and C fixation genes transcription. Besides reinforcing the connection between C and N metabolism, these results support the capacity of *Prochlorococcus* cells to assimilate both N sources (Moore *et al.*, 2002, Berthelot *et al.*, 2019).

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5. Molecular strategies to save nitrogen

N availability limits productivity in vast areas of the oligotrophic oceans. One of the adaptations that helps these microorganisms to survive in low nutrient conditions is their small cell size, which facilitates nutrient transport by increasing the surface to volume ratio and reduces the absolute cellular requirement for nutrients (Chisholm *et al.*, 1992, Zehr *et al.*, 2017). Other adaptations are their small genomes and a proteome with a reduced N content (Grzymski & Dussaq, 2012, Read *et al.*, 2017). The abundance of *Prochlorococcus* in oligotrophic environments is explained on the basis of several adaptative features that reduce its cellular nutrient requirements, thus facilitating growth (Read *et al.*, 2017). One of such genomic adaptations is the preferential utilization of amino acids containing fewer N-atoms, which significantly reduces cellular N requirements (Grzymski & Dussaq, 2012). Furthermore, a low translation rate or shorter functional proteins (Voigt *et al.*, 2014, Read *et al.*, 2017) seem to be also advantageous for a slow-growing microorganism as *Prochlorococcus*, as well as a lower amount of regulatory proteins (Read *et al.*, 2017), using instead a series of non-coding RNAs or mRNAs with short half-lives (see section 3.3). This strategy allows a rapid response of the cells to changes in N availability (Read *et al.*, 2017) while genomic changes can only occur over evolutionary time scales. Moreover, it has been proposed that a relatively constant cellular protein concentration due to slow protein turnover could also be a way of saving N (Karlsen *et al.*, 2021).

Prochlorococcus genomes generally are less GC rich than those from *Synechococcus*, thus requiring less N to grow (Moore *et al.*, 2002, Scanlan *et al.*, 2009), since the amino acids encoded by low GC codons have a lower N content than those encoded by GC rich codons (Bragg & Hyder, 2004, Biller *et al.*, 2015). Besides, surface waters tend to be more nitrogen-limited than deeper waters; this correlates with the fact that HL-adapted strains, which are typically most abundant near the surface, have a lower GC content than LL-adapted strains (Gilbert & Fagan, 2011). The average amount of N in the *Prochlorococcus* proteome is lower than that of coastal bacteria (Grzymski & Dussaq, 2012). These features confer a significant saving of N considering the whole genome of these organisms. This involves, for instance, removing in some strains genes which are considered essential in other freshwater cyanobacteria (such as *narB* or *nirA*, described above; or *kaiC*). Furthermore, the genomes of these oligotrophic microorganisms are observed to encode proteins, on average, with fewer amino acids containing more N in their side chain compared to coastal strains, a critical metabolic

adaptation for these oligotrophic niches (Grzymski & Dussaq, 2012). As an evolutionary trade-off, proteomes from organisms adapted to low N availability often have slightly higher mass or more C atoms, a non-limiting element in these regions (Grzymski & Dussaq, 2012).

930   Some strains have additional signatures of selection for N minimization in the remarkably reduced N content of many N stress-responsive proteins (Biller *et al.*, 2015). In addition, these strains have experienced a genome simplification process in which the number of coding sequences has been reduced, in part, by eliminating many of the regulatory proteins. Rebalancing of macromolecular pools to reduce the quota of N has

935   also been described in areas with a low concentration of nitrogen so that the protein content was reduced, and that of carbohydrates and lipids increased (Casey *et al.*, 2022). Given that biosynthesis of lipids has higher energy requirements than that of carbohydrates, it was observed that allocation of C to lipids was favored close to the ocean surface. In contrast, carbohydrates were preferred at depth (Casey *et al.*, 2022). Besides,

940   it has been reported that under N limitation, some *Prochlorococcus* can use alternative transcription start positions to generate smaller proteins, thus saving N (Read *et al.*, 2017). For instance, genes with essential physiological functions, such as RNA synthesis, glutamate synthesis, fatty acid biosynthesis, and transport of key compounds such as cyanate, have internal transcription sites under N-deprived conditions. The authors

945   proposed that these intra-RNAs may encode functional proteins. Thus the shortened translated proteins would be a good way to reduce the N requirements of the cell when that compound was scarce (Read *et al.*, 2017).

   One group of proteins with an unusual N content is the ribosomal proteins (Acquisti *et al.*, 2009). These proteins are rich in N compared to others, and the genes encoding

950   these ribosomal proteins tend to be down-regulated during N limitation (Domínguez-Martín *et al.*, 2017). Another relevant mechanism in *Prochlorococcus* to avoid utilization of N is the development of a new type of antenna, encoded by the *pcb* genes (Garczarek *et al.*, 2000) instead of phycobilisomes, the protein complexes of light collection utilized by marine *Synechococcus* and freshwater cyanobacteria. Phycobilisomes contain large

955   amounts of nitrogen, reaching up to 50 % of the total N in cells (Krauspe *et al.*, 2021). Recent single-cell studies have shown the occurrence of early representatives in the evolution of *Prochlorococcus,* which possessed phycobilisomes (Ulloa *et al.*, 2021); this study suggests the replacement of phycobilisomes by Pcb antenna in *Prochlorococcus* happened after the divergence of *Prochlorococcus* from other cyanobacteria. This

960 evolutive process was probably driven by the low N availability in oligotrophic oceans inhabited by this genus. Under N limitation, cyanobacteria develop a physiological program that includes dismantling phycobilisomes, which are used as a source of N (Forchhammer & Schwarz, 2019), resulting in a loss of autofluorescence and cell bleaching. This program, called nitrogen chlorosis (Schwarz & Forchhammer, 2005,

965 Klotz *et al.*, 2016, Muro-Pastor *et al.*, 2020), is a highly organized process. Recent studies show that, unlike other cyanobacteria, chlorotic *Prochlorococcus* cells are not viable and do not regrow under axenic conditions when transferred to new media. However, cocultures with a heterotrophic bacterium allowed *Prochlorococcus* to survive for months without nutrients, even without producing resting stages. This dependence on concurrent

970 heterotrophic bacteria underlies the ecological success of *Prochlorococcus* (Roth-Rosenberg *et al.*, 2020) and shows that part of the strategy to survive under N limitation depends on interactions with other organisms.

Mutualistic interactions related to N have also been shown in marine *Synechococcus* (Christie-Oleza *et al.*, 2017). Monocultures of *Synechococcus* showed a

975 decline in cell density at the stationary phase under different N conditions, but when it was grown with *Ruegeria pomeroyi* (heterotrophic bacteria) in co-culture, both organisms could reach similar cell densities as in monoculture, but for a longer period of time. These results showed that heterotrophic bacteria play an essential role in remineralizing N compounds (among other nutrients), which can later be recycled by marine

980 *Synechococcus*, thus supporting the long-term stability of the co-cultures.

The above described strategies to save N are reflected in the elemental composition of *Prochlorococcus* and *Synechococcus* (Bertilsson *et al.*, 2003, Heldal *et al.*, 2003, Lopez *et al.*, 2016, Roth-Rosenberg *et al.*, 2021) (table 3): the N content per cell is lower in *Prochlorococcus*, with only one studied strain (MIT9312) showing values similar to

985 those of *Synechococcus* strains. *Prochlorococcus* evolved in oligotrophic environments, and one of the consequences is that both the cell volume and the N content per cell diminished: a single *Prochlorococcus* cell has N requirements which can be roughly 4-fold lower than marine *Synechococcus* while maintaining its viability and capacity to thrive in those nutrient-limited ocean niches. This comparison is valid even for

990 oligotrophic *Synechococcus* strains, such as WH8102. Nevertheless, when considering the N content per volume, that difference vanishes, as we see similar values for both genera.

6. Evolutionary and ecological aspects

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Low-nutrient conditions in vast areas of the open oceans have driven selection for very small phytoplankton primary producers (Martínez-García & Pinhassi, 2019). It has been estimated that a common ancestor for *Prochlorococcus* could have appeared between 684 and 543 Mya and for marine *Synechococcus* between 550 and 421 Mya

1000 (Sanchez-Baracaldo *et al.*, 2014), providing a long period of time to adapt to such oligotrophic conditions. In those areas, most production is sustained by nutrients like

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ammonium from the remineralization of dissolved organic matter (DOM) (Martínez-García & Pinhassi, 2019).

1005   The marine cyanobacteria *Prochlorococcus* and *Synechococcus* are abundant in many oceanic regions. Still, while *Prochlorococcus* is mainly constrained to the tropical and subtropical open ocean where nutrients are scarce, *Synechococcus* has a wider environmental distribution (Flombaum *et al.*, 2013). Although temperature seems to be the main factor governing the zonal distribution of both microorganisms, the kind and concentrations of nutrients could also influence it. However, the relationship between

1010   nutrient abundance and availability is complex (Flombaum *et al.*, 2013). One of the ecophysiological factors that may explain the differential oceanic distribution of these marine cyanobacteria is biogeochemically significant differences in their capability to assimilate oxidized forms of N (Partensky *et al.*, 1999, Moore *et al.*, 2002). For instance, in a metagenomic analysis in the Indian Ocean, *Prochlorococcus* seems to present a

1015   negative correlation with nitrite concentration but not a clear correlation with other N sources such as nitrate or ammonium (Wang *et al.*, 2021). In the same area, it was also reported that enzymes implicated in DON assimilation were more abundant than those involved in inorganic forms assimilation in *Prochlorococcus* population (Wang *et al.*, 2021). The direct urea utilization could be an useful ecological strategy for high-light

1020   *Prochlorococcus* in ammonia-limiting waters, allowing its acquisition from zooplankton waste product without the need for a heterotrophic intermediary with urease capability (Saito *et al.*, 2014). The *urtA* gene has been amplified from environmental samples and clone libraries, indicating that urea acquisition is common among *Prochlorococcus* or *Synechococcus* strains (Kamennaya *et al.*, 2008); most of them also have the genes coding

1025   for urea hydrolysis (tables 1 and 2). Gene expression analyses have shown that a series of N metabolism genes, not only *urtA* but also *amt* and *nirK* are among the most abundantly expressed genes in low-nutrient environments (Shi *et al.*, 2011, Martínez-García & Pinhassi, 2019).

As described above, nitrate is an expensive source of N for the cell. As a

1030   consequence, cells must compensate for it with an increase in the photochemically generated reducing power required for nitrate assimilation (Thompson *et al.*, 1989). In *Prochlorococcus*, these extra fees apparently provoke a decrease in growth rate under saturating light intensity with nitrate compared to ammonium as the sole N source (Berube *et al.*, 2015). There are two factors related to the capability of a particular

1035   *Prochlorococcus* strain to use nitrate as an N source: one, the abundance of nitrate and/or

other N sources together with nutrient fluxes between different areas; two, the illumination conditions needed to provide the extra energy, that is related with the depth where they thrive (Berube *et al.*, 2016). The frequency of cells that can assimilate nitrate within the HLII clade, the most abundant *Prochlorococcus* clade in subtropical gyres (Malmstrom *et al.*, 2010), is clearly correlated with diminished N availability in surface waters where they dominate. These cells are proposed to have a selective advantage under these conditions with sufficient energy and limiting N (Berube *et al.*, 2016). Besides, *narB*-carrying *Prochlorococcus* can be abundant under potentially N-limiting conditions because they can take advantage of interactions with nitrifying organisms by using nitrite and nitrate produced from ammonia and nitrite oxidation. The situation appears to be more complex for the LLI clade. All previously described *Prochlorococcus* in the LLI clade can assimilate nitrite, but only a fraction can also incorporate the more oxidized nitrate (table 2) (Berube *et al.*, 2015).

Cells belonging to the LLI clade dominate at shallower depths (Zinser *et al.*, 2007) and have characteristics that are intermediate between HL and other LL clades (Campbell *et al.*, 1997, Ahlgren *et al.*, 2006, Yan *et al.*, 2018). Thus, this clade tolerates higher irradiance levels among low-light *Prochlorococcus* so it can get the required, reducing power to support the reduction of nitrate. This may represent a selective advantage for *Prochlorococcus* living close to elevated concentrations of nitrate or nitrite (Berube *et al.*, 2016). However, in the analysis of genomes included in the Cyanorak database (table 2), only one of the six *Prochlorococcus* strains that possess *narB* belongs to the LLI clade. On the other hand, at greater depths within the euphotic zone, the low irradiance would not be able to provide the reducing power needed for nitrate assimilation, even when this is abundant (Berube *et al.*, 2019). The presence of *narB* apparently would not provide any benefit. However, it can also occur that the possible advantage remains unidentified since the streamlining in genome size seems to remove any unnecessary genes.

It was initially proposed that *Prochlorococcus* may have lost the nitrate assimilation genes early after it diverged from *Synechococcus* and reacquired them later through lateral gene transfer mechanisms (Coleman *et al.*, 2006, Martiny *et al.*, 2009, Berube *et al.*, 2015) in the clades that appeared more recently. However, recent studies suggest a more complex model of *Prochlorococcus* evolution: as environmental nutrient levels decrease, the free energy cost of nutrient uptake increases, connecting closely nutrient affinity to the energy flux of cells (Braakman *et al.*, 2017, Berube *et al.*, 2019, Braakman, 2019). It is argued that the diversity and intraspecific distribution of the nitrate

1070 assimilation feature in *Prochlorococcus* is likely driven by a combination of vertical inheritance, gene loss, and homologous recombination, involving only occasionally horizontal gene acquisition (Berube *et al.*, 2019) by mobile gene elements (Hackl *et al.*, in press). Although it has been also standing out that genome rearrangement may provide *Prochlorococcus* with significant advantages for occupying new ecological niches (Yan

1075 *et al.*, 2018). An even more recent model has proposed that the Snowball Earth catastrophe (global glaciation) occurring during the Neoproterozoic Era, and lasting for millions of years, caused a high reduction of *Prochlorococcus* genome (Zhang *et al.*, 2021). The proposal also established that there were a series of spaces able to support the life of microorganisms such as *Prochlorococcus*, but its severe conditions, including low

1080 temperatures, dim light, and limited nutrients, forced to evolve a series of adaptive genomic mechanisms to deal with those stresses. These adaptions involved essential changes in N metabolism genes (Zhang *et al.*, 2021).

Initial genomic studies showed that *Prochlorococcus* and *Synechococcus* have small genomes ranging in size from 1.6 to 2.5 x 10^6 nucleotides (Dufresne *et al.*, 2003,

1085 Palenik *et al.*, 2003, Rocap *et al.*, 2003). MED4 has 364 genes without an orthologue in MIT9313, whereas MIT9313 has 923 that are not present in MED4. These strain-specific genes indicate how well these strains thrive under different environmental conditions. Almost half of the 923 MIT9313-specific genes are present in *Synechococcus* sp. WH8102, suggesting that they have been lost from MED4 by genome reduction (Rocap

1090 *et al.*, 2003). *Prochlorococus* strains appear to share a common gene pool (core) of some 1270 genes (Kettler *et al.*, 2007). In contrast, the rest of the genes principally encode specific functions which allow the adaptation to their particular microenvironments (Scanlan & Post, 2008). Currently, that genome size range has not significantly changed, although it has increased and goes from 1.48 to 2.68 x 10^6 nucleotides (according to

1095 Cyanorak). It is currently believed that genome reduction was driven by selection for the removal of genes providing only a tiny fitness benefit outweighed by the associated costs (Sun & Blanchard, 2014). Niche adaptation induced the diversification of genomes adapting to HL and LL environmental conditions along the water column, leading to the appearance of HL-adapted strains with smaller genomes and low GC content than the LL

1100 strains (Biller *et al.*, 2015). Genomic studies have shown that any *Prochlorococcus* strain has ca. 2,000 genes, but the total pangenome is 40 times bigger (over 80,000 genes) (Biller *et al.*, 2014, Biller *et al.*, 2015). This means there is a vast gene potential for

adaptation to specific niches in the ocean, including a subset of genes involved in N assimilation.

1105   The analysis of the N acquisition capacities of the MIT9313 and MED4 strains showed a progressive loss of the ability to use nitrate and nitrite during *Prochlorococcus* evolution. MIT9313 has lost a 25-gene cluster containing the genes encoding the nitrate/nitrite transporter and nitrate reductase. MIT9313 has retained the nitrite reductase gene, but in a way that suggests it has been acquired by lateral gene transfer. This could

1110   support the genes losses and gains proposed in the Snowball Earth hypothesis (Zhang *et al.*, 2021). MED4 suffered another deletion episode in which the nitrite reductase gene was also lost. So MIT9313 cannot use nitrate, and MED4 cannot utilize nitrate or nitrite (Moore *et al.*, 2002). As nitrite-utilizing strains MIT9313, MIT9303, NATL1A, and NATL2A diverged deeply rooted in time, it appears that the nitrite assimilatory genes

1115   were lost after the loss of the nitrate assimilatory genes. This loss might correspond with the appearance of HL *Prochlorococcus* strains and their success in the ocean surface layers (Scanlan *et al.*, 2009). Each *Prochlorococcus* ecotype can use the N form more abundant under the illumination conditions to which they are best adapted: ammonium in the surface waters and nitrite at depth (Rocap *et al.*, 2003). So, N appears to be an essential

1120   selective agent driving niche differentiation of strains such as MED4 and MIT9313 (Tolonen *et al.*, 2006).

    Genome reduction appears to be a crucial factor underlying the ecological success of *Prochlorococcus*, compared to *Synechococcus* or photosynthetic picoeukaryotes, in oligotrophic oceanic environments (Dufresne *et al.*, 2005, Otero-Ferrer *et al.*, 2018). An

1125   exception to this statement appears in the genomes of five LL *Prochlorococcus* strains, MIT0701, MIT0702, MIT0703 (Cyanorak database), MIT9303, and MIT9313 (Kettler *et al.*, 2007). The genomes of these strains are similar in size to those of *Synechococcus* (Scanlan *et al.*, 2009). A series of genomic analyses of *Prochlorococcus* (Dufresne *et al.*, 2003, Rocap *et al.*, 2003, Berube *et al.*, 2019) and *Synechococcus* (Palenik *et al.*, 2003, Berube *et al.*, 2018) have provided an excellent example of how this kind of studies can

1130   generate comprehension of its ecological capabilities. A detailed analysis of the current, comprehensive marine picocyanobacterial databases (Cyanorak, Integrated Microbial Genomes/*Prochlorococcus* Portal) is helpful for performing comparative studies on this topic. An examination of their genetic capabilities shows *Synechococcus* has a broader range of abilities using N sources (tables 1 and 2). All *Prochlorococcus* and

1135   *Synechococcus* strains can use ammonium. Most *Synechococcus* strains, 47 out of 51, can use NO_3, whereas only 6 *Prochlorococcus*, 2 HL, and 4 LL possess nitrate transport and reduction genes. Something similar occurs with NO_2, while all *Synechococcus* strains have *nirA*, only 11 of the 43 *Prochlorococcus* possess *nirA*, 2 HL, and all the LLI and

1140   LLIV strains, although some of them do not have the transporter gene. Concerning urea

assimilation, again most *Synechococcus* are capable of using it, in this case, most HL and some LL *Prochlorococcus* can assimilate urea. The situation is peculiar with cyanate, most *Synechococcus* have the gene codifying for cyanase, but only 8, all belonging to clade III, have the transporter gene. Once more, a smaller number of *Prochlorococcus* strains have the genes needed for cyanate assimilation. Finally, amino acids transport genes are found in most *Synechococcus* and about half of *Prochlorococcus* strains, although physiological characterization of these genes has not been carried out yet.

It has been postulated that each *Prochlorococcus* ecotype uses the N species most prevalent at the light levels to which they are adapted, NH_4^+ in the surface waters and NO_3^- at depth (Rocap *et al.*, 2003, Martiny *et al.*, 2009, Berube *et al.*, 2015, Berube *et al.*, 2019). On the other hand, *Synechococcus* has retained nitrate reductase and so can bloom during NO_3^- upwelling events (Glover *et al.*, 1988, Dufresne *et al.*, 2003, Rocap *et al.*, 2003), and this may contribute to its relative abundance near the coast. *Synechococcus* is usually less abundant than *Prochlorococcus* in oligotrophic environments, where NO_3^- concentrations are generally low, but has a more comprehensive global distribution (Scanlan & West, 2002, Palenik *et al.*, 2003). Besides that, photosynthetic picoeukaryotes are more abundant than picocyanobacteria in higher latitudes, including the Arctic Ocean (Balzano *et al.*, 2012, Metfies *et al.*, 2018)

There are now several hundreds of sequenced genomes from cyanobacteria (Alvarenga *et al.*, 2017), and a significant part of them belong to the marine picocyanobacteria *Prochlorococcus* and *Synechococcus* (Berube *et al.*, 2018, Garczarek *et al.*, 2021) including a recently described *Synechococcus* strain from the Arctic that has been found at temperatures as low as -29°C (Tang *et al.*, 2019). Together all these genomes are expected to produce a tremendous amount of information, among others, on N acquisition capabilities, N metabolism, and N stress responses in these species. Detailed analysis of 387 single-cell genome assemblies has provided deep insights into the evolution and variability of N assimilation genes in *Prochlorococcus* (Berube *et al.*, 2019). Berube and coworkers proposed that the main driver of the diversity and distribution of nitrate assimilation genes is a combination of vertical inheritance and gene loss, but it is rarely due to non-homologous recombination. Seasonality in environmental conditions (including the availability of N forms) confers a selective advantage to the presence of nitrate assimilation gene in some *Prochlorococcus* ecotypes, facilitating their conservation (Berube *et al.*, 2016). Niche partitioning in this genus included some basal lineages restricted to great depths, which led to the loss of nitrate assimilation due to its higher cost and the availability of other N sources (García-Fernández *et al.*, 2004, Berube *et al.*, 2019). The low-light adapted *Prochlorococcus* strains MIT9313 and NATL2A retained the genes for nitrite utilization and, in fact grow on nitrite as the sole N source

(Moore *et al.*, 2002, Rocap *et al.*, 2003). This phenotype is consistent with the area of the water column where the low-light adapted *Prochlorococcus* thrives, around depths where a nitrite maximum is found (Scanlan & Post, 2008)

The *cynS* gene, codifying cyanase, was also identified among different kinds or organism sequences in public databases. Furthermore, this gene was particularly prevalent among marine cyanobacteria, including numerous *Prochlorococcus* and *Synechococcus* strains (Rocap *et al.*, 2003, Palenik *et al.*, 2006, Scanlan *et al.*, 2009, Kamennaya & Post, 2011). Amino acid residues proposed to be important in the catalytic activity for the *E. coli* CynS protein are fully conserved in all *Synechococcus* and *Prochlorococcus* cyanases (Kamennaya & Post, 2011). It has been proposed that cyanobacterial *cynS* evolved from a common ancestor near the base of the bacterial radiation. Of a total of 90 GOS-derived cyanase sequences, 56 clustered with cyanobacterial CynS, and were related to known *Synechococcus* and *Prochlorococcus* CynS (Kamennaya & Post, 2011).

In this part of the review, we are trying to describe the great complexity of evolutive changes concerning N metabolism in marine cyanobacteria; the determination of biodiversity in this group of microorganisms is also of great interest. In this sense, it is interesting to remark a proposal that can join both facts. That is, the N regulatory gene *ntcA* is a valuable marker to determine the N-status of cyanobacteria, and it has been proposed that its expression may also serve as an excellent biodiversity marker capable of distinguishing among different clades within each genus with a high-resolution (Penno *et al.*, 2006). *ntcA* has two important advantages for use as a cyanobacterial biodiversity marker. The first one is that it is only found in cyanobacteria (Frías *et al.*, 1993, Lindell & Post, 2001, Post, 2005). The second one is that it can be directly connected to cyanobacterial N-status (Vega-Palas *et al.*, 1992, Lindell & Post, 2001). For example, studies carried out in the field have shown the usefulness of proteomic studies related to NtcA and other proteins considered as biomarkers to assess the nutritional status of picocyanobacterial populations in the oceans (Saito *et al.*, 2014)

Picophytoplankton, containing three major groups, *Prochlorococcus*, *Synechococcus*, and picoeukaryotic phytoplankton, is the most abundant phytoplankton component on the oceans (Fuhrman & Campbell, 1998, Partensky *et al.*, 1999). In the future, ocean warming and reduced nutrients are expected to benefit *Prochlorococcus* and *Synechococcus*, at the expense of other groups with greater size (Visintini *et al.*, 2021). It has been proposed that vertical stratification controls the picophytoplankton

community in subtropical regions and that photosynthetic picoeukaryotes dominate in weakly stratified ocean zones, whereas *Prochlorococcus* and other cyanobacteria are more numerous in strongly stratified ocean environments (Otero-Ferrer *et al.*, 2018). On the other hand, *Prochlorococcus* is more abundant in the open sea, whereas *Synechococcus* and picoeukaryotes dominate coastal systems (Massana, 2011).

Prochlorococcus and *Synechococcus* seem to have substantial differences in their abilities to settle abundant populations along gradients of environmental changes. The *Prochlorococcus* HL genotype is abundant in stably stratified, warm, oligotrophic conditions. However, *Prochlorococcus* populations experience a dramatic reduction over the seasonal cycle, indicating that this genotype could not acclimate to the seasonal changes in environmental conditions. Instead, seasonal changes in *Synechococcus* abundance were less dramatic and were accompanied by a significant shift in *Synechococcus* genotypic diversity. Consequently, genotypic diversity among *Synechococcus* populations may explain its ability to occupy marine environments over a broader range of environmental conditions (Penno *et al.*, 2006). On the other hand, it has been proposed that oceans will experience temperature and N supply changes in the future (Flombaum *et al.*, 2013, Kim *et al.*, 2014, Flombaum *et al.*, 2020). The genetics of populations determines how environmental factors affect their ecologies and evolution, and a study has reported that the addition of different N sources favors diverse components of the phytoplankton community in different parts of oligotrophic areas of the North Pacific Ocean, particularly NH_4^+ addition had a significant effect on *Prochlorococcus* and photosynthetic picoeukaryotes, but not on *Synechococcus* abundances (Shilova *et al.*, 2017). A more recent study has shown that the three phytoplankton groups respond to N sources addition by increasing transcription levels of photosynthesis and C fixation genes. Still, only *Prochlorococcus* substantially increased its growth, particularly after ammonium or urea addition, suggesting it could out-compete the two other groups under these conditions (Shilova *et al.*, 2020).

7. Conclusion and perspectives

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The studies here reviewed have provided a large body of knowledge, contributing to unveiling the main adaptive mechanisms which marine picocyanobacteria have evolved to cope with the N conditions of the oceans where they live. This process, spanning several hundred of million years, led to extensive modifications in their

1245 genomes and physiology, which have been reflected in key features such as the low percentage of GC and strong streamlining of their genomes, the simplified regulatory systems (involving the contribution of ncRNAs heavily), the occurrence of truncated versions of proteins or the reduced responsiveness of essential transcriptional regulators, such as NtcA, to the molecule utilized to sense the N/C status by cyanobacteria, 2-OG.

1250 Taking all those mechanisms into account, *Prochlorococcus* and marine *Synechococcus* have demonstrated outstanding flexibility, as a collective, to colonize and thrive in all kinds of oceanic niches, from cold waters of the poles to warm intertropical oceans; from nutrient-rich coastal regions to extremely oligotrophic areas.

Despite this wealth of knowledge in the last two decades, there are still topics in
1255 this field that remain poorly understood and deserve future research. Among them, the actual contribution of the different inorganic N sources available in the oceans to the primary production by these organisms; the ecological relevance of organic compounds as sources of N; the physiological function, affinity and specificity of N transporters, and their involvement in niche adaptation; the potential crosstalk for regulation of N

1260 metabolism between proteins involved in transcriptional control and the recently discovered control systems mediated by ncRNA; the importance of trophic interactions to sustain microbial populations in N limited ocean environments; and the consequences of N limitation on central metabolic pathways, especially photosynthesis and electron transport, in these important organisms. The wealth of data obtained from systematic

1265 molecular sampling in the oceans and the ever-expanding genomic databases on one side, and state-of-the-art technologies focused on the analysis of single cells on the other, will undoubtedly pave the way for significant advances in this field in the coming years.

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The authors declare no conflict of interest.

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Legends for figures

Figure 1. Nitrogen assimilation pathways in cyanobacteria. Different N sources taken up
1295   through permeases and transporters and metabolized to ammonium, which is incorporated
into C backbones through the GS-GOGAT pathway. The tricarboxylic acid cycle (TCA)
in marine picocyanobacteria is incomplete. Enzymes involved in the assimilation of the
different N sources are also highlighted in the scheme. Nitrate reductase (*narB*); nitrite
reductase (*nirA*); glutamine synthetase I (*glnA*); glutamine synthetase III (*glnN*);
1300   glutamate synthase (*glsF*); Aa, aminoacids; nitrate transporter NrtP (*nrtP*), nitrite
permease NitM (*nitM*); cyanate transporter CynABD (*cynABD*); urea transporter
UrtABC (*urtABC*); ammonium transporter Amt1 (*amt1*). * Uptake of amino acids has
been demonstrated in marine picocyanobacteria, but their assimilation and physiological
function of transporters are poorly characterised. See section 2 for further details.

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**Figure 2. Schematic model of the action of the regulatory proteins NtcA, PII, and PipX
in cyanobacteria.** N abundance within the cell and low concentration of 2-OG (2-OG),
NtcA (PDB 2XGX) is inactive, and it does not allow the start of transcription. PII (PDB
4C3L) interacts with PipX (PDB 2XG8). N is low, therefore, the concentration of 2-OG
1310   increases, NtcA2OG is active and can start transcriptions of the genes with the NtcA
promoter. Moreover, NtcA2OG can interact with PipX (PDB 2XKO) and enhance the
activation of the genes with the associated NtcA promoters.

**Figure 3. Isothermal titration calorimetry study of the NtcA-*glnA* promoter interaction in
1315   the *Prochlorococcus* MED4, MIT9313, and SS120 strains.** The apparent dissociation
constant (Kapp) of the interaction of NtcA with the wild-type *glnA* promoter DNA was
determined in the presence of different concentrations of 2OG. Data are replotted from
Domínguez-Martín *et al*, 2018.

1320   ## Legends for tables

**Table 1. Presence of genes related to N metabolism in selected marine
Synechococcus genomes. Syn, *Synechococcus*; Cya, *Cyanobium*.**

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Table 2. Presence of genes related to N metabolism in selected *Prochlorococcus* genomes. Pro, *Prochlorococcus*.

1325   Table 3. Comparison of nitrogen content and cell volume in marine *Synechococcus* and *Prochlorococcus* strains.

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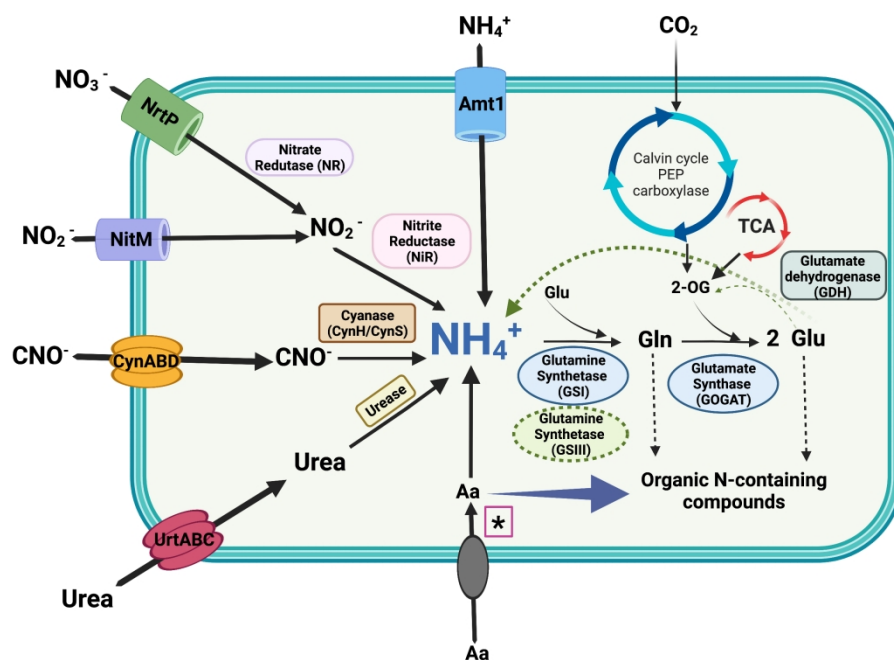
Figure 1. Nitrogen assimilation pathways in cyanobacteria. Different N sources taken up through permeases and transporters and metabolized to ammonium, which is incorporated into C backbones through the GS-GOGAT pathway. The tricarboxylic acid cycle (TCA) in marine picocyanobacteria is incomplete. Enzymes involved in the assimilation of the different N sources are also highlighted in the scheme. Nitrate reductase (*narB*); nitrite reductase (*nirA*); glutamine synthetase I (*glnA*); glutamine synthetase III (*glnN*); glutamate synthase (*glsF*); Aa, aminoacids; nitrate transporter NrtP (*nrtP*), nitrite permease NitM (*nitM*); cyanate transporter CynABD (*cynABD*); urea transporter UrtABC (*urtABC*); ammonium transporter Amt1 (*amt1*). * Uptake of amino acids has been demonstrated in marine picocyanobacteria, but their assimilation and physiological function of transporters are poorly characterised. See section 2 for further details.

645x452mm (118 x 118 DPI)
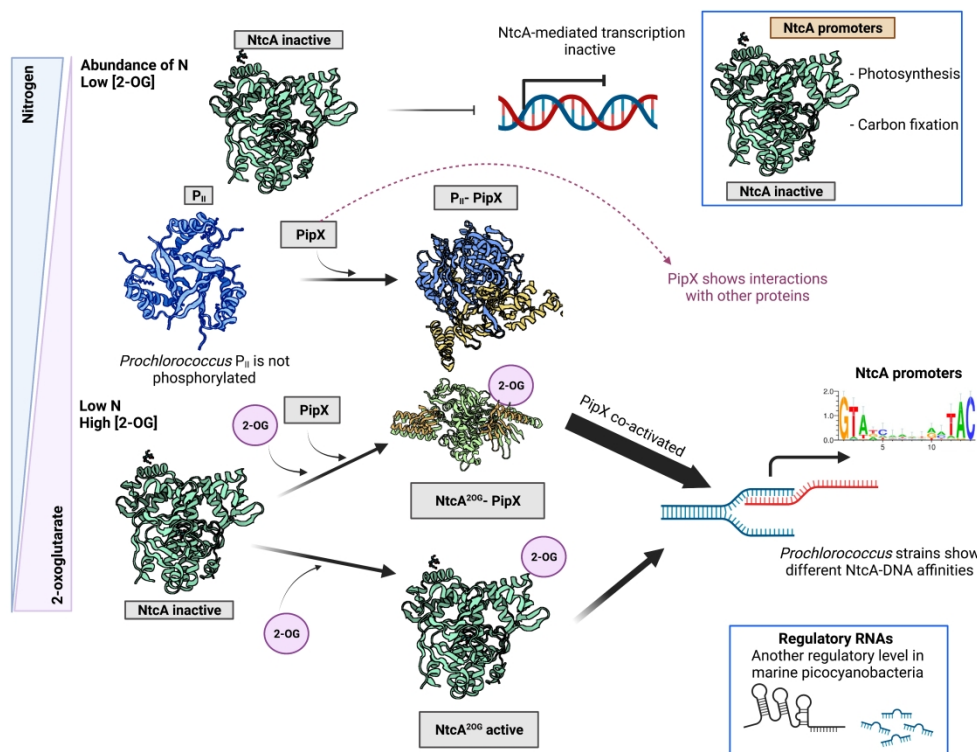
Figure 2. Schematic model of the action of the regulatory proteins NtcA, PII and PipX in cyanobacteria. N abundance within the cell and low concentration of 2-OG (2-OG), NtcA (PDB 2XGX) is inactive, and it can not start transcription. PII (PDB 4C3L) interacts with PipX (PDB 2XG8). N is low, therefore the concentration of 2-OG increases, NtcA2OG is active and can start transcriptions of the genes with NtcA promoter. Moreover, NtcA2OG can interact with PipX (PDB 2XKO) and enhace the activation of the genes with NtcA promoter associated.

838x645mm (118 x 118 DPI)
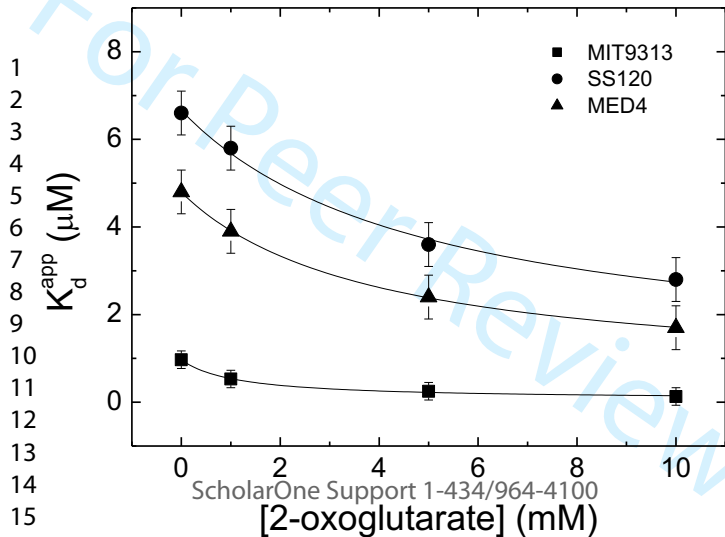
Strain	Clade/Subcluster	ntcA	glnB	pipX	glnA	glsF	glnN	amt1	narB	nirA	nrtP	nitM	ureAC	urtA	urtB	urtC	cynS	cynH	cynABD	gdhA
Syn A15-44	II / 5.1A	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•		
Syn A15-62	II / 5.1A	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn CC9605	II / 5.1A	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn KORDI-52	II / 5.1A	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn M16.1	II / 5.1A	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn PROS-U-1	II / 5.1A	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn RS9902	II / 5.1A	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn RS9907	II / 5.1A	•	•	•	•	•			•	•	•	•	•	•	•	•	•	•		
Syn TAK9802	II / 5.1A	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn WH8109	II / 5.1A	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn A15-24	III / 5.1A	•	•	•	•	•		•	•	•	•	•		•	•	•	•	•	•	
Syn A15-28	III / 5.1A	•	•	•	•	•		•	•	•	•	•		•	•	•	•	•	•	
Syn A18-40	III / 5.1A	•	•	•	•	•		•	•	•	•	•		•	•	•	•	•	•	
Syn A18-46.1	III / 5.1A	•	•	•	•	•		•	•	•	•	•		•	•	•	•	•	•	
Syn BOUM118	III / 5.1A	•	•	•	•	•		•	•	•	•	•		•	•	•	•	•	•	
Syn RS9915	III / 5.1A	•	•	•	•	•		•	•	•	•	•		•	•	•	•	•	•	
Syn WH8102	III / 5.1A	•	•	•	•	•		•	•	•	•	•		•	•	•	•	•	•	
Syn WH8103	III / 5.1A	•	•	•	•	•		•	•	•	•	•		•	•	•	•	•	•	
Syn BL107	IV / 5.1A	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn CC9902	IV / 5.1A	•	•	•	•	•		•	•	•		•	•	•	•	•	•	•		
Syn CC9311	I / 5.1B	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•		
Syn MVIR-18-1	I / 5.1B	•	•	•	•	•	•		•			•	•	•	•	•	•	•		
Syn PROS-9-1	I / 5.1B	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•	•		
Syn ROS8604	I / 5.1B	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•		
Syn SYN20	I / 5.1B	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•		
Syn WH8016	I / 5.1B	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•		
Syn WH8020	I / 5.1B	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•		
Syn BMK-MC-1	V / 5.1B	•	•	•	•	•	•	•	•	•	•	•	•				•	•		
Syn WH7803	V / 5.1B	•	•	•	•	•	•	•	•	•	•	•					•	•		
Syn MEDNS5	VI / 5.1B	•	•	•	•	•	•	•	•	•		•					•	•		
Syn PROS-7-1	VI / 5.1B	•	•	•	•	•	•	•				•		•	•	•	•	•		
Syn WH7805	VI / 5.1B	•	•	•	•	•	•	•				•		•	•	•	•	•		
Syn A15-60	VII / 5.1B	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn A18-25c	VII / 5.1B	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn NOUM97013	VII / 5.1B	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		
Syn RS9909	VIII / 5.1B	•	•	•	•	•		•	•	•		•	•	•	•	•	•	•		
Syn RS9917	VIII / 5.1B	•	•	•	•	•	•		•			•	•	•	•	•	•	•		
Syn WH8101	VIII / 5.1B	•	•	•	•	•		•	•	•		•	•	•	•	•	•	•		
Syn RS9916	IX / 5.1B	•	•	•	•				•	•		•					•	•		
Syn BIOS-E4-1	CRD1 / 5.1B	•	•	•	•			•	•	•	•	•		•	•	•	•	•		
Syn BIOS-U3-1	CRD1 / 5.1B	•	•	•	•			•	•	•	•	•					•	•		
Syn MITS9220	CRD2 / 5.1	•	•	•	•							•					•	•		
Cya NS01	5.2	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•			
Cya PCC6307	5.2	•	•	•	•	•	•	•	•	•	•			•	•	•				
Cya PCC7001	5.2	•	•	•	•	•	•	•	•	•	•	•		•	•	•		•		
Syn WH5701	5.2	•	•	•	•	•	•	•						•	•	•				
Syn CB0101	CB4 / 5.2	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•			
Syn CB0205	CB5 / 5.2	•	•	•	•	•	•	•	•	•	•	•		•	•	•	•			
Syn RCC307	5.3	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•			
Syn MINOS11	5.3	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•			
Syn CC9616	UC-A	•	•	•	•	•		•	•	•	•			•	•	•				
Syn KORDI-100	UC-A	•	•	•	•	•		•	•	•	•			•	•	•				
Syn A15-127	WPCI	•	•	•	•			•	•	•	•	•	•	•	•	•	•	•		
Syn KORDI-49	WPCI	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•		

Strain	Clade	ntcA	glnB	pipX	glnA	glsF	glnN	amt1	narB	nirA	nrtP	nitM	ureAC	urtA	urtB	urtC	cynS	cynH	cynABD	gdhA
Pro MED4	HLI	•	•	•	•	•		•					•	•	•	•	•		•	
Pro MIT9515	HLI	•	•	•	•	•		•												
Pro AS9601	HLII	•	•	•	•	•		•					•	•	•	•				
Pro EQPAC1	HLII	•	•	•	•	•		•					•	•	•	•		•		•
Pro GP2	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT0604	HLII	•	•	•	•	•		•	•	•	•		•	•	•	•				
Pro MIT9107	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9116	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9123	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9201	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9202	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9215	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9301	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9302	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9311	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9312	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9314	HLII	•	•	•	•	•		•					•	•	•	•	•		•	
Pro MIT9321	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9322	HLII	•	•	•	•	•		•					•	•	•	•				
Pro MIT9401	HLII	•	•	•	•	•		•					•	•	•	•				
Pro SB	HLII	•	•	•	•	•		•	•	•	•		•	•	•	•	•		•	
Pro UH18301	HLII	•	•	•	•	•		•					•	•	•	•				
Pro HNLC2	HLIII	•	•	•	•	•		•					•	•	•					
Pro HNLC1	HLIV	•	•	•	•	•		•					•	•	•					
Pro MIT0801	LLI	•	•	•	•	•		•		•		•	•	•	•	•	•			
Pro NATL1A	LLI	•	•	•	•	•		•		•	•		•	•	•	•	•			
Pro NATL2A	LLI	•	•	•	•	•		•		•	•		•	•	•	•	•			
Pro PAC1	LLI	•	•	•	•	•		•	•	•	•		•	•	•	•	•			
Pro LG	LLII	•	•	•	•	•		•												
Pro SS120	LLII	•	•	•	•	•		•												
Pro SS2	LLII	•	•	•	•	•		•												
Pro SS35	LLII	•	•	•	•	•		•												
Pro SS51	LLII	•	•	•	•	•		•												
Pro SS52	LLII	•	•	•	•	•		•												
Pro MIT0602	LLII	•	•	•	•	•		•												
Pro MIT0603	LLII	•	•	•	•	•		•												
Pro MIT0601	LLIII	•	•	•	•	•		•												
Pro MIT9211	LLIII	•	•	•	•	•		•												
Pro MIT0701	LLIV	•	•	•	•	•		•		•	•	•	•	•	•	•				•
Pro MIT0702	LLIV	•	•	•	•	•		•		•	•	•	•	•	•	•				•
Pro MIT0703	LLIV	•	•	•	•	•		•		•	•	•	•	•	•	•				•
Pro MIT9303	LLIV	•	•	•	•	•		•		•		•	•	•	•	•				•
Pro MIT9313	LLIV	•	•	•	•	•		•			•	•	•	•	•	•				•

Table 3. Comparison of nitrogen content and cell volume in marine *Synechococcus* and *Prochlorococcus* strains

Strain	N content per cell (fg)	Cell volume (μm³)	N content/volume (fg/μm³)
Synechococcus WH7803[1]	18 ± 2	0.71±0.08	25.35
Synechococcus WH8103[1]	18 ± 3	1.2±0.2	15
Synechococcus WH8103[2]	50 ± 2		
Synechococcus WH8102 diel changes[3]	16-32		
Synechococcus WH8012[2]	20 ± 3		
Prochlorococcus SARG[1]	4.5 ± 0.2	0.144±0.008	31.25
Prochlorococcus PCC 9511[1]	4.3 ± 0.4	0.22±0.02	19.55
Prochlorococcus MED4[2]	9.4 ± 0.9		
Prochlorococcus NATLI-MIT[1]	4.3 ± 0.2	0.139±0.010	30.94
Prochlorococcus GP2[1]	2.2 ± 0.2	0.13±0.01	16.92
Prochlorococcus SB[1]	3.7 ± 0.2	0.22±0.01	16.82
Prochlorococcus EQPAC1[1]	2.9 ± 0.2	0.077±0.005	37.66
Prochlorococcus MIT9312 days in batch culture changes[4]	15.87-22.36		

[1] Data from Heldal et al, 2003

[2] Data from Bertilsson et al, 2003

[3] Data from Roth-Rosemberg et al, 2021

[4] Data from López et al, 2016
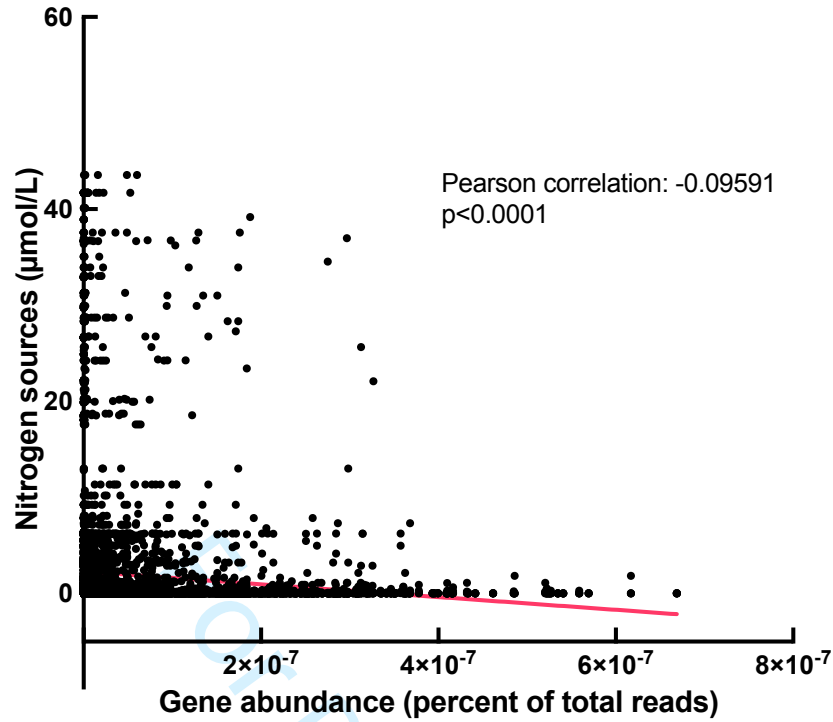
Supplementary figure 1. Pearson correlation between all genes abundance studied in supplementary table 1 and the nitrogen sources measured in each Tara Station. Genes abundance and nitrogen sources were calculated and selected as described in Supplementary table 1.

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ntcA
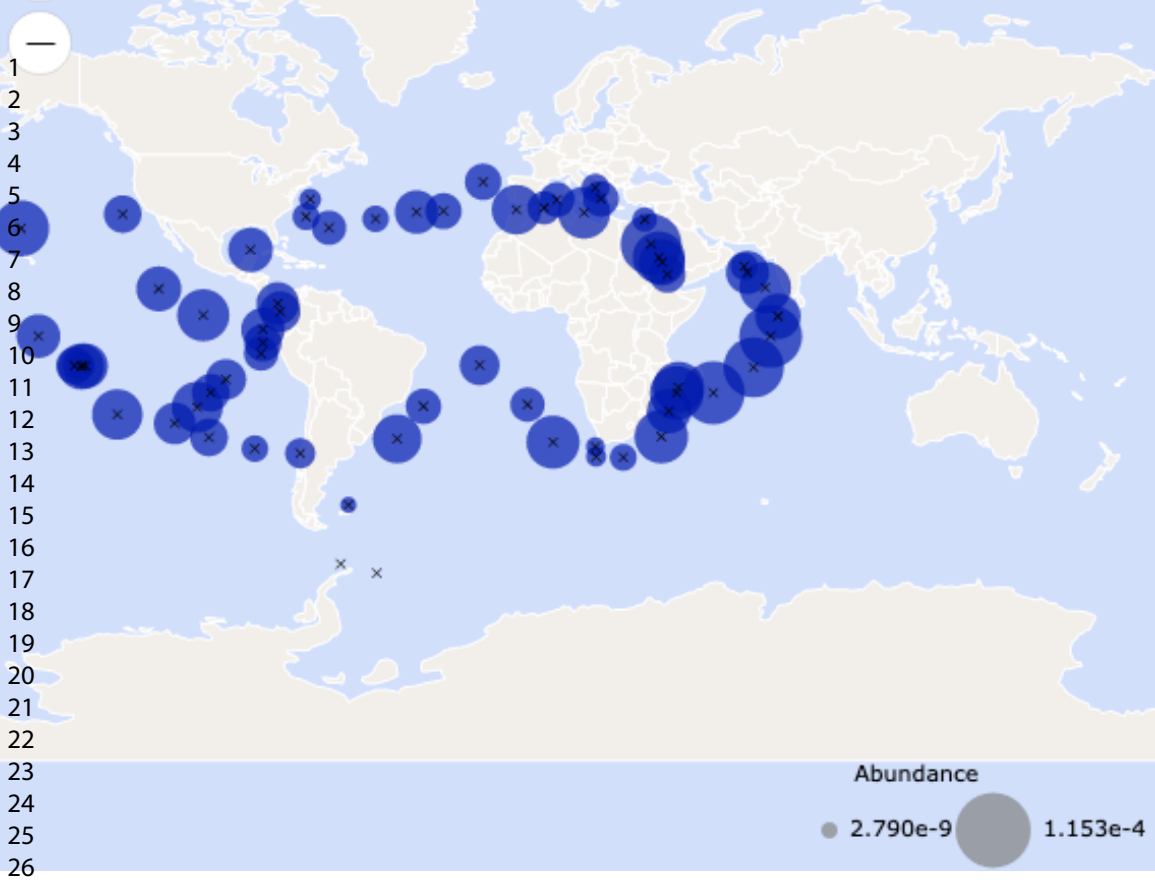
Number of hits: 323
Number of abundance measures: 14,081

(100% Cyanobacteria, 10% *Synechococcales*)

Abundance

2.790e-9   1.153e-4

glnB

Number of hits: 5,729
Number of abundance measures: 62,077

(15% Cyanobacteria, 9% *Synechococcales*)

Abundance

1.340e-7   3.847e-4
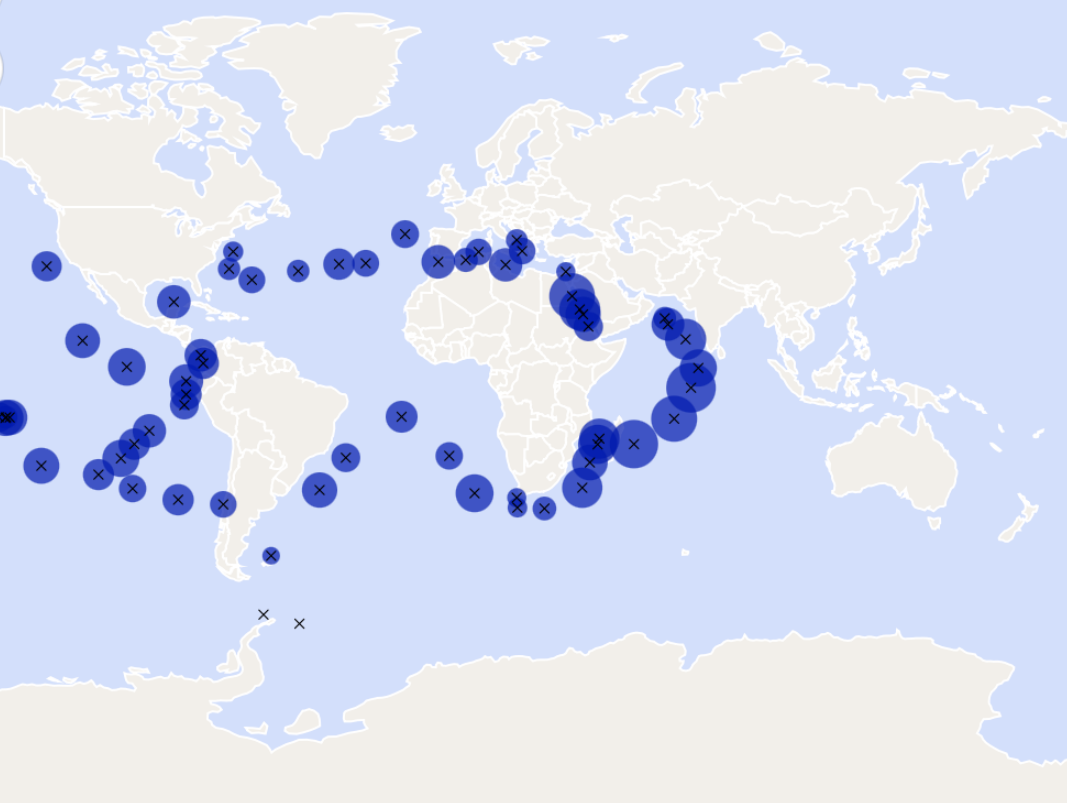
Number of hits: 292
Number of abundance measures: 8,834

(100% Cyanobacteria, 96% *Synechococcales*)

Abundance

● 6.446e-9 ⬤ 1.162e-4

psbO

Number of hits: 343
Number of abundance measures: 13,780

(86% Cyanobacteria, 100% *Synechococcales*)

Abundance

● 9.368e-9 ⬤ 1.668e-4

Supplementary figure 2. Nitrogen-regulated gene abundances based on sequence similarity with environmental genomics databases [1-3]. Nitrogen-regulated genes areshowed at A (*ntcA*), B (*glnB*) and C (*pipX*). D shows the photosystem II gene (*psbO*) abundance. Gene abundance was selected from the OM-RGCv1 catalog gene (1) and estimated by evaluating the coverage of raw sequencing reads mapped to the genes nucleotide sequence. Abundance estimates was expressed using the genes read coverage divided by the total number of reads for the sample ('percent of total reads'). The geographical distribution of the homologs is visualized for each gene. A summary of the similarity search results (number of genes hit and associated with abundance estimates) are also shown in the map.

1) Sunagawa, Shinichi, Luis Pedro Coelho, Samuel Chaffron, Jens Roat Kultima, Karine Labadie, Guillem Salazar, Bardya Djahanschiri, et al. 2015. « Structure and function of the global ocean microbiome ». Science 348 (6237): 1261359. https://doi.org/10.1126/science.1261359
2) The Ocean Gene Atlas v2.0: online exploration of the biogeography and phylogeny of plankton genes. C. Vernette, J. Lecubin, P. Sanchez, Tara Oceans Coordinators, S. Sunagawa, T.O. Delmont, S.G. Acinas, E. Pelletier, P. Hingamp, M. Lescot. (2022) Nucleic Acides Research. gkac420, https://doi.org/10.1093/nar/gkac420
3) The Ocean Gene Atlas: exploring the biogeography of plankton genes online. E. Villar, T. Vannier, C. Vernette, M. Lescot, M. Cuenca, A. Alexandre, P. Bachelerie, T. Rosnet, E. Pelletier, S. Sunagawa, P. Hingamp. (2018). Nucleic Acids Research, Volume 46, Issue W1, 2 July 2018, Pages W289–W295, https://doi.org/10.1093/nar/gky376

Gene	Nitrogen sources	Pearson correlation (r)	P.value
amt	Ammonium*	-0.1741	p<0.05
amt	Nitrate*	-0.2042	p<0.05
amt	Nitrate	-0.3142	p<0.0001
amt	Nitrite*	-0.1437	p<0.05
amt	Nitrite	-0.1822	p<0.05
narB	Ammonium*	-0.07589	p>0.05
narB	Nitrate*	-0.1004	p>0.05
narB	Nitrate	-0.1859	P<0.05
narB	Nitrite*	-0.02462	p>0.05
narB	Nitrite	0.0000279	p>0.05
cynS	Ammonium*	-0.1405	p<0.05
cynS	Nitrate*	-0.1574	p<0.05
cynS	Nitrate	-0.3346	p<0.0001
cynS	Nitrite*	-0.1105	p>0.05
cynS	Nitrite	-0.1031	p>0.05
glnA	Ammonium*	-0.1631	p<0.05
glnA	Nitrate*	-0.1691	p<0.05
glnA	Nitrate	-0.3101	p<0.0001
glnA	Nitrite*	-0.1315	p<0.05
glnA	Nitrite	-0.1894	p<0.05
nirA	Ammonium*	-0.1620	p<0.05
nirA	Nitrate*	-0.1699	p<0.05
nirA	Nitrate	-0.3560	p<0.0001
nirA	Nitrite*	-0.1247	p>0.05
nirA	Nitrite	-0.1334	p>0.05
nrtP	Ammonium*	-0.1545	p<0.05
nrtP	Nitrate*	-0.1898	p<0.05
nrtP	Nitrate	-0.3111	p<0.0001
nrtP	Nitrite*	-0.1466	p<0.05
nrtP	Nitrite	-0.1446	p<0.05
urtA	Ammonium*	-0.1877	p<0.05
urtA	Nitrate*	-0.2260	p<0.001
urtA	Nitrate	-0.3012	p<0.0001
urtA	Nitrite*	-0.1243	p>0.05
urtA	Nitrite	-0.1507	p<0.05
QNI46624.1	Ammonium*	0.03305	p>0.05
QNI46624.1	Nitrate*	-0.03051	p>0.05
QNI46624.1	Nitrate	-0.01291	p<0.05
QNI46624.1	Nitrite*	-0.1650	p>0.05
QNI46624.1	Nitrite	-0.07895	p>0.05

Supplementary table 1. The table shows the Pearson correlation between each gene abundance and the corresponding nutrient concentration (µmol/L) in each Tara Station. Gene abundance was selected from the OM-RGCv1 catalog gene (1) and estimated by evaluating the coverage of raw sequencing reads mapped to the gene's nucleotide sequence. Abundance estimates was expressed using the gene's read coverage divided by the total number of reads for the sample ('percent of total reads') (1). The Pearson correlation showed in this table was calculated plotting the gene abundances for *Prochorococcus* and *Synechococcus* in each Tara Station (n=243) against the environmental samples, as in Supplemental figure X. Environmental variables listed in this table were retrieved from the Ocean Gene Atlas (2,3). Values estimated from
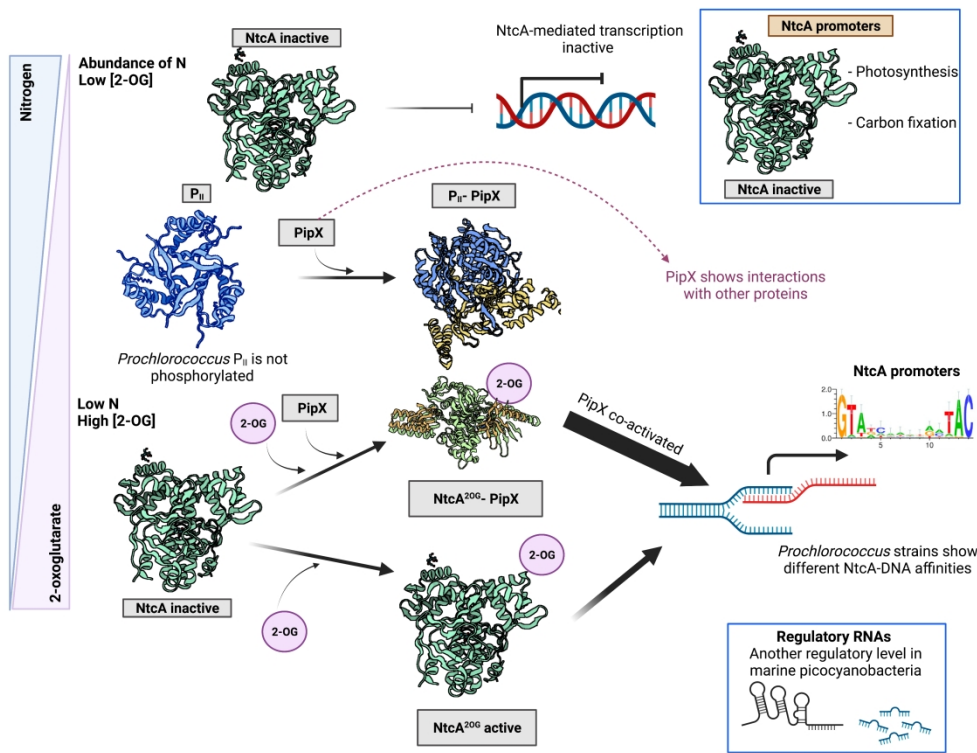
oceanographic models are indicated by a star. The correlation coefficient, r, ranges from -1 to +1 showing on this table values from -1 to 0 (one variable increases as the other decreases). The shaded area represents no correlation between abundances and the environmental variable.

1) Sunagawa, Shinichi, Luis Pedro Coelho, Samuel Chaffron, Jens Roat Kultima, Karine Labadie, Guillem Salazar, Bardya Djahanschiri, et al. 2015. « Structure and function of the global ocean microbiome ». Science 348 (6237): 1261359. https://doi.org/10.1126/science.1261359

2) The Ocean Gene Atlas v2.0: online exploration of the biogeography and phylogeny of plankton genes. C. Vernette, J. Lecubin, P. Sanchez, Tara Oceans Coordinators, S. Sunagawa, T.O. Delmont, S.G. Acinas, E. Pelletier, P. Hingamp, M. Lescot. (2022) Nucleic Acides Research. gkac420, https://doi.org/10.1093/nar/gkac420

3) The Ocean Gene Atlas: exploring the biogeography of plankton genes online. E. Villar, T. Vannier, C. Vernette, M. Lescot, M. Cuenca, A. Alexandre, P. Bachelerie, T. Rosnet, E. Pelletier, S. Sunagawa, P. Hingamp. (2018). Nucleic Acids Research, Volume 46, Issue W1, 2 July 2018, Pages W289–W295, https://doi.org/10.1093/nar/gky376

Strain	Clade/Subcluster	*natF*	*natG*	*natH*
Syn A15-44	II / 5.1A	●	●	●
Syn A15-62	II / 5.1A	●	●	●
Syn CC9605	II / 5.1A	●	●	●
Syn KORDI-52	II / 5.1A	●	●	●
Syn M16.1	II / 5.1A	●	●	●
Syn PROS-U-1	II / 5.1A	●	●	●
Syn RS9902	II / 5.1A	●	●	●
Syn RS9907	II / 5.1A	●	●	●
Syn TAK9802	II / 5.1A	●	●	●
Syn WH8109	II / 5.1A	●	●	●
Syn A15-28	III / 5.1A	●	●	●
Syn A18-40	III / 5.1A	●	●	●
Syn A18-46.1	III / 5.1A	●	●	●
Syn BOUM118	III / 5.1A	●	●	●
Syn RS9915	III / 5.1A	●	●	●
Syn WH8102	III / 5.1A	●	●	●
Syn WH8103	III / 5.1A	●	●	●
Syn BL107	IV / 5.1A	●	●	●
Syn CC9902	IV / 5.1A	●	●	●
Syn CC9311	I / 5.1B	●	●	●
Syn MVIR-18-1	I / 5.1B	●	●	●
Syn PROS-9-1	I / 5.1B	●	●	●
Syn ROS8604	I / 5.1B	●	●	●
Syn SYN20	I / 5.1B	●	●	●
Syn WH8016	I / 5.1B	●	●	●
Syn WH8020	I / 5.1B	●	●	●
Syn BMK-MC-1	V / 5.1B	●	●	●
Syn WH7803	V / 5.1B	●	●	●
Syn MEDNS5	VI / 5.1B	●	●	●
Syn PROS-7-1	VI / 5.1B	●	●	●
Syn WH7805	VI / 5.1B	●	●	●
Syn A15-60	VII / 5.1B	●	●	●
Syn A18-25c	VII / 5.1B	●	●	●
Syn NOUM97013	VII / 5.1B	●	●	●
Syn RS9909	VIII / 5.1B	●	●	●
Syn RS9917	VIII / 5.1B	●	●	●
Syn WH8101	VIII / 5.1B	●	●	●
Syn RS9916	IX /5.1B	●	●	●
Syn BIOS-E4-1	CRD1 / 5.1B	●	●	●
Syn BIOS-U3-1	CRD1 / 5.1B	●	●	●
Syn MITS9220	CRD2 / 5.1	●	●	●
Cya NS01	5.2			
Cya PCC6307	5.2			
Cya PCC7001	5.2			
Syn WH5701	5.2	●	●	●
Syn CB0101	CB4 / 5.2			
Syn CB0205	CB5 / 5.2	●	●	●
Syn RCC307	5.3			
Syn MINOS11	5.3			
Syn CC9616	UC-A	●	●	●
Syn KORDI-100	UC-A	●	●	●
Syn A15-127	WPCI	●	●	●
Syn KORDI-49	WPCI	●	●	●

Strain	Clade	*natF*	*natG*	*natH*
Pro MED4	HLI			
Pro MIT9515	HLI	●	●	
Pro AS9601	HLII			
Pro EQPAC1	HLII			
Pro GP2	HLII	●	●	
Pro MIT0604	HLII			
Pro MIT9107	HLII			
Pro MIT9116	HLII			
Pro MIT9123	HLII			
Pro MIT9201	HLII			
Pro MIT9202	HLII			
Pro MIT9215	HLII			
Pro MIT9301	HLII			
Pro MIT9302	HLII			
Pro MIT9311	HLII	●	●	●
Pro MIT9312	HLII	●	●	●
Pro MIT9314	HLII			
Pro MIT9321	HLII			
Pro MIT9322	HLII			
Pro MIT9401	HLII			
Pro SB	HLII			
Pro UH18301	HLII			
Pro HNLC2	HLIII			
Pro HNLC1	HLIV	●	●	●
Pro MIT0801	LLI	●	●	●
Pro NATL1A	LLI	●	●	●
Pro NATL2A	LLI	●	●	●
Pro PAC1	LLI	●	●	●
Pro LG	LLII	●	●	●
Pro SS120	LLII	●	●	●
Pro SS2	LLII	●	●	●
Pro SS35	LLII	●	●	●
Pro SS51	LLII	●	●	●
Pro SS52	LLII	●	●	●
Pro MIT0602	LLII	●	●	●
Pro MIT0603	LLII	●	●	●
Pro MIT0601	LLIII			
Pro MIT9211	LLIII	●	●	●
Pro MIT0701	LLIV	●	●	●
Pro MIT0702	LLIV	●	●	●
Pro MIT0703	LLIV	●	●	●
Pro MIT9303	LLIV	●	●	●
Pro MIT9313	LLIV	●	●	●

838x645mm (118 x 118 DPI)