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Influence of N, P, and Fe availability on *Braarudosphaera bigelowii*, *Trichodesmium*, *Crocosphaera*, and noncyanobacterial diazotrophs: a review

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Marine biological nitrogen fixation (BNF) is crucial for introducing "new nitrogen" into the oceans. Over the past 30 years, numerous laboratory and on-board culture experiments have been conducted studying the effects of nutrients such as total dissolved nitrogen (TDN), total dissolved phosphorus (TDP), and dissolved iron (DFe) on marine diazotrophs such as *Braarudosphaera bigelowii* (*B. bigelowii*), *Trichodesmium*, *Crocosphaera* and noncyanobacterial diazotrophs (NCDs). Most studies concluded that elevated dissolved inorganic nitrogen levels inhibit nitrogen fixation in *Trichodesmium*, promote its growth, and have minimal effect on *B. bigelowii*. The impact on NCDs is unclear. Moreover, elevated dissolved inorganic phosphorus (DIP) levels can promote individual growth, population growth, and nitrogen fixation in most diazotrophs in P-limited marine environments. Dissolved organic phosphorus is a potential phosphorous source for diazotrophs in low-DIP environments. Elevated DFe can promote population growth and nitrogen fixation in diazotrophs in Fe-limited marine environments. At present, most diazotrophs have yet to achieve pure culture. Moreover, the effect of nutrients on diazotrophs is mainly limited to the study of a single nutrient, which cannot accurately reflect the actual Marine environment where diazotrophs live. As a result, our understanding of the effect of nutrients on diazotrophs is still insufficient. Future research focusing on the issues above and the development of innovative technologies and methodologies to investigate the impact of marine BNF is highly recommended, which will allow for a more precise assessment of the impact of marine BNF on global primary productivity while providing a scientific foundation for rational evaluation of ocean CO_2 uptake and emissions.

KEYWORDS

diazotrophs, nutrients, biological nitrogen fixation, total dissolved nitrogen, total dissolved phosphorus, dissolved iron

1 Introduction

Marine biological nitrogen fixation (BNF) is a microbially-mediated biogeochemical process involving the conversion of atmospheric N_2 into bioavailable NH_4^+ by diazotrophs, thereby providing a continuous supply of "new nitrogen" to producers (Zehr and Capone, 2020; Hutchins and Capone, 2022). This process not only stimulates primary productivity but also enhances the ocean's capacity to absorb CO_2 from the atmosphere (Figure 1). Thus, marine BNF plays a crucial regulatory role in sustaining global primary productivity and driving assimilation and sequestration of carbon in the oceans (Zehr and Capone, 2020).

Marine BNF is influenced by physical and chemical factors (Figure 1). Key physical factors that regulate BNF include temperature, light, and ocean current parameters (Zehr and Capone, 2021b). Ambient temperature impacts the enzyme kinetics and growth rates of diazotrophs, and light affects diazotroph photosynthesis and regulates the ratio of photosynthesis system I (PSI) to photosynthesis system II (PSII). Additionally, ocean currents can induce the mixing of water masses, affecting nutrient structure and diazotroph growth (Zehr and Capone, 2021b). Chemical factors affecting BNF include oxygen, salinity, pH, dissolved organic matter, and various nutrients. Diazotrophs are highly sensitive to oxygen due to the rapid inactivation of nitrogenase enzymes upon oxygen exposure (Zehr and Capone, 2021c). Changes in salinity can alter organismal osmotic pressure, subsequently affecting growth and acclimatization. pH can affect the activities of nitrogenase enzymes in diazotrophs, interfere with their metabolism, and thus affect their community structure (Zehr and Kudela, 2011). The metabolic activity of heterotrophic diazotrophs is closely linked to the presence of dissolved organic matter (Zehr and Capone, 2021b).

Nutrients play a critical role in the growth of marine diazotrophs. Cellular synthesis of various organic compounds, such as amino acids, proteins, cell membranes, nucleic acids, and nitrogenases, requires substantial amounts of specific nutrients or elements, including nitrogen (N), phosphorus (P), and iron (Fe) (Kusano et al., 2011). In the marine environment, N, P, and Fe are often the most essential primary nutrients that affect primary productivity due to their relative availability (Falkowski et al., 1998). However, nutrient distributions exhibit significant variations across different marine environments, and this spatial heterogeneity may contribute to differences in marine BNF (Falkowski et al., 1998; Hutchins and Tagliabue, 2024).

Long-term studies on dinitrogen (N_2) fixation have mainly focused on oligotrophic marine regions, such as tropical and subtropical areas with very low dissolved inorganic nitrogen (DIN) concentration in surface water (Zehr and Capone, 2021a), the North Atlantic gyre area with very low dissolved inorganic phosphorus (DIP) content (Moore et al., 2009), and the North Pacific gyre region with low dissolved iron (DFe) (Yuan et al., 2023). However, recent research has also identified N_2 fixation occurring in some eutrophic marine environments, including nearshore mangroves, upwelling areas, seagrass habitats, and large estuarine plumes (Zilius et al., 2020; Mohr et al., 2021; Kittu et al., 2023; Louchard et al., 2023) (Figure 1).

By comparing eutrophic and oligotrophic areas, researchers discovered that the nutrient structures and the main diazotroph groups differed (Zehr and Capone, 2024). For example, different diazotrophs respond differently to various nutrients due to their physiological differences, enabling them to fix N in various nutrient environments. Despite the comprehensive summaries of the physiological characteristics and N_2 fixation mechanisms of

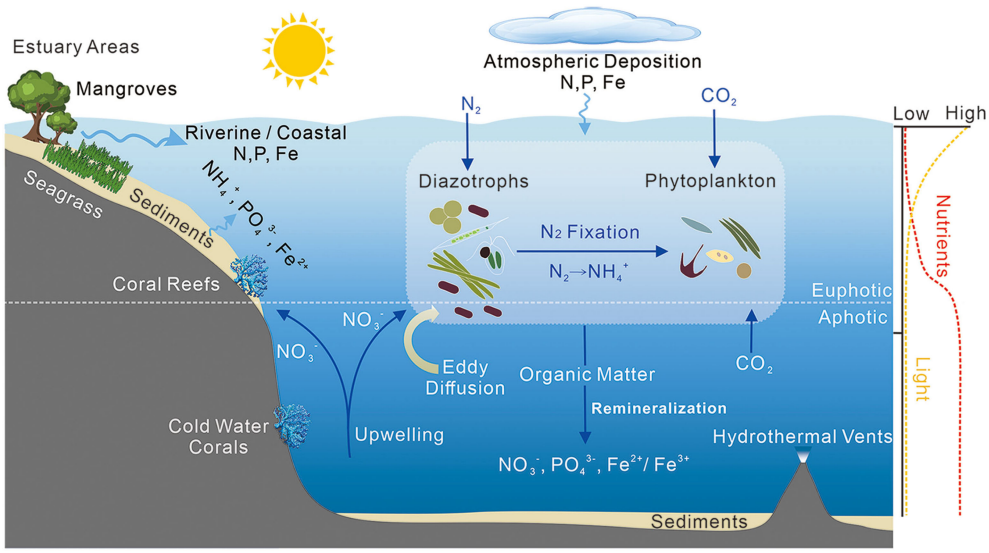

FIGURE 1
Diagram illustrating marine biological nitrogen fixation (BNF) and BNF-associated microbe habitat distribution. Key habitats include estuary areas, mangroves, sediments, seagrass, coral reefs, and hydrothermal vents (Zehr and Capone, 2020). For BNF, marine diazotrophs first absorb and metabolize atmospheric N_2 before converting it to NH_4^+ via nitrogen fixation, thereby providing new nitrogen for phytoplankton (Sohm et al., 2011b). Several nutrients (including nitrogen [N], phosphorus [P], and iron [Fe]) play important roles in this process. Depth profiles (right) show typical light and nutrient distributions (Bristow et al., 2017). N_2, nitrogen gas; CO_2, carbon dioxide; NH_4^+, ammonium; NO_3^-, nitrate; PO_4^{3-}, phosphate; Fe^{2+}, ferrous ion; Fe^{3+}, ferri ion.

diazotrophs (Zehr and Capone, 2020; 2024) as well as research on cellular interactions among diazotrophs (Thompson and Zehr, 2013; Eichner et al., 2023) and factors controlling BNF (Zehr and Capone, 2020) in current studies, there is still a lack of systematic review on the effects of different nutrients on these microorganisms.

Thus, this study presents a comprehensive review of research conducted over the past 30 years on the relationship between diazotrophs such as *Braarudosphaera bigelowii* (*B. bigelowii*), *Trichodesmium*, *Crocosphaera*, and noncyanobacterial diazotrophs (NCDs), as well as total dissolved nitrogen (TDN), total dissolved phosphorus (TDP), and DFe. This study aimed to summarize the response mechanisms of different marine diazotrophs to variations in nutrient abundance, providing a foundation for future research on the role of nutrients in marine BNF.

2 Marine diazotrophs

Research on marine diazotrophs commenced in the 1960s, with initial investigations focusing on *Trichodesmium* due to their microscopic visibility (Dugdale et al., 1961). However, the advent of molecular biology techniques has substantially broadened the exploration of marine nitrogen-fixing biodiversity. Recent research has classified marine diazotrophs into eukaryotic and prokaryotic diazotrophs (Figure 2). *B. bigelowii* is a eukaryotic diazotroph known for containing a nitrogen-fixing organelle—UCYN-A (*Candidatus* Atelocyanobacterium thalassa) (Coale et al., 2024). Furthermore, prokaryotic diazotrophs primarily include cyanobacterial and NCDs. The most common cyanobacterial diazotrophs include *Trichodesmium*, unicellular cyanobacteria, and diatom–diazotroph associations. Typical unicellular cyanobacteria comprise *Crocosphaera* and *Cyanothece* (Zehr and Capone, 2020; 2024). Currently, only *B. bigelowii* (Tschitschko et al., 2024), *Trichodesmium* (Webb et al., 2022), *Crocosphaera* (Masuda et al., 2024) have been isolated and purified, and some isolates from NCDs have been enriched and isolated (Farnelid et al., 2014; Rose et al., 2024); studies on the effects of nutrients on diazotrophs mainly focus on the above microorganisms.

B. Bigelow is a eukaryotic diazotroph associated with the nitrogen-fixing organelle UCYN-A. UCYN-A exists in three sublines UCYN-A1, UCYN-A2, and UCYN-A3 (Figure 3A, B). The sublines can exhibit radius differences up to 10 times, and the volume differences can be as large as 1000 times (Thompson et al., 2014; Cornejo-Castillo et al., 2024). Early studies believed that *B. Bigelow* and UCYN-A exist in "endosymbiosis" because UCYN-A lacks common metabolic pathways such as PSII as well as the entire tricarboxylic acid cycle and remains unaffected by the amount of O_2 produced by photosynthesis. Moreover, it primarily fixes nitrogen during the daytime (Thompson and Zehr, 2013; Zehr and Capone, 2020). Recent studies have demonstrated that *B. bigelowii* controls the division of UCYN-A, and several proteins present in UCYN-A are encoded only in the nucleus of *B. bigelowii* (Coale et al., 2024; Massana, 2024).

Trichodesmium, a nonheteromorphic genus of filamentous cyanobacterium (Figure 3C), comprises six main branches: *T. hildebrantti*, *T. contortum*, *T. tenue*, *T. erythraeum*, *T. thiebautti*, and *T. spiralis* (Webb et al., 2022). *Trichodesmium* can be found in nature in the form of free filaments or can be organized in bundles, thereby forming colonies of various shapes and structures, including spherical puffs, tufts, rafts, and bow ties (Webb et al., 2022). Notably, these cells can simultaneously fix N2 and photosynthesis during daylight hours through spatial and temporal separation of the two processes to prevent interference of nitrogenase by O_2 produced during photosynthesis (Zehr and Capone, 2020; 2024). A recent macro genome-based study has revealed that not all *Trichodesmium* are capable of N_2 fixation, such as *T. miru* and *T. nobis* (Delmont, 2021).

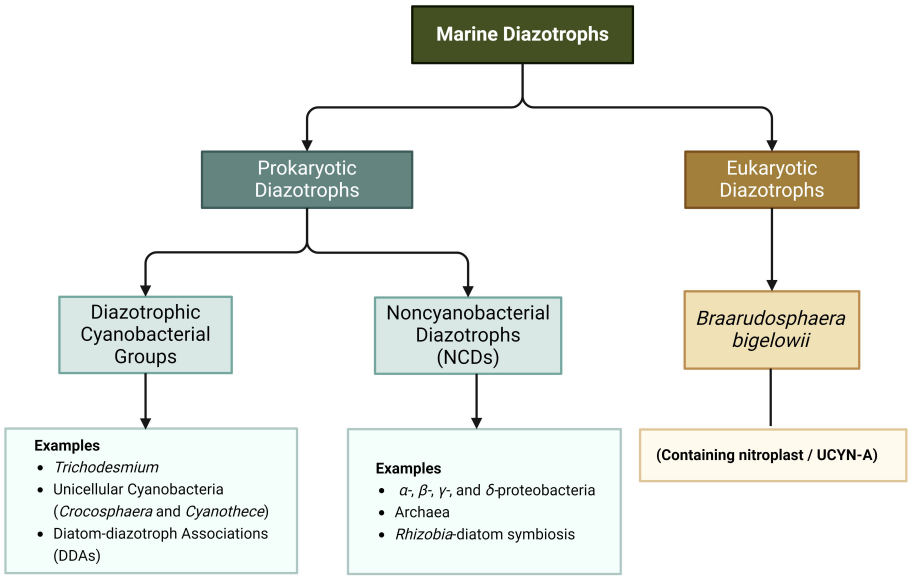

FIGURE 2
Classification of marine diazotrophs. Created in BioRender. Cao, H. (2024) BioRender.com/r84p523.

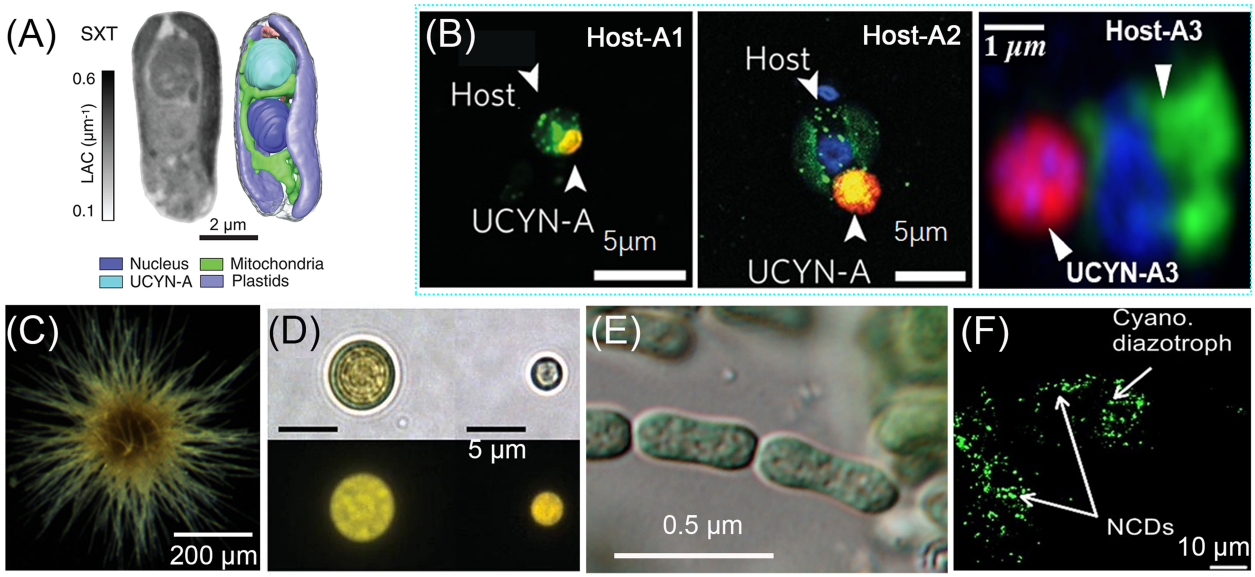

FIGURE 3
Typical marine diazotrophs. **(A)** *Braarudosphaera bigelowii*, orthoslice from SXT data, and colour-coded 3D renderings of subcellular components (Coale et al., 2024), scale bar 2 μm; **(B)** UCYN-A (including A1–A3) as determined using catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) (Martínez-Pérez et al., 2016; Cornejo-Castillo et al., 2019), scale bar 5 or 1 μm; **(C)** A *Trichodesmium* colony photographed in the East China Sea using continuous particle imaging and classification system, scale bar 200 μm; **(D)** UCYN-B *Crocosphaera* (Zehr and Capone, 2021d), scale bar 5 μm; **(E)** *Crocosphaera subtropica* (*Cyanothece* sp. ATCC 51142), with cells embedded in diffluent mucilage visible only by staining (Mareš et al., 2019), scale bar 0.5 μm; **(F)** Enlarged confocal images of NCDs (Riemann et al., 2022), scale bar 10 μm.

Crocosphaera is a unicellular spherical cyanobacterium (Figure 3D, E) and includes *C. watsonii*, *C. subtropica* (previously *Cyanothece* sp. ATCC 51142), *C. chwakensis* (previously *Cyanothece* sp. CCY 0110), and *C. waterburyi* (Masuda et al., 2024). *Crocosphaera* occurs as single cells, in pairs, or amorphous aggregates (Zehr et al., 2022). It undergoes photosynthesis during the day and N_2 fixation at night to prevent exposure to high levels of O_2 (Zehr and Capone, 2020). Furthermore, *Crocosphaera* exists as two distinct cell types: large (≥4 μm) and small (<4 μm); the genomes of these two types exhibit considerable differences, with the larger cell containing more genomes (Bench et al., 2013).

NCDs mainly include α-, β-, γ-, δ-proteobacteria, archaea and non-cyanobacteria (*Candidatus* Tectiglobus diatomicola) symbiosis with Haslea (Figure 3F), whose *nifH* gene is widely distributed in oceans. Moreover, the current research mainly focuses on γ-24774A11 (Turk-Kubo et al., 2022; Tschitschko et al., 2024). It is worth noting that α-proteobacterium (*Rhodopseudomonas* palustris strain BAL398, *Rhodobacteraceae* Sagittula castanea E-37) and γ-proteobacterium (*Pseudomonas stutzeri* strain BAL361, *Raoultella ornithinolytica* strain BAL286, *Candidatus* Thalassolituus haligoni) have been isolated by enrichment (Farnelid et al., 2014; Bentzon-Tilia et al., 2015; Martínez-Pérez et al., 2018; Rose et al., 2024). However, the mechanism of nitrogen fixation by NCDs has not been determined. Previous studies have shown that NCDs may be chemoheterotrophs, which use organic matter in the environment to maintain the energy demand of nitrogen fixation. It may also be adsorbed on particulate matter, providing low-oxygen microzones to avoid poisoning NCDs by O_2 (Bombar et al., 2016; Turk-Kubo et al., 2022).

3 Effect of TDN on marine diazotrophs

TDN in oceans primarily comprises DIN and dissolved organic nitrogen (DON). DIN is predominantly present in the ocean as NO_3^- (accounting for 99%), NH_4^+, and NO_2^- (Katye et al., 2021). Significant components of DON encompass nitrogenous organic compounds such as proteins, amino acids, nucleic acids, amino sugars, and urea (Millero, 2013).

Marine diazotrophs in the ocean can acquire substantial amounts of N from the atmosphere, resulting in minimal constraints on N metabolism (Zehr and Capone, 2021b). Previous research has indicated that elevated DIN levels may partially inhibit N_2 fixation, as the energy required to reduce N_2 to NH_4^+ is 25% greater than that needed to reduce NO_3^- to NH_4^+ (Falkowski, 1983). Consequently, when DIN content can satisfy the demand for biological N sources, diazotrophs tend to undergo assimilation rather than N_2 fixation, thereby limiting N_2 fixation (Karl et al., 2002). However, Knapp suggested that elevated DIN content inhibits the rate of N_2 fixation but does not entirely halt it; moreover, BNF still occurs in environments with NO_3^- concentrations up to 30 μmol L^{-1} or NH_4^+ concentrations up to 200 μmol L^{-1} (Knapp, 2012; Knapp et al., 2012). Studies have also discovered that high DIN content's impact on N_2 fixation may be attributed to (1) environments with high DIN content often being accompanied by relatively high P levels and an increase in nitrogen-fixing biomass due to sufficient P can offset the inhibition of DIN on N_2 fixation (Knapp et al., 2012); and (2) the fact DIN could inhibit most laboratory culture's N_2 fixation is due to adding excessive

amounts of DIN beyond what is found in field environments, which may overestimate high DIN content inhibition on diazotrophs (Knapp, 2012).

In comparison to DIN, the impact of DON on N_2 fixation has been relatively understudied. However, few studies report on diverse mechanisms through which DON regulates N_2 fixation in marine organisms. For example, (1) DON can directly influence nitrogenase activity by inhibiting the synthesis of the Fe and MoFe proteins of nitrogenase, thereby impeding N_2 fixation (Ohki et al., 1991; Mulholland and Capone, 1999; Mulholland et al., 2001). Moreover, (2) DON can serve as a source of N when N_2 fixation is inhibited, and its addition can stimulate the expression of *nifH* genes in diazotrophs while enhancing the rate of N_2 fixation (Benavides et al., 2015, 2017; Walworth et al., 2018; Benavides et al., 2020).

3.1 Effect of changes in DIN content on *B. bigelowii*

UCYN-A, associated with *B. bigelowii* is widely distributed in nutrient-rich estuaries and upwelling areas (Vieyra-Mexicano et al., 2024). Studies based on the nano-SIMS technology show that unlike most Marine phytoplankton with the ability to absorb NO_3^- (Simon and Klotz, 2013), *B. bigelowii* does not assimilate NO_3^- only absorbs a small amount of NH_4^+ in the environment, which is highly unusual in eukaryotic algae (Mills et al., 2020). In laboratory culture, under both limited nitrogen conditions (0.025 μmol L^{-1} NH_4^+/0.015 μmol L^{-1} NO_3^-) as well as relatively nitrogen-sufficient (0.125 μmol L^{-1} NH_4^+/0.2 μmol L^{-1} NO_3^-) condition, *B. bigelowii* has been observed to predominantly rely on N_2 fixation performed by UCYN-A to meet its nitrogen requirements (Mills et al., 2020). Furthermore, a separate study indicated that even after supplying 2 μmol L^{-1} NH_4NO_3 to *B. bigelowii*, almost no change was observed in the rate of N_2 fixation, and no significant alteration was observed in *nifH* expression (Krupke et al., 2015). The results indicated that the change in DIN content had no significant effect on the nitrogen fixation of *B. bigelowii*, which might be related to the unique physiological habit of *B. bigelowii*. Studies have shown that *B. bigelowii* is a mixed-trophic organism, and photosynthesis and nitrogen fixation alone are insufficient to maintain growth needs. Moreover, *B. bigelowii* also needs to obtain additional nutrients by swallowing bacteria (Burns et al., 2018; Mak et al., 2024); nitrogen fixation and ingestion processes may have ingested enough DIN to meet the nitrogen requirements of *B. bigelowii*.

3.2 Effect of changes in DIN content on *Trichodesmium*

The results of laboratory culture showed that adding high NO_3^- (i.e., 10–20 μmol L^{-1}) could reduce the nitrogen fixation rate of *Trichodesmium* by 50%–70% but could not stop the N_2 fixation (Mulholland et al., 2001; Holl and Montoya, 2005; Knapp et al., 2012). This is because high NO_3^- content will inhibit the expression of nitrogenase subunits and thus inhibit nitrogenase synthesis but not nitrogenase activity (Holl and Montoya, 2005; Sandh et al., 2011). However, although the presence of NO_3^- will affect the nitrogen fixation of *Trichodesmium*, it can still absorb a small amount of NO_3^- in the environment for its individual growth (Mulholland et al., 1999; Eichner et al., 2014; Klawonn et al., 2020). For example, Boatman et al. found that adding 100 μmol L^{-1} NO_3^- increased the growth rate of *Trichodesmium* bunchae by 13% (Boatman et al., 2018).

Mulholland et al. found in laboratory studies that there is a tightly coupled relationship between the release and absorption of NH_4^+ and *Trichodesmiu* (Mulholland and Capone, 1999; Mulholland et al., 1999, 2004), high NH_4^+ (i.e., 10–20 μmol L^{-1}) content inhibited the nitrogenase activity, resulting in a decrease in the nitrogen fixation rate of *Trichodesmium* (Ohki et al., 1991; Mulholland et al., 2001), but can promote the individual growth of *Trichodesmium* (Knapp, 2012; Post et al., 2012; Boatman et al., 2018). Klawonn et al. 's study in the waters near ALOHA Station in the subtropical North Pacific also confirmed that adding about 1 μmol L^{-1} of $^{15}NH_4^+$ to collected *Trichodesmium* can rapidly absorb and assimilate NH_4^+ for its growth (Klawonn et al., 2020).

3.3 Effect of changes in DIN content on *Crocosphaera*

Moisander reported that the abundance of *Crocosphaera* exhibited weak or no correlation with NO_3^- in the southwestern Pacific Ocean (Moisander et al., 2010). Furthermore, laboratory studies have demonstrated that high NO_3^- concentrations of 800–1000 μmol L^{-1} did not inhibit *Crocosphaera*'s N_2 fixation; this may be related to the non-expression of the nitrate assimilation gene (*nirA*) in the cells from *Crocosphaera* when cultured (Großkopf and LaRoche, 2012; Dekaezemacker et al., 2013; Rabouille et al., 2021). Consequently, it was concluded that NO_3^- does not impact individual growth, population growth, and N_2 fixation in *Crocosphaera*. However, studies have also shown that increasing NO_3^- concentration in the environment can induce and activate NO_3^- transporters on cell membranes from *Crocosphaera* (Glibert et al., 2016). Thus, small amounts of NO_3^- can still be absorbed from *Crocosphaera* (only 2%–4% of total uptake) (Rabouille et al., 2021; Masuda et al., 2022; Umbricht et al., 2024).

However, as NH_4^+ is a product of N_2 fixation, it can suppress the activity of nitrogenase (Thomas et al., 1982; Dekaezemacker and Bonnet, 2011) and hinder N_2 fixation in *Crocosphaera*. For instance, Dekaezemacker and Bonnet (2011) conducted experiments with NH_4^+ on *Crocosphaera*. They observed that increasing NH_4^+ concentration from 0.2 μmol L^{-1} to 10 μmol L^{-1} resulted in a reduction of N_2 fixation rates by 36% for *C. watsonii* WH8501 and 83% for *C. watsonii* WH0003, respectively, and the total nitrogen fixation was significantly reduced (Dekaezemacker and Bonnet, 2011). Additionally, Garcia and Hutchins (2014) found that adding 0.4 μmol L^{-1} of NH_4^+ decreased the rate of N_2 fixation in *C. watsonii* WH0003 by up to 90% (Garcia and Hutchins, 2014). It is worth noting that NH_4^+ is more accessible to transport across membranes due to the low energy requirements of utilizing NH_4^+ (Ludwig, 1938; Glibert et al., 2016), tends to utilize NH_4^+ rather than NO_3^- first, showing a high absorption rate on NH_4^+ (Masuda et al., 2013, 2022).

3.4 Effect of changes in DIN content on NCDs

Limited studies have shown that the effects of changes in DIN content on NCDs' growth and nitrogen fixation are unclear (Moisander et al., 2017). Rose et al. isolated, enriched and cultured γ-proteobacterial, and their genome study showed that γ-proteobacterial contained genes that absorbed and assimilated NH_4^+ and NO_3^- (Rose et al., 2024). Therefore, when DIN is deficient, the growth rate of NCDs will be significantly reduced (Bentzon-Tilia et al., 2015). Some reports have also found higher abundances of NCDs in DIN-rich environments (Shiozaki et al., 2014). For example, In the Arab Sea with NO_3^- content up to 15 μmol L^{-1} (Bird and Wyman, 2013), in the western Yellow Sea of China with DIN content of 7.29 μmol L^{-1} (Zhang et al., 2015), and in the Benguela upwelling zone with NO_3^- content between 5 and 27 μmol L^{-1} (Sohm et al., 2011a), all detected significant levels of γ-proteobacterial. In addition, through generalized addition model operation on global qPCR copy data of the *nifH* gene of γ-24774A11, it is also found that the abundance of γ-24774A11 rises significantly when NO_3^- is greater than 10 μmol L^{-1} (Shao and Luo, 2022). However, there are few studies on the nitrogen fixation rate of NCDs with the change of NO_3^- content. Limited studies have shown that the nitrogen fixation rate of NCDs will be significantly inhibited when NO_3^- content increases (Bentzon-Tilia et al., 2015).

4 Effect of TDP on marine diazotrophs

TDP in the ocean consists of DIP and dissolved organic phosphorus (DOP). In seawater, DIP is predominantly composed of $H_2PO_4^-$ (1%), HPO_4^{2-} (87%), and PO_4^{3-} (12%) ions (Ruttenberg, 2014). DOP primarily comprises phosphate monomer (C–O–P), phosphate polymers (C–O–P), and phosphonates (C–P) compounds (Karl and Björkman, 2015).

DIP is acknowledged as a potential limiting factor for BNF in most oceans (Deutsch et al., 2007). For example, in the west-central tropical North Atlantic, substantial amounts of DFe are introduced by winds carrying dust from the Sahara Desert. This stimulates N_2 fixation but also results in significant consumption of DIP, leading to P limitation (e.g., 0.2–1.0 nmol L^{-1}) on BNF processes (Sañudo-Wilhelmy et al., 2001; Moore et al., 2009; Martiny et al., 2019). Other studies conducted in the northern part of the subtropical northwest Pacific Gyre have confirmed that atmospheric Fe deposition enhances N_2 fixation and subsequently depletes DIP levels, ultimately resulting in a phosphorous deficit (~20 nmol L^{-1}) (Yuan et al., 2023).

Although diazotrophs primarily utilize DIP, DOP may serve as a potential source of phosphorus for the growth of diazotrophs in environments where DOP dominates TDP components (Karl, 2014; Karl and Björkman, 2015; Duhamel et al., 2021; Adams et al., 2022). Several studies have indicated that alkaline phosphatase genes (i.e., *phoA*, *phoD*, and *phoX*) present in diazotrophs play a role in regulating the microbial uptake and metabolization of DOP (Orchard et al., 2009; Frischkorn et al., 2019). Therefore, diazotrophs can utilize DOP to alleviate the P limitation of growth and N_2 fixation rates in low-DIP environments (Dyhrman et al., 2006; Karl, 2014; Rouco et al., 2018).

4.1 Effects of changes in TDP content on *B. bigelowii*

Elevated DIP content has been observed to contribute to population growth and N_2 fixation in *B. bigelowii*. In a study conducted by Langlois, based on on-board culture experiments using samples collected from the northeastern part of the tropical Atlantic Ocean, the addition of 0.2 μmol L^{-1} DIP was reported to cause an increase in the rate of N_2 fixation and abundance of UCYN-A (Langlois et al., 2012). Similarly, a comparable phenomenon was observed in on-board culture experiments by adding 1 μmol L^{-1} DIP in samples from the southwestern Pacific Ocean (Benavides et al., 2022).

Early studies indicated that UCYN-A lacked genes that are essential for the hydrolysis of the C–O–P phosphate monomer, such as alkaline phosphatase *phoA*, and for the utilization of the phosphonate C–P, such as phosphonate lyase *phnC–P*. Hence, UCYN-A is not likely to utilize DOP directly (Tripp et al., 2010; Thompson et al., 2012). However, experiments conducted by Benavides demonstrated that adding 1 μmol L^{-1} DOP to samples from the southwest Pacific stimulated UCYN-A1 growth (Benavides et al., 2022). This could be attributed to the demand for phosphorus being satisfied by *B. bigelowii* through a unique utilization strategy (Turk-Kubo et al., 2012; Thompson and Zehr, 2013); however, the underlying mechanism for this utilization remains unclear.

4.2 Effect of changes in TDP content on *Trichodesmium*

4.2.1 Effect of changes in DIP content on *Trichodesmium*

Elevated DIP levels in the ocean can stimulate individual growth, population growth and nitrogen fixation of *Trichodesmium*. Laboratory culture showed that under the condition of 26°C, the growth rate and nitrogen fixation rate of *Trichodesmium* with a high content of DIP (10 μmol L^{-1}) were three times and two times higher than those with a low content of DIP (0.2 μmol L^{-1}) (Qu et al., 2019), respectively. Under the pH value 7.69–7.76, a high content of DIP (20μmol L^{-1}) was added to *Trichodesmium*. The results showed that the nitrogen fixation rate and carbon sequestration rate of *Trichodesmium* increased by 35%–120% and 15%–100%, respectively, compared with the addition of low-content DIP (0.2μmol L^{-1}) (Hutchins et al., 2007). In the field culture experiment (MEGA3) of samples collected in the tropical North Atlantic Ocean, 0.2 μmol L^{-1} DIP was added, and the *nifH* gene copy number of *Trichodesmium* was observed to increase by ~2 times (Turk-Kubo et al., 2012). Webb et al. added DIP of 30 μmol L^{-1} to samples collected in the tropical South Atlantic and

found that nitrogen fixation was significantly stimulated (Webb et al., 2007). It is important to note that if DIP is added to non-phosphorus-restricted areas, it may not stimulate individual growth and population growth of *Trichodesmium*. In the South China Sea, according to a study, adding 0.1 μmol L^{-1} was not observed after the DIP of *Trichodesmium* cells abundance increased because the environment here DIP concentration is relatively high (5–20 nmol L^{-1}) (Wu et al., 2003).

Several studies have attempted to explain the mechanism of DIP utilization by *Trichodesmium* from the perspectives of DIP absorption kinetics and molecular biology. In the southwestern Pacific *Trichodesmium* DI^{33}P absorption kinetics experiments, the results show that when the DIP > 9 nmol L^{-1}, surface waters in *Trichodesmium* absorption DIP alone would maintain cell growth (Moutin et al., 2005). Further found that *Trichodesmium* had a higher rate of DIP uptake under P-limited (0.2 μmol L^{-1}) (Fu et al., 2005); this is because, under the condition of phosphorus limitation, *Trichodesmium* can increase intracellular phosphate transporter *sphX* expression of the genes encoding to improve the ability of from extracellular DIP, At the same time, the expression of *nifH* gene was down-regulated, showing a lower rate of nitrogen fixation (Orchard et al., 2009; Held et al., 2020). In addition, the results of DI^{33}P absorption kinetics also showed that the total absorption rate of DIP in *Trichodesmium* was lower than that of environmental microorganisms, indicating that the ability of *Trichodesmium* to compete for DIP was worse than that of other microorganisms in the environment (Sohm and Capone, 2006; Sohm et al., 2008; Orchard et al., 2010a; Glibert, 2024), so *Trichodesmium* need to adopt more strategies to maintain their growth in low-P environments.

Due to the low DIP levels in most surface oceans, *Trichodesmium* has evolved strategies to cope with low concentrations of DIP. In laboratory culture, it was found that under low DIP (0.5 μmol L^{-1}), *Trichodesmium* can increase the DIP absorption area of cells by increasing the individual length (Zhu et al., 2020). Low DIP can also stimulate *Trichodesmium* mobile and induce colony formation (Tzubari et al., 2018), and *Trichodesmium* colonies can sense and the choice of particles that are rich in P to maximize absorption of the DIP from the ocean (Held et al., 2020; Wang et al., 2022a); *Trichodesmium* can also change the demand for P in different cellular processes by reducing the transcription and translation process of ribosomal proteins (with a high demand for P), adjusting ATP concentration, and replacing phospholipids with sulfur lipids (Van Mooy et al., 2009; Spungin et al., 2014; Grosse et al., 2017). In addition, some studies also found that when the *Trichodesmium* is in a DIP-restricted environment, DIP is stored in the cell surface by *Trichodesmium's* surface adsorption (Sañudo-Wilhelmy et al., 2004), and *Trichodesmium* can also store limited DIP in the form of polyP to cells (Orchard, 2010; Orchard et al., 2010b), and directly or indirectly alleviate the effects of DIP restriction on cells (Björkman, 2014; Sanz-Luque et al., 2020). Moreover, *Trichodesmium* can also migrate to the phosphocline and store phosphorus by adjusting its buoyancy and then return to the surface ocean for photosynthesis and nitrogen fixation (Karl et al., 1992; White et al., 2006).

4.2.2 Effect of changes in DOP content on *Trichodesmium*

The change in DOP content can stimulate *Trichodesmium*'s individual growth and nitrogen fixation. Orchard et al. added 0.25–1 μCi DO^{33}P to *Trichodesmium* collected in the North Atlantic Ocean. They found that more than 25% of the P absorbed by *Trichodesmium* at most stations was DOP (Orchard et al., 2010a), indicating that DOP was an important P source for *Trichodesmium* (Fu et al., 2005; Dyhrman et al., 2006). This is because when DIP is restricted, *Trichodesmium* can up-regulate the expression of alkaline phosphatase genes (*phoA*, *phoD*, and *phoX*) (Stihl et al., 2001; Orchard et al., 2009), thereby changing the intracellular protein abundance and enzyme activity of DOP hydrolysis to promote the absorption and utilization of DOP by cells (Frischkorn et al., 2019). It is worth noting that Mulholland et al. 's culture experiment suggested that adding 1–50 μmol L^{-1} DOP could stimulate the growth of *Trichodesmium*. However, the individual growth caused by the addition of high DOP content was not as high as that caused by the addition of low DOP content (Mulholland et al., 2002). This may be due to the inhibition of alkaline phosphatase synthesis by DIP produced by DOP hydrolysis, which leads to the limitation of DOP that *Trichodesmium* can absorb and utilize and small changes in individual growth (Stihl et al., 2001).

Trichodesmium has developed many strategies to fully absorb and utilize DOP under DIP constraints. Epibiotic bacteria within *Trichodesmium* aggregates can sense the P source or, by use of quorum sensing, perceive and choose other alternative metal enzymes to regulate the activity of alkaline phosphatase (Van Mooy et al., 2012; Lee et al., 2017) to promote the absorption and utilization of DOP by *Trichodesmium* under phosphorus restriction. For example, Rouco et al. found that the genes regulating DOP in the North Atlantic and North Pacific are dominated by *phnX* (Ca and Fe as cofactors) and *phoA* (Zn and Mg as cofactors), respectively, and that *Trichodesmium* can switch the relative transcriptional abundance of the required metalloenzymes and change the *PhoA*/*PhoX* ratio for DOP hydrolysis. This stimulates the expression of the alkaline phosphatase gene (Rouco et al., 2018; Wang et al., 2022b).

4.3 Effect of changes in TDP content on *Crocosphaera*

4.3.1 Effect of changes in DIP content on *Crocosphaera*

Laboratory culture studies have demonstrated that increased DIP content helps stimulate individual growth and N_2 fixation in *Crocosphaera* (Zhu et al., 2020). In these cultures, the supplementation of 10 μmol L^{-1} DIP led to a three- to fourfold enhancement in the respective growth and N_2 fixation rates of *Crocosphaera* compared to the addition of 0.1 μmol L^{-1} DIP (Liu et al., 2020). Pereira et al. also found that adding sufficient DIP (60 nmol L^{-1}) can significantly reduce the transcription level of the high-affinity phosphate-binding protein *pstS* from *Crocosphaera* and increase cell abundance by 12% within three days (Pereira

et al., 2016; 2019). However, when DIP concentration is in the threshold of 20–60 nmol L^{-1}, the transcription level of *pstS* in cells is up-regulated, which increases the nitrogen fixation rate (Pereira et al., 2019; Marki et al., 2020; Rahav et al., 2022). Higher temperatures can mitigate the effects of DIP limits on growth and nitrogen fixation (Fu et al., 2014). The gene expressions of *pstS* and *sphX* on *Crocosphaera* are up-regulated to increase DIP uptake under P-limited conditions (0.2 μmol L^{-1}) and high-temperature conditions, so the growth and nitrogen fixation rate of *Crocosphaera* will increase with increasing temperature (Deng et al., 2022).

However, some onboard culture experiments have yielded inconsistent results from observations from DIP additions to laboratory cultures. For example, Turk-Kubo et al. conducted cultures of collected samples in the tropical North Atlantic Ocean following the addition of 0.2 μmol L^{-1} DIP and observed that higher levels of DIP in areas affected by the Amazon River plume already met the P requirements of *Crocosphaera* (Turk-Kubo et al., 2012). Consequently, exogenous DIP did not significantly increase the copy number of the *Crocosphaera nifH* gene in this region. Similar findings were reported by Benavides from an on-board culture study conducted in the tropical southwest Pacific Ocean (Benavides et al., 2022). Notably, a recent study revealed that adding 0.1 μmol L^{-1} DIP to samples collected from P-limited regions such as the eastern Mediterranean resulted in a decrease rather than an increase in *Crocosphaera*'s nitrogen fixation rate, presumably due to non-nitrogen-fixing heterotrophs responding more rapidly to DIP additions. The non-nitrogen-fixing heterotrophs have a faster rate of DIP turnover than *Crocosphaera*, so they consume a large amount of DIP (Rahav et al., 2022).

4.3.2 Effect of changes in DOP content on *Crocosphaera*

As *Crocosphaera* lacks a homologue of the phosphonate-specific transfer protein *phnCDE* in its genome and is unable to utilize phosphonate C–P for growth, fluctuations in the phosphonate C–P content of DOP have minimal impact on *Crocosphaera* growth (Dyhrman and Haley, 2006). Interestingly, Filella et al. observed an enhancement in the rate of N_2 fixation by *Crocosphaera* following the addition of 20 μmol L^{-1} DOP (adenosine monophosphate: AMP, belonging to the phosphate ester monomers C–O–P) to samples collected near the Tonga volcano (Filella et al., 2022), indicating that *Crocosphaera* can utilize the phosphate ester monomer C–O–P in DOP as a source of organophosphorus, consistent with findings from several recent studies (Pereira et al., 2019; Yamaguchi et al., 2020). The differences for *Crocosphaera* in the cracking efficiency of different components in DOP may explain the above differences (Rabouille et al., 2022).

4.4 Effect of changes in TDP content on NCDs

The effect of DIP content change on NCDs' growth and nitrogen fixation is still controversial. Studies have shown that NCDs can increase their ability to obtain DIP from the outside world by regulating gene expression in the cell under DIP restriction. The analysis of the γ-4 genome showed that the γ-4 genome contains *phoBR*, *pstSCAP*, *phnCDE* and *ppK-ppX* genes related to phosphorus clearance, and the *PhoB-PhoR* two-component system in γ-proteobacterial can sense DIP limitation. This activating gene expression encodes high-affinity phosphate transporter (*PstSCAP*) (Chakraborty et al., 2011; Cheung et al., 2021). However, no evidence of the sensitivity of NCDs to DIP has been found in field studies. For example, Zehr et al. added 0.5 μmol L^{-1} DIP to samples collected in the North Pacific subtropical gyre. They found that γ-a nitrogen fixation rate and *nifH* gene transcription were not significantly stimulated (Zehr et al., 2007); Moisander et al. also found little change in the expression of γ-24774A11 *nifH* gene after adding 2 μmol L^{-1} DIP to samples collected in the waters off eastern Australia (Moisander et al., 2012).

5 Effect of DFe on marine diazotrophs

Iron from atmospheric deposition, surface runoff, seafloor hydrothermal fluids, sediment inputs, and glacial melting are key nutrient elements that influence the primary productivity of the global oceans (Tagliabue et al., 2017; Jiang et al., 2024). Fe in the ocean exists in granular (>0.4 μm), colloidal (0.02–0.4 μm), and dissolved (<0.02 μm) forms. DFe is an essential form of iron that is available to diazotrophs. Diazotroph nitrogenase consists of a Fe-protein and a FeMo protein encoded by *nifHDK* (Zhang et al., 2020), with diazotrophs requiring fivefold more Fe for N_2 fixation than for ammonia assimilation and tenfold more than other phytoplankton (Kustka et al., 2003; Sohm et al., 2011b). However, DFe content is relatively low in most marine environments, leading to its long-standing recognition as a limiting factor for marine BNF.

In laboratory and on-board culture experiments, higher DFe content is frequently associated with elevated rates of N_2 fixation. An onboard DFe addition experiment conducted at the ALOHA station revealed a 128% increase in the rate of N_2 fixation in response to the addition of 2 nmol L^{-1} of DFe (Grabowski et al., 2008). Studies in the Atlantic affected by Fe inputs from Saharan desert wind and dust have demonstrated a positive correlation between DFe content and N_2 fixation rates (Orcutt et al., 2001; Tyrrell et al., 2003; Moore et al., 2009). Similarly, regions with higher N_2 fixation rates in the northwestern Pacific Ocean and the North Pacific gyre correspond to those with higher atmospheric dust deposition fluxes (Shiozaki et al., 2009, 2010; Yuan et al., 2023). These correlations suggest that atmospheric Fe deposition may influence N_2 fixation. Furthermore, recent studies have also indicated that DFe (i.e., up to 66 nmol L^{-1}) released by shallow hydrothermal fluids has a significant impact on N_2 fixation, establishing the southwest tropical Pacific as a new N_2 fixation hotspot (Bonnet et al., 2023; Mériguet et al., 2023).

5.1 Effects of changes in DFe content on *B. bigelowii*

An elevated DFe content is moderately beneficial for population growth and N_2 fixation in *B. bigelowii*. Despite the absence of PSII,

UCYN-A retains genes that regulate Fe^{3+} transporter proteins (*afuABC*), which may play a crucial role in oligotrophic ocean areas with limited DFe content (Zehr et al., 2016). In an on-board culture experiment, adding 400 nmol L^{-1} DFe to samples collected from eastern Australian waters significantly increased *nifH* gene copy numbers in UCYN-A (Moisander et al., 2012). Another experiment conducted with samples from the temperate northeast Atlantic Ocean showed that adding 200 nmol L^{-1} DFe led to a twofold increase in the rate of N_2 fixation by UCYN-A1 (Li et al., 2018). Furthermore, a recent study near the Bering Strait demonstrated a notable increase in UCYN-A abundance in response to elevated content of Fe sourced from riverine inputs (Cheung et al., 2022).

Notably, adding DFe to collected samples did not increase *nifH* expression in UCYN-A sampled from DFe-rich marine areas. For instance, Turk-Kubo et al. conducted a DFe addition experiment at sampling locations across the tropical North Atlantic; following the addition of 2 nmol L^{-1} DFe, the authors observed an increase in the *nifH* copy number of UCYN-A only in samples obtained from the Amazon River plume, and no significant increase was observed at any other station. This could be attributed to the fact that the ambient DFe levels in other regions (0.74 ± 0.01 nmol L^{-1}) already meet UCYN-A's Fe requirements (Turk-Kubo et al., 2012).

5.2 Effects of changes in DFe content on *Trichodesmium*

The addition of 1 μmol L^{-1} of DFe in laboratory culture studies has resulted in a nearly tenfold increase in the rates of N_2 fixation in *Trichodesmium* (Rueter, 1988; Paerl et al., 1994). In an on-board culture experiment conducted in Fe-limited waters, a significant increase in the rate of N_2 fixation was observed with the addition of 50 nmol L^{-1} DFe to samples of *Trichodesmium* collected from the western part of the southern equatorial Atlantic (Webb et al., 2007). Additionally, outbreaks of *Trichodesmium* blooms have also been documented on the continental shelf off the west coast of Florida, which are known as areas with high DFe levels (16 nmol kg^{-1}) (Lenes et al., 2001). *Trichodesmium* can absorb and utilize DFe because it can produce a reducing agent (superoxide) and release it into vitro (Roe and Barbeau, 2014), reducing Fe^{3+} to Fe^{2+} *in vitro*. Fe^{2+} is transported into the *Trichodesmium* by the high-affinity Fe^{2+} transporters *FeoA* and *FeoB* on the plasma membrane of *Trichodesmium* (Chappell and Webb, 2010).

Some studies have reported the possible adaptation mechanism of *Trichodesmium* in the absence of DFe content. Under the condition of limited DFe content, the abundance of proteins involved in the absorption of DFe increased, while the abundance of iron-binding proteins involved in photosynthesis and the activity of nitrogenases decreased rapidly, indicating that *Trichodesmium* can rapidly redistribute limited DFe resources and transfer iron from the photosynthetic system, respiratory transport chain and other cellular pathways. It is used to increase the synthesis of iron-rich nitrogenase to cope with the decrease in nitrogen fixation efficiency caused by iron restriction (Küpper et al., 2008; Snow et al., 2015; Zhang et al., 2019). *Trichodesmium* colonies can also directly use particulate iron or sand dust to maintain the iron demand in the body under the condition of DFe content limitation (Rubin et al., 2011; Polyviou et al., 2018; Eichner et al., 2023). This process may include the following steps: (1) *Trichodesmium* can migrate and aggregate and form puff-like colonies under Fe restriction (Roe et al., 2012; Tzubari et al., 2018) because puff-like colonies have higher efficiency of iron uptake (Achilles et al., 2003; Roe et al., 2012); (2) The particulate iron and sand are attached to the filaments of the *Trichodesmium* colony and transferred to the centre of the colony along the filaments (Rubin et al., 2011; Basu and Shaked, 2018); (3) Iron complex molecules produced by bacteria parasitizing the centre of their colonies can promote the dissolution of particulate iron (Frischkorn et al., 2017; Lee et al., 2017; Basu et al., 2019). Individual studies have also found that H_2 may provide a source of electrons for mineral iron reduction in *Trichodesmium* cells, thereby promoting Fe uptake by *Trichodesmium* colonies (Eichner et al., 2019).

It is worth noting that adding sufficient DFe (925 pmol L^{-1}) or limited DFe (35 pmol L^{-1}) to *Trichodesmium* at pH 7.81 showed lower growth and nitrogen fixation rates compared with pH 8.02 (Zhang et al., 2019). It is suggested that pH change may affect the effect of DFe on *Trichodesmium*. It has been shown that under acidification conditions, *Trichodesmium* cells mitigate the acidification-induced reduction in nitrogen fixation rate by up-regulating the expression of nitrogenase genes. However, the processes of nitrogenase synthesis and energy production are iron-demanding so that iron limitation (as low as 0.04 nmol L^{-1}) exacerbates the suppression of *Trichodesmium* nitrogen fixation rate by ocean acidification (Shi et al., 2012; Hong et al., 2017). Therefore, when *Trichodesmium* faces the effects of both iron limitation and ocean acidification, it needs to divert iron from cellular pathways, such as the photosynthetic system and respiratory transport chain, and use it for nitrogenase synthesis in response to decreased nitrogen-fixing efficiency due to acidification (Zhang et al., 2019).

5.3 Effects of changes in DFe content on *Crocosphaera*

Elevated DFe content typically enhances the expression of the *nifH* gene and N_2 fixation in *Crocosphaera*. In laboratory cultures, *Crocosphaera* can efficiently utilize added DFe (i.e., 250–450 nmol L^{-1}), resulting in a substantial increase in N_2 fixation rates of up to 17-fold (Fu et al., 2008; Jacq et al., 2014; Garcia et al., 2015; Yang et al., 2021). Lory et al. found that high DFe content (~1.6 nmol L^{-1}) near the volcano of Tonga in the western tropical South Pacific significantly stimulates nitrogen fixation on *Crocosphaera* and added 0.2 nmol L^{-1} $D^{55}Fe$ to the collected samples (Lory et al., 2022). The results showed that Fe: C from *Crocosphaera* is higher than that from other non-nitrogen-fixing organisms, and the cell-specific iron uptake rate (0.46–1.21 amol Fe $cell^{-1}$ d^{-1}) is more than two times higher than that from other non-nitrogen-fixing organisms. It suggests that DFe can be actively absorbed from *Crocosphaera* to maintain cell growth (Tuit et al., 2004; Lory et al., 2022).

However, since large *Crocosphaera* cells (≥4 μm) have relatively higher copy numbers of the iron-related genes *isiA* and *feoB* than small cells (<4 μm), the effect of adding DFe to samples sourced from different regions with different *Crocosphaera* cell's size can vary (Bench et al., 2013; Masuda et al., 2022; Zehr et al., 2022). For instance, the addition of DFe at a concentration of 10 nmol L^{-1} resulted in nearly unchanged N_2 fixation rates and cellular abundance in small *Crocosphaera* cells (<4 μm) during culture experiments conducted in the Southern Ocean (Benavides et al., 2022). Conversely, adding 400 nmol L^{-1} of DFe to samples collected from eastern Australian waters led to a significant increase in the *nifH* gene-copy number in large *Crocosphaera* cells (≥4 μm) (Moisander et al., 2012; Bench et al., 2013).

In addition, strategies have evolved from *Crocosphaera* to adapt to Fe-limiting environments, such as re-using Fe or reducing cell size (Saito et al., 2011; Jacq et al., 2014). Saito et al. found that Fe demand from cells can be reduced by efficient diurnal cycling of nitrogen fixation and photosynthetic-related Ferri proteins, and this strategy reduces the total iron requirement of cells by about 40% (Saito et al., 2011; Masuda et al., 2022); *Crocosphaera* can also keep enough energy on cells by reducing cell size under Fe constraints (1–3.3 nmol L^{-1}) (Jacq et al., 2014; Marki et al., 2020), and the reduction of cell size can ease the absorption rate limit and diffusion limit caused by Fe element (Garcia et al., 2015), to maintain the optimal nitrogen fixation rate and carbon sequestration rate from *Crocosphaera*.

5.4 Effect of changes in DFe content on NCDs

Studies have shown that the genes *afuABC* and *fhuDCB*, which regulate the Fe^{3+}*ABC* transporter, were found in γ-24774A11 cells (Cheung et al., 2021), indicating that NCDs have the potential to utilize DFe to meet its own growth and nitrogenase synthesis needs. For example, Moisander et al. observed a significant increase in the *nifH* gene copy number of γ-24774A11 after adding 400 nmol L^{-1} DFe to the samples collected in the eastern waters of Australia (Moisander et al., 2012). A similar phenomenon was observed by Langlois et al. after the addition of 2 nmol L^{-1} DFe along the subtropical South Atlantic coast (Langlois et al., 2012). However, a generalized additive model study on the qPCR copy data of the *nifH* gene of γ-24774A11 in the world showed that the abundance of γ-A decreased with the increase of DFe content, which may be because γ-A has a competitive advantage compared with autotrophic nitrogen-fixing cyanobacteria in low-iron environments (Shao and Luo, 2022).

6 Conclusions and perspectives

We have observed that most researchers support several general patterns by analysing the impacts of nutrients such as TDN, TDP, and DFe on B. bigelowii, Trichodesmium, Crocosphaera, and NCDs. First, it was observed that changes in DIN levels had little effect on *B. bigelowii*; increased DIN levels had a negative effect on nitrogen fixation by *Trichodesmium*, although it did not wholly inhibit N_2 fixation. Additionally, elevated NO_3^- levels did not impact *Crocosphaera*, but elevated NH_4^+ significantly hindered its ability to fix N; the effect on NCDs is unclear. Under phosphorus-limited conditions, high DIP content can enhance individual growth, population growth, and N_2 fixation in diazotrophs; however, in nonphosphorus-limited areas, increased DIP content may have limited effects on diazotrophs. The influence of changes in DOP content on most diazotrophs remains unclear; nevertheless, DOP could still serve as a potential source of P under DIP-constrained conditions. Elevated DFe content is often linked to higher rates of N_2 fixation due to the correlation between the structural composition of nitrogenase and Fe demand. However, if ambient DFe levels already meet the Fe requirements of diazotrophs, further increases in DFe content may not lead to significant increases in N_2 fixation rates.

Following years of research, we have developed a basic understanding of how nutrients affect the growth of diazotrophs and their BNF. However, due to the complexity of the marine environment and limited knowledge regarding diazotrophs, our understanding of this mechanism is limited and requires future elucidation.

6.1 Advance the cultivation of as-yet uncultivated marine diazotrophs

The application of molecular biology techniques to the study of marine diazotrophs has contributed significantly to the understanding of the role of marine BNF, especially in recent years when the development of high-throughput sequencing technologies such as macro genomes and macro transcriptomes (Delmont et al., 2022; Tschitschko et al., 2024) has broadened the understanding of uncultured marine diazotrophs. For example, Delmont et al. found from the TARA Oceans macro genome that NCDs express the *nifH* gene *in situ* and that their macroeconomic abundance exceeds that of most open-ocean cyanobacterial diazotrophs (Delmont et al., 2018, 2022).

However, due to the low abundance of most NCDs, no direct evidence has been obtained to date that NCDs can perform nitrogen fixation in the *in situ* ocean (Turk-Kubo et al., 2022). To address the challenge that it is difficult to achieve the detection of low-abundance cells with the current level of detection (Bombar et al., 2016), scholars have begun to carry out research on the metabolic pathways and physiological and ecological characteristics of NCDs by integrating the application of enrichment and isolation with molecular techniques. For example, Martínez-Pérez et al. enriched *S. castanea* among α-proteobacteria from an active nitrogen-fixing region in the Peruvian upwelling zone and detected its active metabolic processes (Martínez-Pérez et al., 2018); Rose et al. enriched γ- proteobacterial diazotrophs *Candidatus* Thalassolituus haligoni from Halifax Harbour, and the cell-specific nitrogen-fixing rate of the γ-proteobacterium was obtained from measurements made by ^{15}N tracer technology (Rose et al., 2024). Although they enriched cultures of several isolates, the rate of nitrogen fixation by NCDs in the *in situ* ocean is still unavailable. For this reason, there

is an urgent need to accelerate the realisation of pure cultures of these enriched isolates and to select optimal model organisms for physiological and ecological studies (Lewis et al., 2021) to confirm the ability of NCDs to fix nitrogen in the *in situ* environment aggressively.

Isolation to obtain pure strains is the best method to study microorganisms' metabolic activity and physiological properties (Liu et al., 2022), but it comes with significant challenges. New technologies may accelerate the realisation of pure cultures of diazotrophs such as NCDs. For example, the reverse genomics approach is essential for targeting the culture of marine microorganisms, which provides technical support for the targeted isolation and culture of functional microorganisms (Cross et al., 2019); automated Raman-based sorting techniques allow for the functional classification of stable isotope-labelled living cells, which can generate living cells suitable for subsequent single-cell genomics, micro-macro-genomics, and pure culture (Lee et al., 2019).

6.2 Strengthen the investigation into the synergistic effects of multiple nutrients on marine BNF

Current research on the role of nutrients in marine BNF often focuses on a single nutrient, and studies of single-nutrient limitation may fail to capture the complexity of the *in situ* physiological ecology of diazotrophs (Held et al., 2020). Indeed, in the surface layer of the open ocean, where multiple nutrients are often simultaneously deficient (Saito et al., 2008; Moore et al., 2013), co-limitation of nutrients often occurs (Garcia et al., 2015; Held et al., 2020; Cerdan-Garcia et al., 2022; Yang et al., 2022; Browning and Moore, 2023). In addition, global warming and ocean acidification and nutrients can synergistically affect marine BNF (Deutsch et al., 2024; Hutchins and Tagliabue, 2024). For example, DFe limitation exacerbates ocean acidification's adverse effects on *Trichodesmium*'s growth and nitrogen fixation (Hong et al., 2017; Zhang et al., 2019). Thus, it is necessary and urgent to research the synergistic effects of nutrients on marine BNF.

Studies have reported the effects of global warming and ocean acidification on nitrogen fixation in synergy with nutrients through in-laboratory simulations and ecological modelling studies (Deutsch et al., 2024; Hutchins and Tagliabue, 2024). However, these synergistic effects are difficult to confirm through field culture studies due to the relatively large timescales of global warming and ocean acidification and small culture timescales (Gruber, 2016), as well as the complexity and variability of marine environments and the multiple resistances to obtaining high-resolution sampling data (Benavides and Robidart, 2020). The recent broadening of the understanding of marine nitrogen-fixing biodiversity has further increased the complexity and difficulty of studies (Hutchins and Capone, 2022).

The integrated application of laboratory simulations, genomics techniques, *in situ* cultures and long-term observations, combined with artificial intelligence techniques such as machine learning (Tang et al., 2019) and the construction of Earth system models (Yao et al., 2022), ecological models, can help to comprehensively and integratively assess the synergistic effects of nutrients as well as the impacts of global changes on marine BNF, respectively (Zehr and Capone, 2024).

6.3 Enhance the advancement of innovative technologies and methodologies for investigating marine BNF

With the application of technologies such as flow cytometry (Geisler et al., 2023), macro genomics and macro transcriptomics (Delmont, 2021), nano-SIMS (Mills et al., 2020), and soft X-ray tomography (SXT) (Coale et al., 2024), the physiological and ecological study of marine nitrogen-fixing organisms has refreshed our understanding of marine nitrogen-fixing biodiversity. For example, UCYN-A, long thought to be 'endosymbiotic', is an organelle-nitroplast within the host, and marine diazotrophs have been corrected from containing only prokaryotes to both prokaryotes and eukaryotes (Coale et al., 2024; Massana, 2024).

Around the determination of nitrogen fixation rates, Großkopf et al. proposed a $^{15}N_2$ enriched water incubation method in 2012 (Großkopf et al., 2012). Comparative experiments have revealed that because $^{15}N_2$ bubbles do not dissolve sufficiently in incubation bottles (Mohr et al., 2010), the $^{15}N_2$ bubble injection method, which has been traditionally used for the past 20 years, potentially underestimates the nitrogen fixation rate of $^{15}N_2$-dominated marine waters dominated by *Trichodesmium* (Großkopf et al., 2012). Although the $^{15}N_2$ enriched water method can introduce potential nutrient and trace metal contamination due to its operational complexity (Klawonn et al., 2015), it is still the most accurate and direct estimate of nitrogen fixation rates available (White et al., 2020). Recently, new sensitive methods such as stable-isotope probing (Kapili et al., 2020) and isotopic acetylene reduction assay (Haynes et al., 2022) have begun to be applied to the determination of oceanic nitrogen fixation rates, which are expected to obtain rates closer to the real ones.

In addition, almost all the studies on marine BNF are based on shipboard culture after water harvesting or laboratory culture, which makes it challenging to fully reflect the actual marine environment and limits our comprehensive understanding of the role of marine diazotrophs. Researchers have begun to develop marine *in situ* culture techniques. For example, the Microbial Sampler-Submersible Incubation Device (MS-SID) applies the $^{15}N_2$ enriched water incubation method to achieve *in situ* culture of BNF as well as collection and preservation of *in situ* diazotrophs, but it is limited to *in situ* deployment of short-term time series due to the limited sample preservation time (Bombar et al., 2015; Edgcomb et al., 2016); the Environmental Sample Processor (ESP) enables *in situ* genetic analyses of diazotrophs and *in situ* assessment of nitrogen-fixing biodiversity, an abundance of specific genes and metabolites through DNA probes, protein

arrays and qPCR techniques, but its large size and deployment and recovery require significant logistical assistance (Robidart et al., 2014; Scholin et al., 2017). Due to the complexity of the marine environment, the integrated technology of *in situ* culture and biodiversity analysis for marine nitrogen fixation still faces many technical challenges that need to be broken through. Nevertheless, advances in *in situ* culture and analysis techniques are expected to advance our understanding of marine BNF.

Author contributions

WC: Writing – review & editing, Writing – original draft, Visualization, Investigation. QY: Writing – review & editing, Writing – original draft, Visualization, Project administration, Funding acquisition, Conceptualization. FJ: Writing – review & editing, Supervision. CL: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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