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Effects of CO_2 on the Nitrogen Isotopic Composition of Marine Diazotrophic Cyanobacteria

Zuozhu Wen[1], Ruotong Jiang[1], Tianli He[1], Thomas J. Browning[2], Haizheng Hong[1], Shuh-Ji Kao[3], Jin-Yu Terence Yang[1], and Dalin Shi[1]

[1]State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, PR China, [2]Marine Biogeochemistry Division, GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany, [3]State Key Laboratory of Marine Resource Utilization in South China Sea, School of Marine Science and Engineering, Hainan University, Haikou, PR China

Abstract Biological N_2 fixation has been crucial for sustaining early life on Earth. Very negative $\delta^{15}N$ values detected in Archean sediments, which are not observed in present-day environments, have been attributed to the low efficiency of proto-nitrogenases. Alternatively, variations in early atmospheric CO_2 may also play a role. Here we examine the effects of CO_2 concentrations on the biomass $\delta^{15}N$ signatures of the diazotrophs *Trichodesmium erythraeum* and *Crocosphaera watsonii*, which utilize Mo-Fe nitrogenase (the most common form of the enzyme). Our results show that these organisms produce biomass with $\delta^{15}N$ values up to ~3‰ lower under both decreased and elevated CO_2 concentrations compared to modern levels (~380 µatm). These deviations from modern CO_2 levels reduce nitrogenase enzyme efficiency, leading to increased organismal isotopic fractionation during N_2 fixation. This study offers an alternative explanation for the observed fluctuations in geological $\delta^{15}N$ records and provides new insights into the past nitrogen cycle on Earth.

Plain Language Summary The isotope effect of biological N_2 fixation is crucial for understanding the nitrogen cycle, but its regulation under different atmospheric CO_2 levels as appeared in Earth's history is not well understood. Our research shows that CO_2 levels significantly influence the nitrogen isotope composition in the biomass of the diazotrophic cyanobacteria *Trichodesmium* and *Crocosphaera* by affecting the nitrogenase efficiency and thus the growth rate. This study sheds light on the geological changes in the $\delta^{15}N$ records and provides new insights into the historical nitrogen cycle on Earth.

Zuozhu Wen and Ruotong Jiang these authors contributed equally to this work.

Key Points:

- The effects of CO_2 on the biomass $\delta^{15}N$ signatures of the diazotrophs *Trichodesmium erythraeum* and *Crocosphaera watsonii* are examined
- Both species produce biomass with $\delta^{15}N$ values lower under both decreased and elevated CO_2 concentrations compared to modern CO_2 levels
- CO_2-controlled nitrogenase efficiency significantly influences organismal isotopic fractionation during N_2 fixation

Supporting Information:

Supporting Information may be found in the online version of this article.

Correspondence to:

D. Shi,
dshi@xmu.edu.cn

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1. Introduction

Nitrogen fixation by diazotrophic bacteria converts abundant dinitrogen (N_2) gas into ammonia, which sustains the oceanic N reservoir and thereby ocean productivity (Altabet, 2007; Gruber & Galloway, 2008; Stueken et al., 2015). This process is catalyzed by the metalloenzyme nitrogenase and produces organic N that is isotopically depleted in ^{15}N ($\delta^{15}N$, $[(^{15}N/^{14}N_{sample}/^{15}N/^{14}N_{air}) - 1] \times 1{,}000$ when expressed in per mil) relative to source N_2. This is opposite to denitrification and anammox, which return lighter N to the atmosphere and hence leave the marine nitrate (NO_3^-) pool and resulting biomass relatively enriched in ^{15}N (Brunner et al., 2013; Casciotti, 2016). Therefore, the isotope effect of N_2 fixation (i.e., the fractionation factor for N_2 fixation, ε_{fix}) is a key parameter for isotope-based studies of present and past N cycling on Earth (Casciotti, 2016; Lloyd et al., 2020).

The isotopic effect of N_2 fixation on nitrogen was traditionally assumed to be invariant with environmental conditions and equivalent to the in vivo isotope effect of molybdenum (Mo)-nitrogenase (~−2‰) (Bauersachs et al., 2009; Carpenter et al., 1997; Minagawa & Wada, 1986). Researchers have therefore often adopted a $\delta^{15}N$ value of ~−1‰ (~2‰ lower than the $\delta^{15}N$ of dissolved N_2 substrate, +0.7‰) for biomass produced by diazotrophs (Brandes & Devol, 2002; Montoya, 2008; Sigman et al., 2009). Geologists have used N inputs primarily from N_2 fixation and the subsequent recycling of ammonium from diazotrophic biomass decay, to account for the low $\delta^{15}N$ values (centered around 0‰) found in ancient sediments dating back approximately 3.2 billion years (Gyr) (Stueken et al., 2015). However, the presence of extremely negative bulk $\delta^{15}N$ values (<~−2‰, can low to −7‰) in the Cretaceous Oceanic Anoxic Events (OAEs, 145–166 Myr) and the early Archean (>3.2 Gyr ago), which are not observed in modern marine sediments (Altabet, 2007; Altabet & Francois, 1994; Shen et al., 2006), challenges the current understanding of the marine nitrogen isotope budget (Brandes & Devol, 2002).

The low $\delta^{15}N$ values observed during OAEs have been previously explained as the assimilation of ammonium by dominant eukaryotes supplemented by diazotrophy (Higgins et al., 2012). Alternatively, recent studies have demonstrated that changes in environmental conditions significantly impact the $\delta^{15}N$ values in diazotrophic biomass, providing new insights into the variability of the $\delta^{15}N$ records. For instance, the heterocystous cyanobacteria *Anabaena* shows up to a 3‰ increase in $\delta^{15}N$ of biomass when grown in Fe-limited versus Fe-enriched media (Zerkle et al., 2008), likely due to the release of isotopically lighter N in siderophores under Fe limitation (McRose et al., 2019). Additionally, increased N_2 partial pressure in *Anabaena* cultures resulted in significantly lighter $\delta^{15}N$ biomass, although the difference was relatively minor (less than 0.5‰) (Silverman et al., 2019). A critical discovery potentially explaining the extremely low $\delta^{15}N$ values in ancient sediments was the use of vanadium (V) and iron (Fe)-only alternative nitrogenases, which yield significantly lower $\delta^{15}N$ biomass (−6 to −7‰) (Rowell et al., 1998; Zhang et al., 2014). However, the mechanism by which the use of alternative nitrogenase enzymes alters isotope fractionation remains uncertain. Given the lower efficiency of the V- and Fe-only nitrogenases compared to the Mo-nitrogenases (Eady, 1996; Miller & Eady, 1988), it is possible that the commitment to N_2 catalysis is decreased due to (a) a decline in N_2 reduction efficiency post-binding to the active site; (b) or an increased competition at the active site between N_2 and H_2 (Guth & Burris, 1983; Yang et al., 2013), with the latter being more abundantly produced by alternative nitrogenases (Eady, 1996). The decreased commitment to catalysis may thus lead to a more complete expression of the isotopic effect associated with the subsequent N_2 bond-breaking step of the nitrogenase catalyzation.

Recent studies show that ocean acidification resulting from elevated atmospheric carbon dioxide (CO_2) above the modern levels (~380 µatm vs. ~800 µatm) significantly reduces the efficiency of Mo-nitrogenase in the filamentous diazotrophic cyanobacterium *Trichodesmium* (Hong et al., 2017; Zhang et al., 2019). This is probably attributed to a greater allocation of electrons to protons (H^+) rather than N_2 at low pH (8.1 vs. 7.8), as evidenced by an enhanced production of H_2 (Pham & Burgess, 1993; Shi et al., 2012). Should this phenomenon be consistent across all diazotroph species and nitrogenase types, past fluctuations in atmospheric CO_2 (which exceeded 1,500 ppm prior to 420 Myr or even higher in Archean, dropping to pre-industrial levels [ca. 280 ppm] after ~20 Myr [Figure S1 in Supporting Information S1]) might have notably impacted nitrogenase efficiency, and consequently, the isotopic fractionation during N_2 fixation.

In this study, we examined the effect of changing CO_2 concentrations on biomass $\delta^{15}N$ of the important cyanobacterial diazotrophs *Trichodesmium erythraeum* and *Crocosphaera watsonii*. We reported the first evidence that changes in CO_2 levels significantly alter the biomass $\delta^{15}N$ of both species by impacting nitrogenase efficiency. Our findings provide new insights into the fluctuations observed in the $\delta^{15}N$ records, and thus the Earth's past nitrogen cycle.

2. Materials and Methods

2.1. Diazotroph Strains and Culture Conditions

The marine cyanobacteria *Trichodesmium erythraeum* IMS101 and *Crocosphaera watsonii* WH8501 were grown in Aquil-tricho medium prepared with 0.22 µm-filtered and microwave-sterilized oligotrophic South China Sea surface water (Hong et al., 2017). Cultures underwent an acclimation period spanning at least 20 generations prior to starting the experiments. The medium was enriched with 10 µM chelexed and filter-sterilized NaH_2PO_4, filter-sterilized vitamins and trace metals buffered with 20 µM EDTA (Sunda et al., 2005), and a replete concentration of Fe (1 µM). Cultures were grown under five pCO_2/pH (~180–1,400 µatm, pH ~7.5 to 8.3, Table S1 in Supporting Information S1) conditions at 27°C and 80 µmol photons m^{-2} s^{-1} (14 hr:10 hr light-dark cycle). All experiments were carried out with three biological replicates. Cultures of *C. watsonii* were axenic, however, cultures of *T. erythraeum* were not. For both of the two species, sterile techniques were applied for culturing and experimental manipulations. Heterotrophic bacteria abundance in *T. erythraeum* culture was measured using flow cytometry. The total bacterial N biomass was less than 0.32% ($n = 3$) of *T. erythraeum* N biomass, calculated by assuming an oceanic bacterial cell content of 2.1 fg N $cell^{-1}$ (Fukuda et al., 1998). All data of the measured parameters including carbonate chemistry, growth and N_2 fixation rates, biomass $\delta^{15}N$, and nitrogenase efficiency are shown in Table S1 in Supporting Information S1 and a data set (Shi & Wen, 2024).

2.2. Carbonate Chemistry Manipulation

pCO_2/pH in media was manipulated by adding different amounts of ultra-pure HCl and NaOH (both from Sigma-Aldrich Chemical) (Shi et al., 2009). The pH of media was monitored daily for *T. erythraeum* and every 2 days for *C. watsonii*, using a spectrophotometric method (H. Zhang & Byrne, 1996) and each manipulated pH remained stable throughout the experimental period (Figure S2 in Supporting Information S1). The dissolved inorganic carbon (DIC) concentration was analyzed using a CO_2 analyzer (LI 7000, Apollo SciTech). Calculations of alkalinity and pCO_2 were made using the CO2Sys program (Pierrot et al., 2011).

2.3. Chl *a*, Cell Concentrations and Growth Rate

The growth of *T. erythraeum* was monitored by measuring Chl *a* concentration daily, while the growth of *C. watsonii* was monitored through the measurement of cell abundance every 2 days. *T. erythraeum* Chl *a* concentration was determined via extraction in 90% methanol, followed by analysis using a spectrophotometer according to de Marsac and Houmard (1988). *C. watsonii* cell densities were determined using a Z2 Coulter Counter (Beckman Coulter). Specific growth rates were calculated from linear regressions of the natural logarithm of Chl *a* or cell concentration versus time for the exponential growth phase and four data points were included in each growth curve.

2.4. N_2 Fixation Rates and $\delta^{15}N$ Signal of Biomass

N_2 fixation rates were measured using the $^{15}N_2$ gas dissolution method (Mohr et al., 2010). The test for potential contamination of $^{15}N_2$ gas (Cambridge Isotope Laboratories, Lot #: I-19197/AR0586172) has been reported by Lu et al. (2018). The values of $\delta^{15}N$ of total dissolved nitrogen of the blank seawater and $^{15}N_2$ gas pre-dissolved seawater were 4.7 and 5.0‰, respectively, suggesting no contamination of the $^{15}N_2$ gas (Lu et al., 2018). The $^{15}N_2$-enriched water was prepared by dissolving 5 mL $^{15}N_2$ gas into 500 mL degassed seawater. After that, 25 mL $^{15}N_2$-enriched water was added to each 250 mL polycarbonate bottle containing diazotroph culture, and then incubated under the same culture conditions for 2 hr. The measurement was conducted at the midpoint of the light period and dark period, respectively, for *T. erythraeum* and *C. watsonii*. After incubation, diazotrophs were filtered onto 25 mm pre-combusted GF/F filters. Diazotrophs not enriched with $^{15}N_2$ were also collected by filtration concurrently with the N_2 fixation rate measurement to determine the natural biomass $\delta^{15}N$ signal. All filter samples were then dried and analyzed using a Thermo Scientific Flash 2,000 HT elemental analyzer coupled with a Thermo Finnigan Deltaplus Advantage isotope ratio mass spectrometer. The average reproducibility of USGS-40 standard was ±0.3‰ for $\delta^{15}N$. The N_2 fixation rate was calculated according to Montoya et al. (1996).

2.5. Quantification of *NifH* Proteins

For the analysis of nitrogenase (NifH) protein abundance, *T. erythraeum* and *C. watsonii* cells were collected onto polycarbonate membrane filters at the midpoint of the light and dark period respectively. Proteins were extracted and denatured in an extraction buffer (50 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% v/v glycerol, and 1% v/v β-mercaptoethanol) under heating at 100°C for 10 min, followed by centrifugation at 20,000 g for 5 min to remove insoluble material. Total protein in the supernatant was measured using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, California, USA). Equal amounts of proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for 30 min at 200 V. Proteins were transferred to a PVDF membrane in ice-could transfer buffer (25 mM Tris, 192 mM glycine, and 2.5% [vol/vol] methanol). The membrane was blocked with 5% milk powder in TBST buffer (Tris-buffered saline containing 0.25% [vol/vol] Tween-20, pH 7.5) for 1 hr. Then, the membrane was incubated for 1 hr with primary antibodies (Agrisera: NifH, Art no. AS01 021S), followed by 1 hr incubation with Goat anti Chicken lgY (Agrisera: Art no. AS09 606) with TBST washes before and after. The membrane then was probed with alkaline phosphatase (AP stock solution, pH 9.5), and visualized with NBT/BCIP (Roche, Indianapolis). Protein bands were quantified by densitometry and protein levels were calculated from the standard (Agrisera: NifH, Art no. AS01 021S) curves.

2.6. Statistical Analysis

Data was analyzed using MATLAB R2022b to determine the statistical significance of differences via either *t*-test or one-way ANOVA. A significance level of $p < 0.05$ was applied.

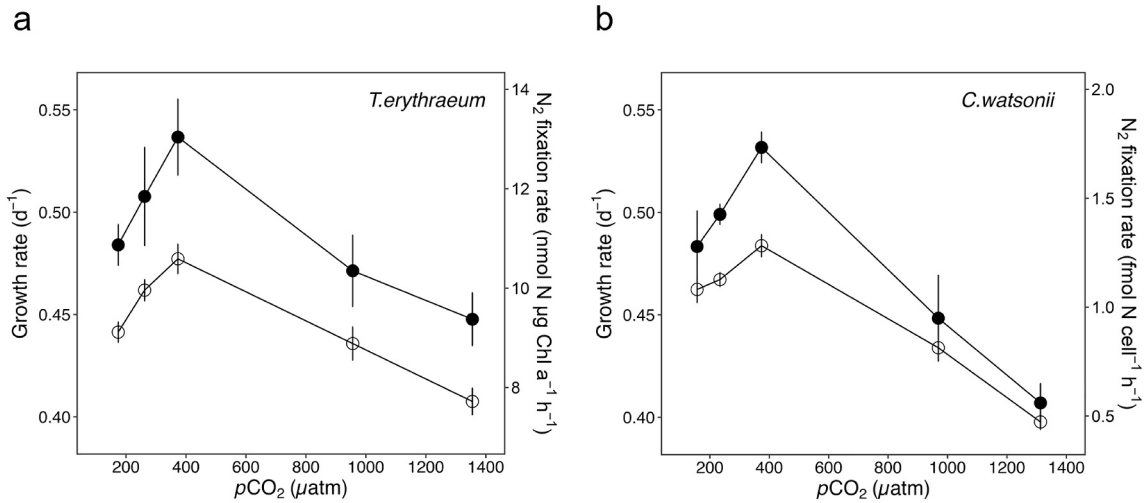

Figure 1. Growth and N_2 fixation rates of *T. erythraeum* (a) and *C. watsonii* (b) in response to CO_2 perturbations. Filled dots stand for growth rate; The open circles are N_2 fixation rates. Note that N_2 fixation rates of *T. erythraeum* and *C. watsonii* are measured in different units. The values of growth rates and N_2 fixation rates are presented as mean ± SD ($n = 3$).

3. Results and Discussion

3.1. Growth and N_2 Fixation Rates Response to CO_2 Perturbations

In our study, *T. erythraeum* and *C. watsonii* were cultivated under a range of pCO_2/pH conditions, with pCO_2 varying from approximately ∼180–1,400 µatm and accordingly pH levels ranging from about 7.5 to 8.3. These conditions were selected to reflect the environmental variability over the past 420 million years (Figure S1 in Supporting Information S1), as outlined in Foster et al. (2017). The growth and N_2 fixation rates exhibited by both species varied markedly across the different pCO_2 levels, displaying a unimodal-like response to pCO_2 perturbations (Figure 1 and Table S1 in Supporting Information S1). Notably, these rates were at their peak under modern pCO_2 levels (∼380 µatm) and decreased significantly at both lower and higher pCO_2 concentrations.

The response of phytoplankton to CO_2 perturbations lies in the simultaneous changes in seawater pCO_2 and pH, which can have conflicting effects on their physiology and growth. For instance, the carbon-fixing enzyme RubisCO of cyanobacteria (including *Trichodesmium* and *Crocosphaera*) generally has a half-saturation concentration an order of magnitude higher (>100 µM) than the current seawater CO_2 level (∼10 µM) (Badger et al., 1998). This forces them to invest substantial resources and energy into carbon concentrating mechanisms (CCMs) to increase intracellular CO_2, particularly at low seawater pCO_2 levels (e.g., ∼180 µatm). Elevated environmental CO_2 concentrations are expected to downregulate CCMs, and thus benefit cell growth and N_2 fixation (Badger et al., 2006; Hong et al., 2017; Kranz et al., 2011). Conversely, the concurrent decrease in seawater pH may reduce the cytosolic pH and nitrogenase efficiency, and thus the growth and N_2 fixation rates (Hong et al., 2017). Given that the cytosolic pH of *T. erythraeum* is about 1 unit lower than its environmental conditions as previously reported by Hong et al. (2017), and assuming a similar condition of *Crocosphaera*, the increasing CO_2 levels from ∼180 to 1,400 µatm may decrease the cytosolic pH of both diazotroph species from levels of ∼7.3 to 6.5, with two lowest pHs (CO_2 levels ∼1,000 and 1,400 µatm) deviating from the optimal pH range (7–8) for nitrogenase activity (Hadfield & Bulen, 1969; Imam & Eady, 1980; Pham & Burgess, 1993; Schneider et al., 1995). Therefore, the observed increases in growth and N_2 fixation rates from ∼180 µatm to modern CO_2 levels (Figure 1) indicate an overall dominance of beneficial effects of elevated pCO_2, with minimal impact of reduced pH as the cytosolic pH is still within the optimal range. In contrast, the substantial declines in growth and N_2 fixation at high CO_2 levels above the modern levels (∼1,000 and 1,400 µatm, Figure 1) reflect the detrimental impacts of reduced pH, which outweigh the positive effects of elevated pCO_2. Our results on the two diazotrophic species thus provide further evidence supporting the tradeoff between the effects of pCO_2 and pH on diazotrophs in response to CO_2 perturbation.

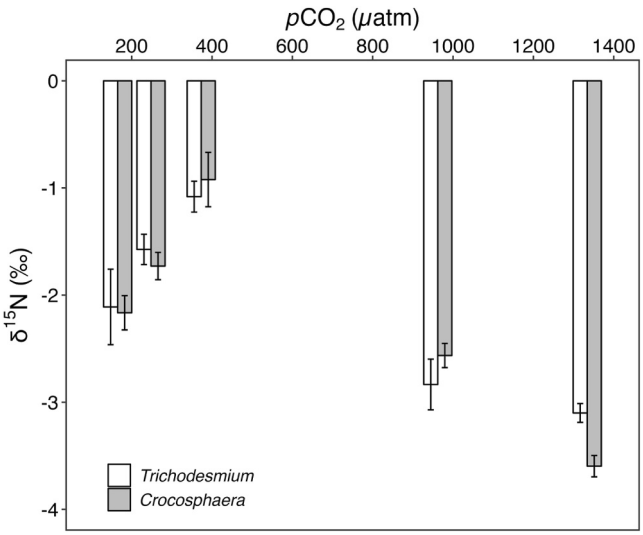

Figure 2. Biomass $\delta^{15}N$ values of *T. erythraeum* and *C. watsonii* produced when grown in variable CO_2 concentrations. White, *Trichodesmium*; Gray, *Crocosphaera*. The values of biomass $\delta^{15}N$ are presented as mean ± SD ($n = 3$).

3.2. Biomass $\delta^{15}N$ Responses to CO_2 Perturbations

The biomass $\delta^{15}N$ of both *T. erythraeum* and *C. watsonii* exhibit a response to pCO_2/pH perturbations that is analogous to their growth and N_2 fixation rates. Under modern CO_2 conditions, the biomass $\delta^{15}N$ values peaked at around −1‰ for both species (Figure 2). These values align well within the range of −0.7 to −0.25‰ previously reported for biomass produced by *Trichodesmium* collected from Sargasso Sea and Caribbean Sea (Carpenter et al., 1997), and are also consistent with the biomass $\delta^{15}N$ values typically associated with organisms utilizing the "canonical" Mo-nitrogenase (Zhang et al., 2014). Our data reveal that any deviation from the modern pCO_2 levels, either an increase or a decrease, results in a significant reduction ($p < 0.01$, one-way ANOVA) in biomass $\delta^{15}N$. Specifically, at ∼1,400 μatm pCO_2, the biomass $\delta^{15}N$ decreased by a maximum of 2.0‰ for *T. erythraeum* and 2.7‰ for *C. watsonii*.

Our study shows for the first time that CO_2 exerts important controls on the N isotopic composition of diazotrophic biomass. Moreover, we observed that both N_2 fixation and growth rates were significantly positively correlated with biomass $\delta^{15}N$ (R^2 for both species are higher than 0.9, $p < 0.05$, Figure S3 in Supporting Information S1). The mechanisms behind these strong correlations remain unclear. We propose a quasi-steady-state cellular model for *Trichodesmium* and *Crocosphaera* (Figure S4 in Supporting Information S1) to help understand the relationship between organismal $\delta^{15}N$ and N_2 fixation rate/growth rates, which was adapted from the approach on the heterocystous cyanobacteria *A. cylindreica* employed by Silverman et al. (2019). This cellular model relates the observed organismal isotope fractionation $\varepsilon_{N.Biomass/N2.gas}$ (defined here as $\delta^{15}N_{Biomass}$–$\delta^{15}N_{N2.gas}$, with $\delta^{15}N_{N2.gas} = 0‰$) to the equilibrium fractionation factor between the aqueous and gaseous phase of N_2 ($\varepsilon_{aq/g}$), the intrinsic kinetic isotope fractionation of nitrogenase during the N_2 fixation reaction (ε_{fix}), the extracellular concentration of N_2 ($C_{N2.ext}$), the N_2 diffusion rate constant (K_1), and the rate of N_2 fixation by nitrogenase in the cells (Φ_{fix}) (for more detailed descriptions, see Supporting Information):

$$\varepsilon_{\frac{N_{Biomass}}{N_{2.gas}}} = \delta_{N_{biomass}} = \varepsilon_{\frac{aq}{g}} + \varepsilon_{fix} - \frac{\varepsilon_{fix}}{K_1 \times C_{N2\cdot ext}} \times \Phi_{fix}$$

where $\varepsilon_{aq/g}$, K_1, and $C_{N2.ext}$ were constant in our culture system. If ε_{fix} was assumed to be stable between the different CO_2 treatments, the rate of N_2 fixation by nitrogenase in the cells (Φ_{fix}), which was constrained by the measured N_2 fixation rates, was then the key variable that determined the organismal isotope effect ($\varepsilon_{N.Biomass/N2.gas}$). Consistently, our results showed that the organismal isotope effects were strongly correlated with N_2 fixation rates (Figures S3c and S3d in Supporting Information S1). In addition, as the N_2 fixation rates of both species were significantly correlated with the growth rates ($R^2 > 0.95$, $p < 0.01$, Figure S5a in Supporting Information S1), $\varepsilon_{N.Biomass/N2.gas}$ can also be related to growth rate (μ) by replacing Φ_{fix} with its function of μ (Equation S5 in Supporting Information S1). In this regard, our results were consistent with the previous finding of a growth rate-dependent organismal isotope effect during N_2 fixation in the heterocystous cyanobacteria *A. cylindreica* (Silverman et al., 2019).

Furthermore, previous research has indicated that a decrease in nitrogenase efficiency at elevated pCO_2 levels above 400 μatm leads to reduced growth and N_2 fixation rates of *T. erythraeum* (Hong et al., 2017; Shi et al., 2012). More importantly, it has been inferred that changes in nitrogenase efficiency can also impact isotopic fractionation during N_2 fixation (Zhang et al., 2014). We thus hypothesized that in our study the correlations of growth and N_2 fixation rates with biomass $\delta^{15}N$ were mediated by nitrogenase efficiency under CO_2 perturbation. We therefore calculated the nitrogenase efficiency of *T. erythraeum* and *C. watsonii* by normalizing $^{15}N_2$-based N_2 fixation rates to the abundance of nitrogenase reductase (NifH) protein. In alignment with previous findings, we observed significant reductions in nitrogenase efficiency for both species under elevated CO_2 conditions (corresponding to decreased pH) above the modern levels ($p < 0.05$, One-way ANOVA, Figure 3). This reduction was attributed to a decrease in N_2 fixation rates coupled with an increase in NifH protein production (Figures 1 and 3). As discussed above, the concurrent decrease in seawater pH with elevated pCO_2 may reduce the cytosolic

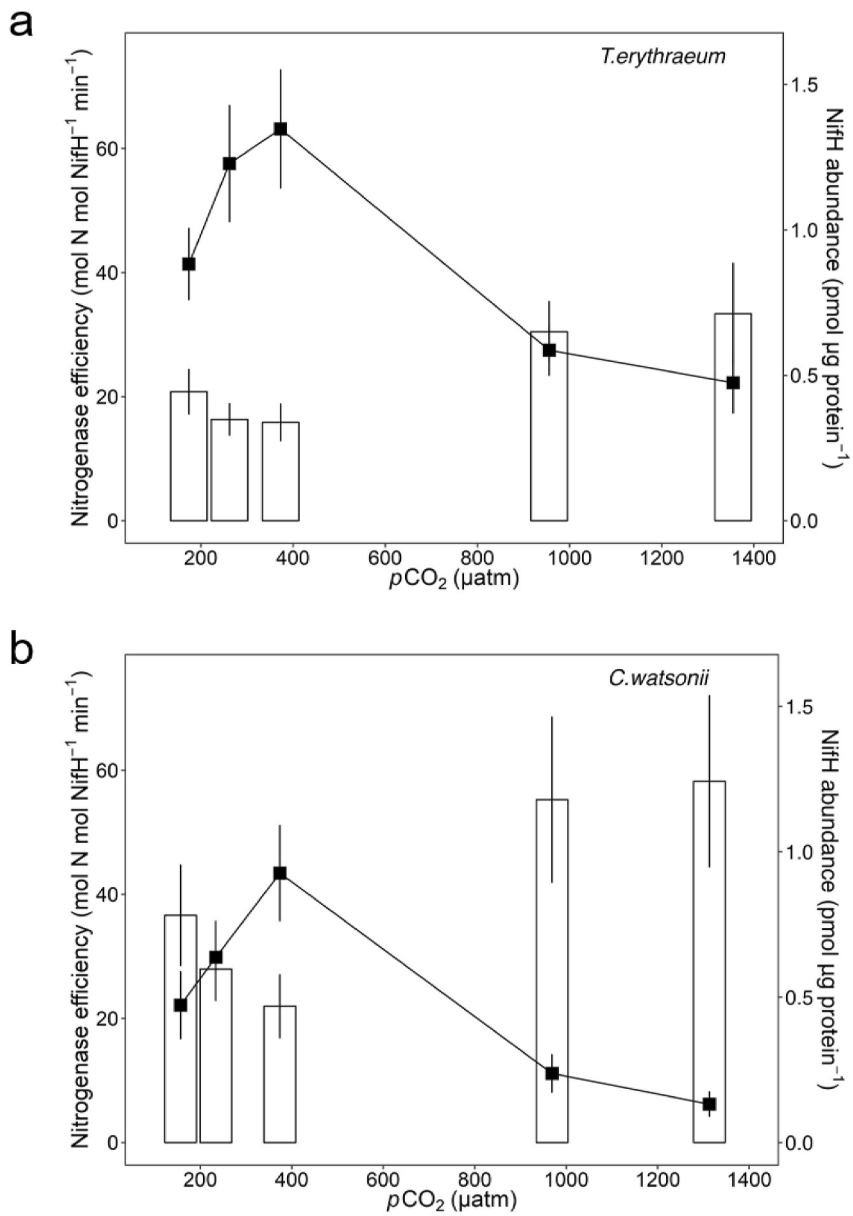

Figure 3. Nitrogenase efficiency of *T. erythraeum* (a) and *C. watsonii* (b) in response to CO_2 perturbations. The filled squares stand for the nitrogenase efficiency; The hollow bars are nitrogenase reductase (NifH) protein abundances. Data shows the mean ± standard deviation of $n = 3$ biological replicates.

pH that deviates from the optimal pH range (7–8) for nitrogenase activity, resulting in lower nitrogenase efficiency (Hong et al., 2017). We also noted a significant decrease in nitrogenase efficiency at ∼180 µatm pCO_2 compared to modern pCO_2 levels ($p < 0.05$, One-way ANOVA, Figure 3). Under low CO_2 concentrations, *T. erythraeum* and *C. watsonii* would need to invest substantial resources and energy into CCMs to enhance the efficiency of RubisCO carboxylation, thereby alleviating carbon limitation (Badger et al., 2006; Giordano et al., 2005; Kaplan & Reinhold, 1999). Upregulation of CCMs may thus divert energy and resources that would otherwise be allocated to other cellular metabolic pathways, such as N_2 fixation (Barcelos e Ramos et al., 2007; Kranz et al., 2009; Levitan et al., 2007). Therefore, it is plausible that CO_2 limitation may reduce energy and reductant supply to nitrogenase and decrease the enzyme efficiency. Regardless of the specific mechanisms responsible for variations in nitrogenase efficiency, we found that CO_2-controlled nitrogenase efficiency was significantly positively correlated with N_2 fixation and the biomass $\delta^{15}N$ of *T. erythraeum* and *C. watsonii* ($R^2 > 0.9$, $p < 0.05$, Figure 4 and Figure S5b in Supporting Information S1). Given these strong correlations,

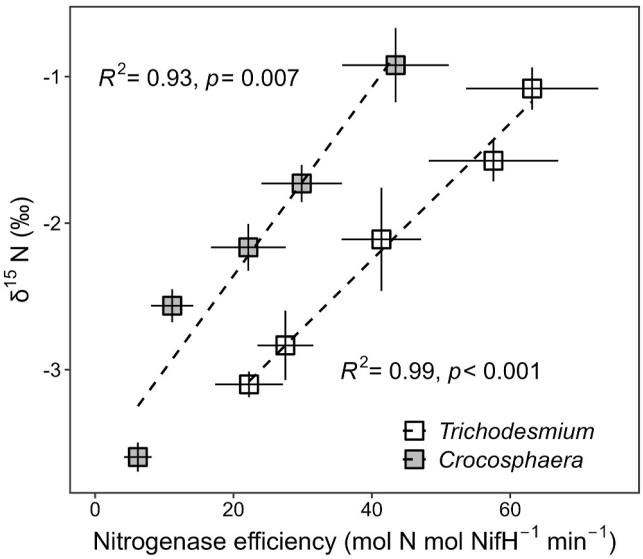

Figure 4. Relationships between nitrogenase efficiency and biomass δ^{15}N. Open squares, *T. erythraeum*; Gray squares, *C. watsonii*. Data shows the mean ± standard deviation of n = 3 biological replicates.

$\varepsilon_{N.Biomass/N2.gas}$ can be related to nitrogenase efficiency (E) by replacing Φ_{fix} with its function of E (Equation S6 in Supporting Information S1). Our results thus point toward the important role of nitrogenase efficiency in regulating the organismal isotopic effect of N_2 fixation along the CO_2 gradients. It should be noted that in the above discussion, ε_{fix} was assumed to be constant, despite nitrogenase efficiencies of *T. erythraeum* and *C. watsonii* changing with the varying CO_2 concentrations in the culture system. It was suggested that the lower efficiency of alternative V- and Fe-only nitrogenases could result in a more pronounced isotopic effect during N_2 fixation, compared to the canonical Mo-nitrogenase (Eady, 1996; Miller & Eady, 1988). The possibility that the ε_{fix} may be altered potentially due to the change of nitrogenase efficiency could not be ruled out, although the exact effect and mechanisms remained unclear.

Interestingly, despite the different nitrogenase efficiencies between *T. erythraeum* and *C. watsonii*, their biomass δ^{15}N values were not significantly different (Figures 2 and 3). These results imply that other factors controlling the organismal isotope effect had different impacts between the two species. For instance, N_2 diffusion transport could be more complicated in filamentous *T. erythraeum* due to its segregation of N_2 fixation and oxygenic photosynthesis (Berman-Frank et al., 2001), while unicellular diazotroph *C. watsonii* may be less affected by N_2 diffusion issues. In addition, given the genetic differences between *T. erythraeum* and *C. watsonii* (Zehr & Kudela, 2011), it is inferred that the values of ε_{fix}, which is essential to determine the overall organismal isotope effect, may differ in the two species. Overall, the observed organismal isotope effects are the combined results of all the factors mentioned above, which need to be fully understood in the future.

4. Conclusions

Our research has established that CO_2 perturbation significantly affects the N isotopic fractionation during cyanobacterial N_2 fixation. We observed significant ^{15}N-depleted biomass in cyanobacterial diazotrophs grown under CO_2 concentrations either below or above modern levels. These findings appear to be strongly linked to the efficiency of nitrogenase. This study suggests a mechanism through which fluctuations in CO_2 could influence trends in δ^{15}N values preserved in ancient organic matter found in sediments. For example, if our experimental observations are indicative of diazotroph growth patterns in the geological past, it could be inferred that the elevated CO_2 (up to ∼ 5,000 μatm at 201 Myr) above modern levels (∼380 μatm) prior to the Miocene epoch (>20 million years ago, Figure S1 in Supporting Information S1) (Hönisch et al., 2012) might have led to the production of isotopically lighter biomass and the CO_2 influence on δ^{15}N can be preserved in ancient organic matter found in sediments. Our findings could offer additional insights into the anomalously low δ^{15}N values (<−2‰) observed in sediments from the OAEs of the Cretaceous period (145–166 Myr), suggesting that if extant diazotrophs utilizing alternative nitrogenases during these periods responded similarly to the changes in CO_2 as observed in our study, it could partly explain those low δ^{15}N sediment values (Lloyd et al., 2020). In addition to CO_2, environmental factors such as light, temperature and nutrients, which have undergone significant changes throughout Earth's history, are expected to be critical co-drivers that may interactively impact nitrogenase efficiency and consequently nitrogen isotope fractionation. These interactions warrant further investigation.

Acknowledgments
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Data Availability Statement

All data needed to evaluate the conclusions in the paper has been published in Figshare (Shi & Wen, 2024).

References

Altabet, M. A. (2007). Constraints on oceanic N balance/imbalance from sedimentary ^{15}N records. *Biogeosciences*, *4*(1), 75–86. https://doi.org/10.5194/bg-4-75-2007

Altabet, M. A., & Francois, R. (1994). Sedimentary nitrogen isotopic ratio as a recorder for surface ocean nitrate utilization. *Global Biogeochemical Cycles*, *8*(1), 103–116. https://doi.org/10.1029/93gb03396

Badger, M. R., Andrews, T. J., Whitney, S. M., Ludwig, M., Yellowlees, D. C., Leggat, W., & Price, G. D. (1998). The diversity and coevolution of Rubisco, plastids, pyrenoids, and chloroplast-based CO_2-concentrating mechanisms in algae. *Canadian Journal of Botany*, *76*(6), 1052–1071. https://doi.org/10.1139/b98-074

Badger, M. R., Price, G. D., Long, B. M., & Woodger, F. J. (2006). The environmental plasticity and ecological genomics of the cyanobacterial CO_2 concentrating mechanism. *Journal of Experimental Botany*, *57*(2), 249–265. https://doi.org/10.1093/jxb/eri286

Barcelos e Ramos, J., Biswas, H., Schulz, K. G., LaRoche, J., & Riebesell, U. (2007). Effect of rising atmospheric carbon dioxide on the marine nitrogen fixer *Trichodesmium*. *Global Biogeochemical Cycles*, *21*(2). https://doi.org/10.1029/2006gb002898

Bauersachs, T., Schouten, S., Compaoré, J., Wollenzien, U., Stal, L. J., & Sinninghe Damsteé, J. S. (2009). Nitrogen isotopic fractionation associated with growth on dinitrogen gas and nitrate by cyanobacteria. *Limnology & Oceanography*, *54*(4), 1403–1411. https://doi.org/10.4319/lo.2009.54.4.1403

Berman-Frank, I., Lundgren, P., Chen, Y. B., Kupper, H., Kolber, Z., Bergman, B., & Falkowski, P. (2001). Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. *Science*, *294*(5546), 1534–1537. https://doi.org/10.1126/science.1064082

Brandes, J. A., & Devol, A. H. (2002). A global marine-fixed nitrogen isotopic budget: Implications for Holocene nitrogen cycling. *Global Biogeochemical Cycles*, *16*(4), 67–61. https://doi.org/10.1029/2001gb001856

Brunner, B., Contreras, S., Lehmann, M. F., Matantseva, O., Rollog, M., Kalvelage, T., et al. (2013). Nitrogen isotope effects induced by anammox bacteria. *Proceedings of the National Academy of Sciences of the United States of America* (Vol. 110(47), 18994–18999). https://doi.org/10.1073/pnas.1310488110

Carpenter, E. J., Harvey, H. R., Fry, B., & Capone, D. G. (1997). Biogeochemical tracers of the marine cyanobacterium *Trichodesmium*. *Deep-Sea Research, Part A: Oceanographic Research Papers I*, *44*(1), 27–38. https://doi.org/10.1016/s0967-0637(96)00091-x

Casciotti, K. L. (2016). Nitrogen and oxygen isotopic studies of the marine nitrogen cycle. *Annual Review of Marine Science*, *8*(1), 379–407. https://doi.org/10.1146/annurev-marine-010213-135052

de Marsac, N. T., & Houmard, J. (1988). Complementary chromatic adaptation: Physiological conditions and action spectra, In *Methods enzymol* (pp. 318–328). Academic Press.

Eady, R. R. (1996). Structure-function relationships of alternative nitrogenases. *Chemistry Review*, *96*(7), 3013–3030. https://doi.org/10.1021/cr950057h

Foster, G. L., Royer, D. L., & Lunt, D. J. (2017). Future climate forcing potentially without precedent in the last 420 million years. *Nature Communications*, *8*(1), 14845. https://doi.org/10.1038/ncomms14845

Fukuda, R., Ogawa, H., Nagata, T., & Koike, I. (1998). Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environments. *Applied and Environmental Microbiology*, *64*(9), 3352–3358. https://doi.org/10.1128/aem.64.9.3352-3358.1998

Giordano, M., Beardall, J., & Raven, J. A. (2005). CO_2 concentrating mechanisms in algae: Mechanisms, environmental modulation, and evolution. *Annual Review of Plant Biology*, *56*(1), 99–131. https://doi.org/10.1146/annurev.arplant.56.032604.144052

Gruber, N., & Galloway, J. N. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature*, *451*(7176), 293–296. https://doi.org/10.1038/nature06592

Guth, J. H., & Burris, R. H. (1983). Inhibition of nitrogenase-catalyzed ammonia formation by hydrogen. *Biochemistry*, *22*(22), 5111–5122. https://doi.org/10.1021/bi00291a010

Hadfield, K. L., & Bulen, W. A. (1969). Adenosine triphosphate requirement of nitrogenase from *Azotobacter vinelandii*. *Biochemistry*, *8*(12), 5103–5108. https://doi.org/10.1021/bi00840a064

Higgins, M. B., Robinson, R. S., Husson, J. M., Carter, S. J., & Pearson, A. (2012). Dominant eukaryotic export production during ocean anoxic events reflects the importance of recycled NH_4^+. *Proceedings of the National Academy of Sciences of the United States of America* (Vol. 109(7), pp. 2269–2274). https://doi.org/10.1073/pnas.1104313109

Hong, H., Shen, R., Zhang, F., Wen, Z., Chang, S., Lin, W., et al. (2017). The complex effects of ocean acidification on the prominent N_2-fixing cyanobacterium *Trichodesmium*. *Science*, *356*(6337), 527–531. https://doi.org/10.1126/science.aal2981

Hönisch, B., Ridgwell, A., Schmidt, D. N., Thomas, E., Gibbs, S. J., Sluijs, A., et al. (2012). The geological record of ocean acidification. *Science*, *335*(6072), 1058–1063. https://doi.org/10.1126/science.1208277

Imam, S., & Eady, R. R. (1980). Nitrogenase of Klebsiella pneumoniae: Reductant-independent ATP hydrolysis and the effect of pH on the efficiency of coupling of ATP hydrolysis to substrate reduction. *FEBS Letters*, *110*(1), 35–38. https://doi.org/10.1016/0014-5793(80)80016-0

Kaplan, A., & Reinhold, L. (1999). CO_2 concentrating mechanisms in photosynthetic microorganisms. *Annual Review of Plant Physiology and Plant Molecular Biology*, *50*(1), 539–570. https://doi.org/10.1146/annurev.arplant.50.1.539

Kranz, S. A., Eichner, M., & Rost, B. (2011). Interactions between CCM and N_2 fixation in *Trichodesmium*. *Photosynthesis Research*, *109*(1–3), 73–84. https://doi.org/10.1007/s11120-010-9611-3

Kranz, S. A., Sültemeyer, D., Richter, K. U., & Rost, B. (2009). Carbon acquisition by *Trichodesmium*: The effect of pCO_2 and diurnal changes. *Limnology & Oceanography*, *54*(2), 548–559.

Levitan, O., Rosenberg, G., Setlik, I., Setlikova, E., Grigel, J., Klepetar, J., et al. (2007). Elevated CO_2 enhances nitrogen fixation and growth in the marine cyanobacterium *Trichodesmium*. *Global Change Biology*, *13*(2), 531–538. https://doi.org/10.1111/j.1365-2486.2006.01314.x

Lloyd, M. K., McClelland, H. L. O., Antler, G., Bradley, A. S., Halevy, I., Junium, C. K., et al. (2020). The isotopic imprint of life on an evolving planet. *Space Science Reviews*, *216*(7), 112. https://doi.org/10.1007/s11214-020-00730-6

Lu, Y., Wen, Z., Shi, D., Chen, M., Zhang, Y., Bonnet, S., et al. (2018). Effect of light on N_2 fixation and net nitrogen release of *Trichodesmium* in a field study. *Biogeosciences*, *15*(1), 1–12. https://doi.org/10.5194/bg-15-1-2018

McRose, D. L., Lee, A., Kopf, S. H., Baars, O., Kraepiel, A. M. L., Sigman, D. M., et al. (2019). Effect of iron limitation on the isotopic composition of cellular and released fixed nitrogen in *Azotobacter vinelandii*. *Geochimica et Cosmochimica Acta*, *244*, 12–23. https://doi.org/10.1016/j.gca.2018.09.023

Miller, R. W., & Eady, R. R. (1988). Molybdenum and vanadium nitrogenases of *Azotobacter Chroococcum*: Low temperature favors N_2 reduction by vanadium nitrogenase. *Biochemical Journal*, *256*(2), 429–432. https://doi.org/10.1042/bj2560429

Minagawa, M., & Wada, E. (1986). Nitrogen isotope ratios of red tide organisms in the east China Sea: A characterization of biological nitrogen fixation. *Marine Chemistry*, *19*(3), 245–259. https://doi.org/10.1016/0304-4203(86)90026-5

Mohr, W., Großkopf, T., Wallace, D. W., & LaRoche, J. (2010). Methodological underestimation of oceanic nitrogen fixation rates. *PLoS One*, *5*(9), e12583. https://doi.org/10.1371/journal.pone.0012583

Montoya, J. P. (2008). Chapter 29 - nitrogen stable isotopes in marine environments. In D. G. Capone, D. A. Bronk, M. R. Mulholland, & E. J. Carpenter (Eds.), *Nitrogen in the marine environment* (2nd ed., pp. 1277–1302). Academic Press.

Montoya, J. P., Voss, M., Kähler, P., & Capone, D. G. (1996). A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. *Applied and Environmental Microbiology*, *62*(3), 986–993. https://doi.org/10.1128/aem.62.3.986-993.1996

Pham, D. N., & Burgess, B. K. (1993). Nitrogenase reactivity: Effects of pH on substrate reduction and CO inhibition. *Biochemistry-Us*, *32*(49), 13725–13731. https://doi.org/10.1021/bi00212a043

Pierrot, D., Wallace, D. W. R., Lewis, E. R., Pierrot, D., Wallace, R., Wallace, D. W. R., & Wallace, W. E. (2011). MS excel program developed for CO2 system calculations.

Rowell, P., James, W., Smith, W. L., Handley, L. L., & S, C. M. (1998). ^{15}N discrimination in molybdenum- and vanaduim-grown N_2-fixing *Anabaena variabilis* and *azotobacter vinelandii*. *Soil Biology and Biochemistry*, *31*(14), 2177–2180.

Schneider, K., Müller, A., Krahn, E., Hagen, W. R., Wassink, H., & Knüttel, K. H. (1995). The molybdenum nitrogenase from wild-type Xanthobacter autotrophicus exhibits properties reminiscent of alternative nitrogenases. *European Journal of Biochemistry*, *230*(2), 666–675. https://doi.org/10.1111/j.1432-1033.1995.tb20608.x

Shen, Y., Pinti, D. L., & Hashizume, K. (2006). Biogeochemical cycles of sulfur and nitrogen in the Archean ocean and atmosphere, in *Archean geodynamics and environments* (pp. 305–320).

Shi, D., Kranz, S. A., Kim, J.-M., & Morel, F. M. M. (2012). Ocean acidification slows nitrogen fixation and growth in the dominant diazotroph *Trichodesmium* under low-iron conditions. *Proceedings of the National Academy of Sciences of the United States of America* (Vol. 109(45), pp. E3094–E3100). https://doi.org/10.1073/pnas.1216012109

Shi, D., & Wen, Z. (2024). Effects of CO_2 on diazotroph biomass $\delta^{15}N$ [Dataset]. *Figshare*. https://doi.org/10.6084/m9.figshare.25709310.v2

Shi, D., Xu, Y., & Morel, F. M. M. (2009). Effects of the pH/CO_2 control method on medium chemistry and phytoplankton growth. *Biogeosciences*, *6*(7), 1199–1207. https://doi.org/10.5194/bg-6-1199-2009

Sigman, D. M., DiFiore, P. J., Hain, M. P., Deutsch, C., Wang, Y., Karl, D. M., et al. (2009). The dual isotopes of deep nitrate as a constraint on the cycle and budget of oceanic fixed nitrogen. *Deep-Sea Research, Part A: Oceanographic Research Papers I*, *56*(9), 1419–1439. https://doi.org/10.1016/j.dsr.2009.04.007

Silverman, S. N., Kopf, S. H., Bebout, B. M., Gordon, R., & Som, S. M. (2019). Morphological and isotopic changes of heterocystous cyanobacteria in response to N_2 partial pressure. *Geobiology*, *17*(1), 60–75. https://doi.org/10.1111/gbi.12312

Stueken, E. E., Buick, R., Guy, B. M., & Koehler, M. C. (2015). Isotopic evidence for biological nitrogen fixation by molybdenum-nitrogenase from 3.2 Gyr. *Nature*, *520*(7549), 666–669. https://doi.org/10.1038/nature14180

Sunda, W. G., Price, N. M., & Morel, F. M. M. (2005). Trace metal ion buffers and their use in culture studies, In *Algal culturing techniques* (pp. 35–63). Academic Press.

Yang, Z.-Y., Khadka, N., Lukoyanov, D., Hoffman, B. M., Dean, D. R., & Seefeldt, L. C. (2013). On reversible H_2 loss upon N_2 binding to FeMo-cofactor of nitrogenase. *Proceedings of the National Academy of Sciences of the United States of America* (Vol. 110(41), pp. 16327–16332). https://doi.org/10.1073/pnas.1315852110

Zehr, J. P., & Kudela, R. M. (2011). Nitrogen cycle of the open ocean: From genes to ecosystems. *Annual Review of Marine Science*, *3*(1), 197–225. https://doi.org/10.1146/annurev-marine-120709-142819

Zerkle, A. L., Junium, C. K., Canfield, D. E., & House, C. H. (2008). Production of ^{15}N-depleted biomass during cyanobacterial N_2-fixation at high Fe concentrations. *Journal of Geophysical Research*, *113*(G3). https://doi.org/10.1029/2007jg000651

Zhang, F., Hong, H., Kranz, S. A., Shen, R., Lin, W., & Shi, D. (2019). Proteomic responses to ocean acidification of the marine diazotroph *Trichodesmium* under iron-replete and iron-limited conditions. *Photosynthesis Research*, *142*(1), 17–34. https://doi.org/10.1007/s11120-019-00643-8

Zhang, H., & Byrne, R. H. (1996). Spectrophotometric pH measurements of surface seawater at in-situ conditions: Absorbance and protonation behavior of thymol blue. *Marine Chemistry*, *52*(1), 17–25. https://doi.org/10.1016/0304-4203(95)00076-3

Zhang, X., Sigman, D. M., Morel, F. M. M., & Kraepiel, A. M. L. (2014). Nitrogen isotope fractionation by alternative nitrogenases and past ocean anoxia. *Proceedings of the National Academy of Sciences of the United States of America* (Vol. 111(13), pp. 4782–4787). https://doi.org/10.1073/pnas.1402976111