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Effects of CO_2 on the nitrogen isotopic composition of *Trichodesmium* and *Crocosphaera*

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Abstract

Biological nitrogen (N_2) fixation is the main input of fixed nitrogen to ecosystems on Earth. Nitrogen isotope fractionation during this process is a key parameter for understanding the nitrogen cycle, however, relatively little is known about its regulatory mechanisms. Here we examine the effects of varying CO_2 concentrations on biomass $\delta^{15}N$ signatures of the cyanobacterial diazotrophs *Trichodesmium erythraeum* and *Crocosphaera watsonii*. We show that these organisms produce biomass up to ~3‰ lower in $\delta^{15}N$ under either decreased (~180 µatm) or elevated (~1400 µatm) CO_2 concentrations in comparison to modern levels (~380 µatm). Our results pointed towards changes in nitrogenase enzyme efficiency in response to CO_2 perturbations impacting isotopic fractionation during N_2 fixation and thus the biomass $\delta^{15}N$. This study contributes to an improved interpretation of the observed fluctuations in the $\delta^{15}N$ records, and thus the past nitrogen cycle on Earth.

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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 (Stueken et al., 2015; Gruber and Galloway, 2008; Altabet, 2007). 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, leaving the marine nitrate (NO_3^-) pool and resulting biomass relatively enriched in ^{15}N (Casciotti, 2016; Brunner et al., 2013). 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 (Lloyd et al., 2020; Casciotti, 2016).

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 (ε_{fix}^{Mo} is ~+2‰) (Carpenter et al., 1997; Bauersachs et al., 2009; Minagawa and Wada, 1986). Researchers have therefore often adopted a $\delta^{15}N$ value of ~ -1‰ for biomass produced by diazotrophs (Brandes and 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 2.7 billion years (Gyr) (Lloyd

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et al., 2020). However, the presence of extremely negative bulk $\delta^{15}N$ values (<~-2‰, can low to -7‰) in the Cretaceous Oceanic Anoxic Events (OAEs, 145–66 Myr) and the early Archean (>3.2 Gyr ago), which are not observed in modern marine sediments (Altabet and Francois, 1994; Altabet, 2007; Shen et al., 2006), challenges the current understanding of the marine nitrogen isotope budget (Brandes and Devol, 2002).

Until recently, 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, *Anabaena*, a filamentous cyanobacterium, 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), mainly 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 revealed that higher pressures resulted in significantly lighter $\delta^{15}N$ biomass, although the difference was relatively minor (less than 0.5‰) (Silverman et al., 2019). Additionally, a critical discovery potentially explaining the extremely low $\delta^{15}N$ values in ancient sediments was the use of vanadium (V-) and Fe-only alternative nitrogenases, which yield significantly lower $\delta^{15}N$ biomass (-6 to -7‰) (Rowell et al., 1998; Zhang et al., 2014). The mechanism by which the use of alternative nitrogenase enzymes alters isotope fractionation remains unclear. Given the lower efficiency of the V- and Fe-only nitrogenases compared to the Mo-nitrogenases (Eady, 1996; Miller and Eady, 1988),

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it is possible that the commitment to N_2 catalysis is decreased due to 1) a decline in N_2 reduction efficiency post-binding to the active site; 2) or an increased competition at the active site between N_2 and H_2 (Guth and 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 pCO_2 above the modern levels (~380 µatm *vs.* ~800 µatm) significantly reduces the efficiency of Mo-nitrogenase in the filamentous diazotrophic cyanobacterium *Trichodesmium* (Shi et al., 2012; Hong et al., 2017; Zhang et al., 2019; Zhang et al., 2022). 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 (Shi et al., 2012; Pham and Burgess, 1993). Should this phenomenon be consistent across all diazotroph species and nitrogenase types, past fluctuations in atmospheric CO_2 [which exceeded 1500 ppm prior to 420 Myr, dropping to pre-industrial levels (ca. 280 ppm) after ~20 Myr (Fig. S1)] might have notably impacted nitrogenase efficiency, and consequently, the isotopic fractionation during N_2 fixation. Here we examine the effect of changing CO_2 concentrations on biomass $\delta^{15}N$ of model cyanobacterial diazotrophs *Trichodesmium erythraeum* IMS101 and *Crocosphaera watsonii* WH8501. We present the first evidence that changes in CO_2 levels significantly alter the biomass

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$\delta^{15}N$ 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. Methods

2.1 Bacterial strains and culture conditions

The marine cyanobacterium *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 (cation exchange resin) 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 unialgal and grown under five pCO_2/pH (~180 to 1400 µatm, pH ~7.5 to 8.3) conditions at 27°C and 80 µmol photons m^{-2} s^{-1} (14 h: 10 h light–dark cycle) in an AL-41L4 algae chamber (Percival). The experimental pCO_2/pH was chosen to fall within the range in the last 420 million years (Fig. S1) (Foster et al., 2017). All experiments were carried out with three biological replicates. Sterile techniques were applied for culturing and experimental manipulations. 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 and supplementary data.

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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 *Trichodesmium* and every two days for *Crocosphaera*, using a spectrophotometric method (Zhang and Byrne, 1996) and each manipulated pH remained stable throughout the experimental period (Fig. S2). 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 *Trichodesmium* was monitored by measuring Chl*a* concentrations daily, while the growth of *Crocosphaera* was monitored through the measurement of cell abundance every two days. *Trichodesmium* Chl*a* concentration was determined via extraction in 90% methanol, followed by analysis using a spectrophotometer according to De Marsac and Houmard (1988). *Crocosphaera* 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 *vs.* time for the exponential growth phase and four data points were included in each growth curve.

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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). Briefly, $^{15}N_2$ enriched water was prepared according to Wen et al. (2022) by fully dissolving 5 mL 98% pure $^{15}N_2$ gas (Cambridge Isotope Laboratories) 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 at the same culture conditions for 2 h. The measurement was conducted at the midpoint of the light period and dark period, respectively, for *Trichodesmium* and *Crocosphaera*. After incubation, diazotrophs were filtered onto 25 mm pre-combusted GF/F filters (Millipore). Diazotrophs were collected by filtration concurrently with the N_2 fixation rate measurement, but not enriched with $^{15}N_2$, to determine the natural biomass $\delta^{15}N$ signal. All filter samples were then dried and analyzed using a Thermo Scientific Flash 2000 HT elemental analyzer coupled with a Thermo Finnigan Deltaplus Advantage isotope ratio mass spectrometer. The N_2 fixation rate was calculated according to Montoya et al. (1996).

2.5 Quantification of *NifH* proteins

Trichodesmium and *Crocosphaera* cells were collected onto polycarbonate membrane filters (Millipore) 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

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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 h. Then, the membrane was incubated for 1 h with primary antibodies (Agrisera: NifH, Art no. AS01 021S), followed by 1 h 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.

3. Results and discussion

3.1 Growth and N_2 fixation rates response to CO_2 perturbations

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In our study, *Trichodesmium* and *Crocosphaera* were cultivated under a range of pCO_2/pH conditions, with pCO_2 varying from approximately ~180 to 1400 µatm and 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 (Fig. S1), as outlined in Foster et al. (2017). N_2 served as the sole nitrogen source for these organisms. 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 (Fig. 1). 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.

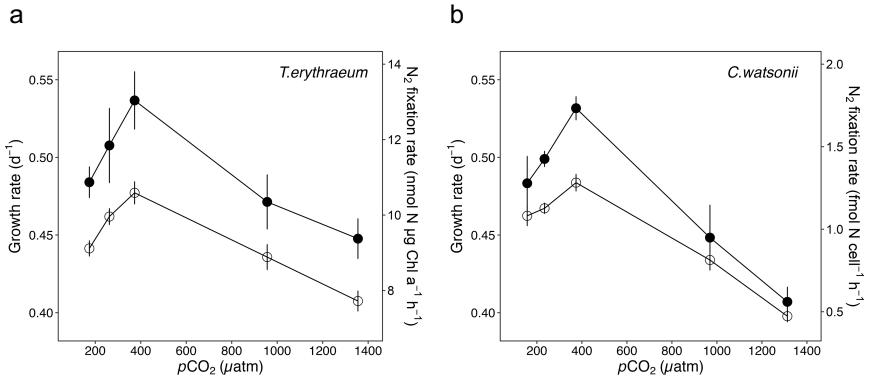

Figure 1. Growth and N_2 fixation rates of *T. erythraeum* (a) and *C. wastonii* (b) in response to CO_2 perturbations. Filled dots stand for growth rate; The open circles are N_2 fixation rates. Note that N_2 fixation rates of *Trichodesmium* and *Crocosphaera* are measured in different units. The values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

The response of phytoplankton to CO_2 perturbations lies in the simultaneous changes in pCO_2 and pH, which can have conflicting effects on their physiology and growth.

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For instance, the C-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 concentration 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 (Kranz et al., 2011; Badger et al., 2006). Conversely, the concurrent decrease in seawater pH may significantly 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 *Trichodesmium* 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 elevated CO_2 levels from ~180 to 1400 µatm may decrease the cytosolic pH of both species to levels of ~7.3 to 6.5, with two lowest pHs (CO_2 levels ~1000 and 1400 µatm) deviating from the optimal pH range (7–8) for nitrogenase activity (Hadfield and Bulen, 1969; Imam and Eady, 1980; Schneider et al., 1995; Pham and Burgess, 1993). Therefore, the observed increases in growth and N_2 fixation rates from ~180 µatm to modern CO_2 levels of 400 µatm (Fig. 1) indicate an overall dominance of beneficial effects from elevated pCO_2, with minimal impact from 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 beyond the modern levels (~1000 and 1400 µatm, Fig. 1) reflects the detrimental impacts of reduced pH,

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which outweigh the positive effects of elevated pCO_2.

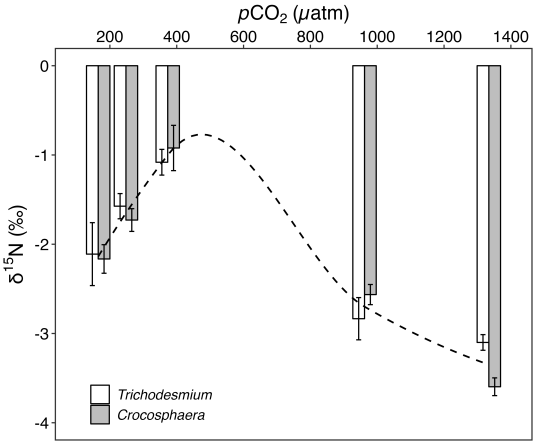

Figure 2. Biomass $\delta^{15}N$ values of *T. erythraeum* and *C. wastonii* produced when grown in variable CO_2 concentrations. White, *Trichodesmium*; Grey, *Crocosphaera*. The values of biomass $\delta^{15}N$ are presented as mean ± SD (n = 3). The dashed line is the overall trend of biomass $\delta^{15}N$ of both species in response to CO_2 perturbations.

3.2 Biomass $\delta^{15}N$ responses to CO_2 perturbations

The biomass $\delta^{15}N$ of both *Trichodesmium* and *Crocosphaera* 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 (Fig. 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 decrease, results in a significant reduction ($p < 0.01$, determined by one-way ANOVA) in biomass $\delta^{15}N$. Specifically, at ~1400 µatm pCO_2, the

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biomass $\delta^{15}N$ decreased by a maximum of 2.0‰ for *Trichodesmium* and 2.7‰ for *Crocosphaera*.

Our study shows for the first time that CO_2 exerts important controls on the N isotopic composition of diazotrophic biomass. Additionally, we observed that both growth and N_2 fixation rates were significantly positively correlated with biomass $\delta^{15}N$ (R^2 for both species are higher than 0.9, $p < 0.05$, Fig. S3). The mechanisms behind these strong correlations remain unclear. Regarding the mechanism behind the ocean acidification effect on *Trichodesmium*, previous research has indicated that a decrease in nitrogenase efficiency at elevated pCO_2 levels above 400 µatm, is a primary factor leading to reduced growth and N_2 fixation rates (Hong et al., 2017; Shi et al., 2012). Additionally, it has been inferred that changes in nitrogenase efficiency can also impact isotopic fractionation during N_2 fixation. For instance, the lower efficiency of alternative V- and Fe-only nitrogenases compared to the canonical Mo-nitrogenase (Miller and Eady, 1988; Eady, 1996) is hypothesized to result in a more pronounced isotopic effect during N_2 fixation, thereby leading to more negative $\delta^{15}N$ values in biomass (Zhang et al., 2014). We thus hypothesize that the correlations of growth and N_2 fixation rates with biomass $\delta^{15}N$ in our study, are mediated by nitrogenase efficiency under CO_2 perturbation.

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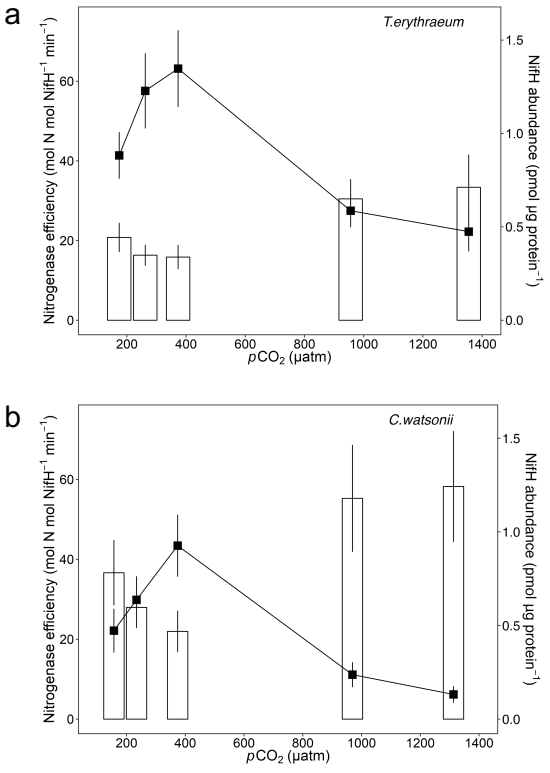

Figure 3. Nitrogenase efficiency of *T. erythraeum* (a) and *C. wastonii* (b) in response to CO_2 perturbations. The filled squares stand for the nitrogenase efficiency; The hollow bars are nitrogenase reductase (NifH) protein abundances. Data shows the mean ± standard deviation of n = 3 biological replicates.

We therefore calculated the nitrogenase efficiency of *Trichodesmium* and *Crocosphaera* by normalizing $^{15}N_2$-based N_2 fixation rates to the abundance of nitrogenase reductase (NifH) protein. In alignment with previous finding, 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, Fig. 3). This reduction was attributed to a decrease in N_2 fixation rates coupled with an increase in NifH protein production (Figs. 1 and 3). We

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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, Fig. 3). As discussed above, under low CO_2 concentrations (< 380 µatm), *Trichodesmium* and *Crocosphaera* will need to invest substantial resources and energy into CCMs to enhance the efficiency of RubisCO carboxylation, thereby reducing the risk of carbon limitation (Giordano et al., 2005; Badger et al., 2006; Kaplan and Reinhold, 1999). Enhanced expression of CCMs may thus divert energy and resources that would otherwise be allocated to other cellular metabolic pathways, such as N_2 fixation (Levitan et al., 2007; Kranz et al., 2009; Barcelos E Ramos et al., 2007). We therefore hypothesize that CO_2 limitation may reduce energy and reductant supply to nitrogenase and decrease the enzyme efficiency, though the mechanism remained unsolved. Irrespective of the specific mechanisms driving variations in nitrogenase efficiency, we found significant positive correlations between CO_2-controlled nitrogenase efficiency and biomass $\delta^{15}N$ ($R^2 = 0.99$, $p < 0.001$ and $R^2 = 0.93$, $p = 0.007$ for *Trichodesmium* and *Crocosphaera* respectively, Fig. 4). Our results therefore point towards the potential roles of nitrogenase efficiency in regulating the kinetic isotopic effect of N_2 fixation.

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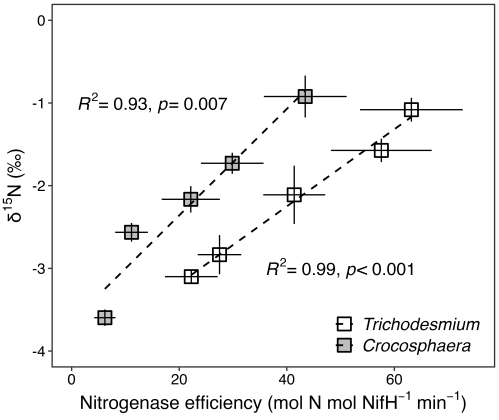

Figure 4. Relationships between nitrogenase efficiency and biomass $\delta^{15}N$. Open squares, *Trichodesmium*; Grey squares, *Crocosphaera*. Data shows the mean ± standard deviation of n = 3 biological replicates.

4. Conclusion and implications

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 $\delta^{15}N$ values preserved in ancient organic matter found in sediments.

During glacial/interglacial cycles over the past 800,000 years (as illustrated in Fig. S1), the periodic presence of lighter sediment $\delta^{15}N$ [as low as ~2–4‰ in the (sub)tropical regions] during interglacial periods has been previously associated with

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changes in upper water column structure, sea level, and ocean circulation, which lead to the import of 'excess' phosphorus into the ocean surface and consequently enhance N_2 fixation (Ren et al., 2009; Straub et al., 2013; Li et al., 2019). If our short-term experimental observations are indicative of diazotroph growth patterns in the geological past, it could be inferred that the elevated CO_2 levels during the interglacial periods (rising from ~180 µatm to 300 µatm) might have resulted in 0.5 to 1‰ lesser isotopic effect of Mo-nitrogenase and thus isotopically heavier N biomass by cyanobacterial diazotrophs. This suggests that isotope-based study into the contribution of N_2 fixation to the overall N inputs during glacial/interglacial periods needs to take into account the impact of environmental changes on the isotopic fractionation of N_2 fixation. Furthermore, prior to the Miocene epoch (> 20 million years ago, as shown in Fig. S1), elevated CO_2 (up to ~ 5000 µatm at 201 Myr) above modern levels (~380 µatm) (Hönisch et al., 2012) might have also led to the production of isotopically lighter biomass. This could offer additional insights into the anomalously low $\delta^{15}N$ values (< –2‰) observed in sediments from the Oceanic Anoxic Events of the Cretaceous period (145–66 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 $\delta^{15}N$ sediment values (Lloyd et al., 2020).

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Data availability. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplement. Additional data associated with the paper are available from the corresponding authors upon request.

Author contributions. DS and HH designed the research. TH and ZW performed the experiments. RJ, ZW, TH and DS analyzed the data. ZW, RJ, TJB and DS wrote the manuscript. All authors discussed the results and commented and edited the manuscript.

Competing interests. The authors declare that they have no conflict of interests.

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