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Changes in isotope fractionation during nitrate assimilation by marine eukaryotic and prokaryotic algae under different pH and CO_2 conditions

Yawen Chen,[1] Jin-Yu Terence Yang ,[1*] Jin-Ming Tang ,[1] Haizheng Hong,[2] Shuh-Ji Kao ,[1,3] Minhan Dai,[1] Dalin Shi [1*]

[1]State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen, China

[2]State Key Laboratory of Marine Environmental Science, College of the Environment and Ecology, Xiamen University, Xiamen, China

[3]State Key Laboratory of Marine Resource Utilization in South China Sea, School of Marine Science and Engineering, Hainan University, Haikou, China

Abstract

The impact of environmental factors on nitrogen (N) and oxygen (O) isotope effects during algal nitrate assimilation causes uncertainty in the field application of sedimentary N isotope records and nitrate isotopes to understand the marine nitrogen cycle. Ocean acidification is predicted to change nitrogen cycling including nitrate assimilation, but how N and O isotope effects during algal nitrate assimilation vary in response to changes in seawater pH and partial pressure CO_2 (pCO_2) remains unknown. We measured N and O isotope effects during nitrate assimilation and physiological states of the marine diatom *Thalassiosira weissflogii* and *Synechococcus* under different pH (8.1 or 7.8) and pCO_2 (400 or 800 μatm) conditions. Low pH and/or high pCO_2 equally decreased N and O isotope effects during nitrate assimilation by diatoms possibly due to reducing cellular nitrate efflux/uptake ratio and decreased isotope effects for nitrate uptake, whereas they did not affect those by *Synechococcus* with low intracellular nitrate concentration and limited nitrate efflux. Our results provide compelling experimental evidence showing different changes in N and O isotope effects during nitrate assimilation by marine eukaryotic and prokaryotic phytoplankton at low pH and/or high pCO_2. These findings suggest new insight into environmental controls on variability in the isotope effect during algal nitrate assimilation, and have implications for improving a predictive understanding of N and O isotope tools in acidified oceans.

Nitrogen (N) isotopic compositions of oceanic nitrate and bulk sediments are powerful indicators for evaluating surface nitrate utilization in the modern and past oceans, facilitating the understanding of spatial and temporal changes in the strength of the biological pump (Altabet and Francois 1994; Robinson et al. 2020). The key to the application of stable N isotope tools is to understand the magnitude of the isotope effect (i.e., a phenomenon associated with the isotopes of a given element exhibiting different rates during reactions, which is reported as $\varepsilon = ({}^{light}k/{}^{heavy}k - 1) \times 1000$, expressed in ‰, in which k refers to the specific reaction rate constants of light and heavy isotopes) for nitrate assimilation. On the other hand, culture studies suggest that nitrate consumption via algal nitrate assimilation and marine heterotrophic denitrification fractionates N and O isotopes of nitrate to a similar extent (i.e., N and O isotope effects are comparable), resulting in equal increases in nitrate N and O isotopic composition (Granger et al. 2008, 2010). This pattern of nitrate N and O isotopes varying in unison has also been observed in natural seawaters where nitrate consumption is dominated (DiFiore et al. 2009; Fawcett et al. 2015; Rafter and Sigman 2016). In contrast to nitrate consumption, nitrate production via nitrification has different effects on the dual isotopes of nitrate; the N isotopic composition of the newly nitrified nitrate is dependent on the source of N being nitrified, whereas its O isotopic composition is mainly set by incorporation of the O isotopic signals from the ambient water (Casciotti et al. 2002). This difference generally leads to environmental deviations in changes in O vs. N isotopic composition of nitrate from a trajectory of 1 expected for the nitrate-consuming processes

*Correspondence: jyyang@xmu.edu.cn; dshi@xmu.edu.cn

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

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aforementioned. The signature with consistent N and O isotope effects during nitrate consumption revealed from culture- and field-based studies provides a benchmark for using coupled nitrate N and O isotope measurements to disentangle otherwise overprinting processes that commonly co-occur in natural oceanic environments (e.g., Wankel et al. 2007; Granger and Wankel 2016; Buchwald et al. 2018).

Understanding how the changing environmental factors impact the magnitude and ratio of N and O isotope effects for nitrate assimilation by phytoplankton could reduce uncertainties in the environmental applications of isotope tracers. Culture experiments have proven advances in providing a mechanistic understanding of the isotope effect for nitrate assimilation. For instance, prior studies have investigated the potential changes in the N isotope effect for nitrate assimilation under different culture conditions, showing that the N isotope effect for nitrate assimilation increased significantly under light limitation and remained constant under phosphate-, temperature-, and iron-limited conditions (Needoba et al. 2004; Karsh 2014). These results suggest that environmental factors other than irradiance have insignificant influences on the N isotope effect for nitrate assimilation. However, there is little understanding of how the O isotope effect for nitrate assimilation changes with environmental factors. Whether the environmental changes would influence the invariant coupling of N and O isotope effects during nitrate assimilation observed in phytoplankton cultures remains equivocal, which requires better evaluation for accurate interpretation of environmental nitrate isotope distributions.

Ocean acidification caused by anthropogenic CO_2 input, referring to an increase in partial pressure CO_2 (pCO_2) and a concomitant decrease in the pH of seawater, has been suggested to have appreciable effects on marine biogeochemistry and pose a threat to ocean ecosystems (Doney et al. 2012). Among them, the responses of the marine N cycle to ocean acidification that are related to shaping the nitrogen pools and their biogeochemical cycles have been receiving extensive attention (Wannicke et al. 2018). However, little is known about how nitrate assimilation responds to ocean acidification. Limited studies have shown that the responses of nitrate assimilation to ocean acidification may vary among phytoplankton species. For example, acidification slightly reduced the nitrate uptake rates of the marine diatoms (Shi et al. 2015), but promoted those of the dinoflagellate *Amphidinium carterae* (Bausch et al. 2019). The changing physiology associated with nitrate assimilation by phytoplankton under ocean acidification may imply changes in its isotope fractionation. The marine eukaryotic and prokaryotic phytoplankton possess different pathways of nitrate assimilation, such as different nitrate transporters (NRT) and reductases (Granger et al. 2010), possibly resulting in different responses of the isotope effect for nitrate assimilation under ocean acidification. Additionally, the rising pCO_2 and reducing pH may give rise to different impacts on marine N cycling processes and algal physiology. For example, Hong et al. (2017) found that growth and N_2 fixation rates of the cyanobacterium *Trichodesmium* decreased under low pH conditions, despite a positive effect of high pCO_2. Similarly, increasing pCO_2 for diatoms down-regulated the CO_2-concentrating mechanism to save energy for growth, while reducing pH increased the photosynthetic carbon fixation rate of diatoms (Shi et al. 2019). Identifying the interactive effects of low pH and high pCO_2 would help the mechanistic understanding of the complex responses of algal nitrate assimilation to ocean acidification. Therefore, it is worth probing how the N and O isotope effects during nitrate assimilation by different phytoplankton species change at low pH and/or high pCO_2.

In this study, the eukaryotic marine diatom *T. weissflogii* and the prokaryotic *Synechococcus* were cultured under four different pH-pCO_2 conditions to differentiate the effects of decreased pH and increased pCO_2. These two ubiquitous pelagic phytoplankton strains are widely used to study the mechanisms (including intracellular nitrate reduction, nitrate uptake into cells, and nitrate efflux from cells) of isotope fractionation during nitrate assimilation (e.g., Granger et al. 2010; Karsh et al. 2014). The main goal of this study is to comparatively assess the interactive effects of decreased pH and elevated pCO_2 on N and O isotope effects during nitrate assimilation for cultures of eukaryotic and prokaryotic strains. Furthermore, combined with their physiological responses to ocean acidification, we discuss the major mechanisms for any changes in N and O isotope effects for algal nitrate assimilation induced by reducing pH and/or elevating pCO_2 based on culture observations and highlight the implications for field understandings of N and O isotope signals in acidified oceans.

Materials and methods

Algal culturing and experimental design

Axenic cultures of *T. weissflogii* (CCMP1336) and *Synechococcus* sp. CCMP2370 (Provasoli-Guillard National Center for Marine Algae and Microbiota, USA) were grown semi-continuously in the filtered and sterilized synthetic ocean water medium Aquil* under continuous light at 20°C in an AL-41 L4 algal chamber (Percival Scientific, USA) (Sunda et al. 2005). The light intensities were 100 and 45 μmol photons m^{-2} s^{-1} for *T. weissflogii* and *Synechococcus*, respectively. The irradiance levels used are characteristic of those prevailing in the lower euphotic zone and thus relevant to natural environments such as nitracline with nitrate utilization. Nitrate was the only N source, and its concentrations are shown in Supporting Information Table S1. The carbonate chemistry of the medium was manipulated by adding different amounts of $NaHCO_3^-$ with 2 mmol L^{-1} 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid (Sigma), to obtain the targeted pH and pCO_2 levels, i.e., ambient (pH 8.1, pCO_2 400 μatm), carbonated (pH 8.1, pCO_2 800 μatm), acidified (pH 7.8, pCO_2 400 μatm), and ocean acidification (pH 7.8, pCO_2 800 μatm) conditions (Supporting Information Text S1; Table S2; Shi et al. 2019). We implemented sterile

culture techniques throughout the experiments to avoid bacterial contamination.

After being preacclimated to the experimental conditions for more than 100 generations (corresponding to ~ 186 days for *T. weissflogii* and ~ 137 days for *Synechococcus*), triplicate cultures of algae were harvested at ~ 4 days for *T. weissflogii* and at ~ 7 days for *Synechococcus* in the mid-exponential phase. Cell numbers and volumes of the diatom and *Synechococcus* were measured daily with a Z2 Coulter Counter and a Multisizer 4e Coulter Counter (Beckman Coulter), respectively. Their specific growth rates were determined by linear regression of the natural logarithm of cell numbers vs. time.

Nitrate reductase activity and short-term nitrate uptake rate

Nitrate reductase (NR) activity was determined by the spectrophotometric method (Shi et al. 2015). The short-term nitrate uptake rate was measured by the N isotope labelling method (Shi et al. 2015). Detailed descriptions of these methods are shown in Supporting Information Text S2 and S3.

Dissolved organic nitrogen (DON), and intracellular and medium nitrate

The cultures were filtered through polycarbonate membranes (1.2 μm pore size for *T. weissflogii* and 0.4 μm pore size for *Synechococcus*) and the filtrate was collected for measuring medium nitrate and DON. Medium nitrate was determined using a nitrogen oxide (NO_x) chemiluminescence analyzer (Teledyne) with a detection limit of 10 nmol L^{-1} (Fawcett et al. 2015). DON was converted into nitrate using the "persulfate oxidation" method and estimated by subtracting medium nitrate (Knapp et al. 2005). The intracellular nitrate was extracted using an ultrasonication method. The cells on membranes were eluted with a fixed volume of Milli-Q water (1.1 mL for *T. weissflogii* and 4 mL for *Synechococcus*) and disrupted using the Bioruptor (UCD-200, Diagenode), a non-contact sonicator, followed by centrifugation for 5 min. We subsequently measured nitrate in the 1 mL supernatant with a NO_x analyzer and calculated intracellular nitrate concentration accordingly. Considering that the presence of nitrite in the culture system may influence the calculation of the isotope effect for nitrate assimilation (Knöller et al. 2011), intracellular nitrite was measured but was not detectable (i.e., < 10^{-4} fmol $cell^{-1}$). In addition, we randomly monitored the presence of nitrite using the Griess-Ilosvay reagent during the cultures (Becker et al. 2020), and found that medium nitrite was not accumulated in significant concentrations (< ~0.5 μmol L^{-1}). These results suggested that nitrite was reduced efficiently in the algal cells during the experiments. Likewise, previous culture studies on isotope fractionation during nitrate assimilation by *T. weissflogii* and *Synechococcus* under similar culture conditions to our study have shown that nitrite concentration is generally close to the detection limit in the medium and intracellular samples (e.g., Needoba et al. 2004; Granger et al. 2010; Karsh et al. 2014). Nitrite was thus not removed from samples before concentration and isotopic measurements.

Isotope analysis

Particle samples from cultures and uptake incubation were collected onto precombusted GF/F filters. The N content and isotope composition on particles were measured using the continuous flow elemental analyzer (Vario PYRO Cube)—isotope ratio mass spectrometer (Isoprime 100) system (EA-IRMS). Three international N isotope standards, including USGS40, USGS41, and IAEA-600, were used for calibration.

N and O isotope composition of nitrate was determined by the denitrifier method (Sigman et al. 2001; Casciotti et al. 2002). Nitrate was quantitatively reduced to nitrous oxide (N_2O) by denitrifying bacteria lacking N_2O reductase. The N and O isotope ratios of the N_2O gas were then measured using a GasBench II connected to an IRMS (Thermo Scientific DELTA V advantage). Isotope ratios are reported using the delta (δ) notation expressed in per mil (‰):

$$\delta^{15}N_{sample}\,(‰ vs.\,air) = \left[\left({}^{15}N/{}^{14}N\right)_{sample}/\left({}^{15}N/{}^{14}N\right)_{standard} - 1\right] \times 1000‰ \quad (1)$$

$$\delta^{18}O_{sample}\,(‰ vs.\,VSOMW) = \left[\left({}^{18}O/{}^{16}O\right)_{sample}/\left({}^{18}O/{}^{16}O\right)_{standard} - 1\right] \times 1000‰ \quad (2)$$

where air N_2 is the $^{15}N/^{14}N$ standard and Vienna standard mean water is the $^{18}O/^{16}O$ standard. Sample nitrate $\delta^{15}N$ and $\delta^{18}O$ were calibrated using the international isotope standards, IAEA-N-3 and USGS34. Replicate for individual standards was < 0.2‰ for $\delta^{15}N$ and < 0.5‰ for $\delta^{18}O$.

Isotope effects during nitrate assimilation

To derive the organism-level N and O isotope effects during nitrate assimilation ($^{15}\varepsilon_{org}$ and $^{18}\varepsilon_{org}$; i.e., the net isotope effect expressed in the environment), the Rayleigh Model for a closed system was applied to fit the changes in $\delta^{15}N$ and $\delta^{18}O$ of medium nitrate (Granger et al. 2004):

$$\delta^{15}N_{rem}\left(\text{or}\,\delta^{18}O_{rem}\right) = \delta^{15}N_{ini}\left(\text{or}\,\delta^{18}O_{ini}\right) - {}^{15}\varepsilon_{org}\left(\text{or}\,{}^{18}\varepsilon_{org}\right) \times \ln f \quad (3)$$

where f is the ratio of remaining nitrate to initial nitrate, and $\delta^{15}N_{rem}$ (or $\delta^{18}O_{rem}$) and $\delta^{15}N_{ini}$ (or $\delta^{18}O_{ini}$) are the N (or O) isotope ratios of remaining and initial medium nitrate, respectively.

Statistical analysis

The significance of differences ($p < 0.05$, except as otherwise noted) among four pH-pCO_2 culture conditions was analyzed by one-way ANOVA, followed by Tukey test. The

significant effects of pH and pCO_2 were tested by two-way ANOVA. Different superscript letters indicate data with significant differences among different culture conditions, while the same superscript letter indicates data with no significant difference ($p \geq 0.05$). These statistical analyses were performed by IBM SPSS Statistics 26.0.

Results

Growth rates of *T. weissflogii* and *Synechococcus* sp. CCMP2370 were 1.31 d^{-1} and 0.49 d^{-1}, respectively, and were unchanged at low pH and/or high pCO_2 (Supporting Information Table S3). Both low pH and high pCO_2 reduced the cell volume of *T. weissflogii*. By contrast, low pH increased the cell volume of *Synechococcus* (Supporting Information Table S3). Meanwhile, low pH and high pCO_2 reduced the C and N quotas of *T. weissflogii* by a similar extent, while low pH equally increased the C and N quotas of *Synechococcus*. Their cellular C : N ratios thus remained unchanged at low pH and/or high pCO_2 (Supporting Information Table S3). Intracellular nitrate concentration stored by *T. weissflogii* was ~ 32 fmol $cell^{-1}$, considerably higher than that by *Synechococcus* (0.006 fmol $cell^{-1}$). Intracellular nitrate concentrations of both algae were unaffected under the low pH and/or high pCO_2 conditions (Supporting Information Fig. S1). Low pH slightly though not significantly decreased the nitrate uptake rates of *T. weissflogii*, whereas low pH increased those of *Synechococcus*. Similarly, low pH and high pCO_2 did not significantly change the NR activities in *T. weissflogii*, but remarkably inhibited those in *Synechococcus* (Supporting Information Fig. S1). The $\delta^{15}N$ and $\delta^{18}O$ of the medium nitrate ($\delta^{15}N\text{-}NO_3^-{}_{ex}$ and $\delta^{18}O\text{-}NO_3^-{}_{ex}$) increased concomitantly over time among all treatments, along with increases in N isotope values in particulate N (Supporting Information Figs. S2 and S3). The sum of DON and particulate N was comparable to nitrate consumption (Supporting Information Table S1), suggesting closed systems during our experiments.

Under the ambient condition (pH 8.1, pCO_2 400 μatm), $^{15}\varepsilon_{org}$ for *T. weissflogii* and *Synechococcus* were 7.8‰ and 1.9‰, respectively, and their $^{18}\varepsilon_{org}$ were 8.6‰ and 2.2‰, respectively (Fig. 1; Supporting Information Table S3). We found linear covariations in $\delta^{15}N$ and $\delta^{18}O$ of intracellular and medium nitrate with ratios of ~ 1 (i.e., $\Delta\delta^{18}O\text{-}NO_3^-{}_{ex} : \Delta\delta^{15}N\text{-}NO_3^-{}_{ex}$ of ~1 and $\Delta\delta^{18}O\text{-}NO_3^-{}_{in} : \Delta\delta^{15}N\text{-}NO_3^-{}_{in}$ of ~1) during nitrate assimilation by two algae (Fig. 1; Supporting Information Table S3). Compared with the ambient condition, $^{15}\varepsilon_{org}$ and $^{18}\varepsilon_{org}$ for *T. weissflogii* were concurrently reduced at low pH and/or high pCO_2, whereas those for *Synechococcus* were influenced insignificantly under different pH-pCO_2 conditions. Specifically, $^{15}\varepsilon_{org}$ for *T. weissflogii* decreased from 7.8‰ to 5.1‰, and their $^{18}\varepsilon_{org}$ decreased from 8.6‰ to 5.9‰ under the low pH and high pCO_2 condition (Fig. 1a; Supporting Information Table S3). By contrast, $^{15}\varepsilon_{org}$ and $^{18}\varepsilon_{org}$ for *Synechococcus* remained at ~2‰ in spite of changes in pH and/or pCO_2 (Fig. 1c; Supporting Information Table S3). The effects of low pH and/or high pCO_2 led to consistent changes in $^{18}\varepsilon_{org}$ and $^{15}\varepsilon_{org}$ for two algae. Thus, the $\Delta\delta^{18}O : \Delta\delta^{15}N$ of ~1 in the intracellular and medium nitrate was not changed significantly by decreasing pH and/or increasing pCO_2 (Fig. 1b,d).

Discussion

Responses of isotope fractionation during algal nitrate assimilation to low pH and high pCO_2

The observed $^{15}\varepsilon_{org}$ values for *T. weissflogii* and *Synechococcus* sp. CCMP2370 grown under the ambient condition are within previously reported ranges for *T. weissflogii* (6‰-13‰; Needoba et al. 2003, 2004; Granger et al. 2004) and *Synechococcus* (2‰–6‰; Needoba et al. 2003; Granger et al. 2010) previously obtained under similar culture conditions. Our results support the fact that marine eukaryotic phytoplankton expresses higher $^{15}\varepsilon_{org}$ relative to prokaryotic phytoplankton (Granger et al. 2010). Moreover, we found $\Delta\delta^{18}O\text{-}NO_3^-{}_{ex} : \Delta\delta^{15}N\text{-}NO_3^-{}_{ex}$ of ~ 1 during nitrate assimilation by both the eukaryotic and prokaryotic algae, further confirming the invariant $^{18}\varepsilon_{org} : {}^{15}\varepsilon_{org}$ of ~ 1 as previously reported in laboratory cultures and field studies (DiFiore et al. 2009; Granger et al. 2010; Fawcett et al. 2015). These findings obtained under the ambient condition are consistent with the current view of isotope fractionation during nitrate assimilation by marine phytoplankton.

This study attempts to uncover the interactive effects of reducing pH and rising pCO_2 on $^{15}\varepsilon_{org}$ and $^{18}\varepsilon_{org}$, as well as on their ratio, by marine eukaryotic vs. prokaryotic phytoplankton. Our results suggest different changes in $^{15}\varepsilon_{org}$ and $^{18}\varepsilon_{org}$ by marine eukaryotic and prokaryotic algae under the low pH and high pCO_2 condition, with pronounced changes only by marine eukaryotes due largely to the effect of reducing pH (Fig. 1; Supporting Information Table S4). The different changes in $^{15}\varepsilon_{org}$ observed under the low pH and high pCO_2 condition between eukaryotic and prokaryotic phytoplankton reflect that their regulation of nitrate metabolism is affected in different ways, as indicated by different physiological responses of *T. weissflogii* and *Synechococcus* to low pH and high pCO_2 (Supporting Information Fig. S1; Table S3). These findings differ from the consistent impacts of increased pCO_2 on carbon isotope fractionation during inorganic carbon assimilation by both marine eukaryotic and prokaryotic phytoplankton, which show elevated carbon isotope fractionation during inorganic carbon assimilation at high pCO_2 by increasing the availability of aqueous CO_2 (Gervais and Riebesell 2001; Hurley et al. 2021). However, the algae grown in our experiments was not limited by nitrogen availability because abundant nitrate remained in the medium (Supporting Information Table S1). We thus expect other mechanisms to trigger different changes in $^{15}\varepsilon_{org}$ and $^{18}\varepsilon_{org}$ for the marine eukaryotic and prokaryotic phytoplankton at low pH and high pCO_2.

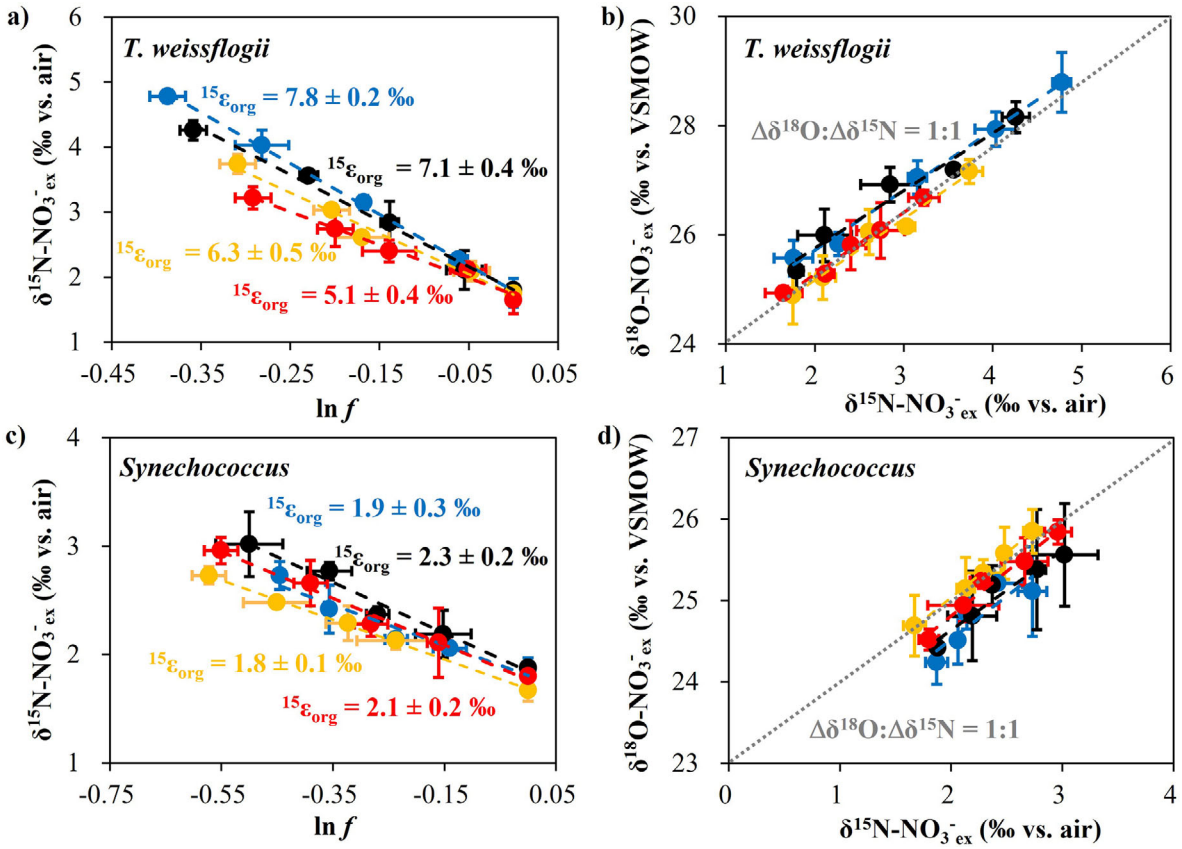

Fig. 1. Plots of $\delta^{15}N$ of medium nitrate ($\delta^{15}N\text{-}NO_3^-{}_{ex}$) vs. the natural log of fractional remaining nitrate (ln*f*) and plots of $\delta^{18}O$ of medium nitrate ($\delta^{18}O\text{-}NO_3^-{}_{ex}$) vs. $\delta^{15}N\text{-}NO_3^-{}_{ex}$ for (**a**, **b**) *T. weissflogii* and (**c**, **d**) *Synechococcus* sp. CCMP2370 under different pH-pCO_2 conditions. Color symbols indicate the ambient (pH 8.1, pCO_2 400 μatm; blue), carbonated (pH 8.1, pCO_2 800 μatm; black), acidified (pH 7.8, pCO_2 400 μatm; orange), and ocean acidification (pH 7.8, pCO_2 800 μatm; red) conditions. In (a, c), the colored dashed line shows the linear regression for each pH-pCO_2 condition, with its slope as $^{15}\varepsilon_{org}$. In (b, d), the colored dashed line shows the linear regression between $\delta^{18}O\text{-}NO_3^-{}_{ex}$ and $\delta^{15}N\text{-}NO_3^-{}_{ex}$ for each pH-pCO_2 condition (Supporting Information Table S3), and the gray dotted lines denote the trajectories for the $\Delta\delta^{18}O : \Delta\delta^{15}N$ of 1 : 1.

Potential mechanisms of changes in ε_{org} induced by low pH and high pCO_2

Isotopic fractionation during nitrate assimilation is suggested to associate with the individual isotope effects for intracellular nitrate reduction (ε_{NR}), nitrate uptake (ε_{in}), and nitrate efflux (ε_{out}), as well as the relative proportion of nitrate efflux to nitrate uptake (E/U) (Karsh et al. 2014). It can be calculated by (Granger et al. 2010):

$$\varepsilon_{org} = \varepsilon_{in} + E/U \times (\varepsilon_{NR} - \varepsilon_{out}) \quad (4)$$

The ε_{NR} previously measured for eukaryotic NR (26.6‰; Karsh et al. 2012) is significantly higher than the reported ε_{in} and ε_{out} (2.0‰ and 1.2‰) for *T. weissflogii* (Karsh et al. 2014). The ε_{org} is thus mainly driven by isotope fractionation for the intracellular NR enzyme and is less influenced by isotope fractionation associated with cellular nitrate uptake and efflux. It is also determined by the degree of its external expression in the medium through changes in the ratio of nitrate efflux to nitrate uptake (e.g., E/U), showing that a lower ε_{org} is related to lesser efflux (Needoba et al. 2004). However, as the ε_{org} decreases, ε_{in} may increasingly contribute to the expression of the isotope fractionation. By considering the key processes in the reductase-driven model aforementioned, we will assess the potential mechanisms that affect ε_{org} for *T. weissflogii* and *Synechococcus* at low pH and high pCO_2, respectively (Fig. 2).

For eukaryotic diatom T. weissflogii

$^{15}\varepsilon_{NR}$ can be obtained by the $\delta^{15}N$ difference between intracellular ($\delta^{15}N\text{-}NO_3^-{}_{in}$) and medium nitrate ($\delta^{15}N\text{-}NO_3^-{}_{ex}$), along with the addition of $^{15}\varepsilon_{org}$ (Karsh 2014):

$$^{15}\varepsilon_{NR} = \left(\delta^{15}N - NO_3^-{}_{in} - \delta^{15}N - NO_3^-{}_{ex}\right) + {}^{15}\varepsilon_{org} \quad (5)$$

$^{18}\varepsilon_{NR}$ is calculated in the same way. The estimated $^{15}\varepsilon_{NR}$ of *T. weissflogii* was roughly constant under four pH-pCO_2 conditions, varying within 22‰–23‰, and the corresponding $^{18}\varepsilon_{NR} : {}^{15}\varepsilon_{NR}$ ratios remained at ~1 (Table 1). These results indicate that ε_{NR} of *T. weissflogii* is insensitive to changes in pH or pCO_2. The unchanged NR activities and intercellular

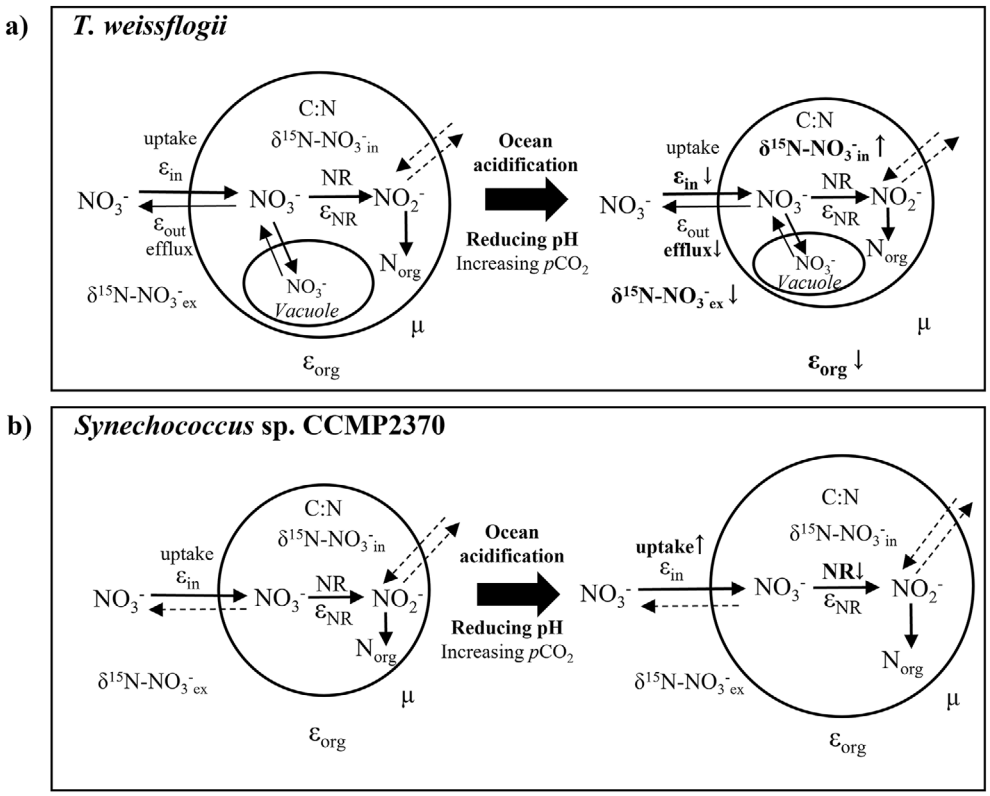

Fig. 2. Schematic of physiological states and isotope effects during nitrate assimilation by (**a**) *T. weissflogii* and (**b**) *Synechococcus* sp. CCMP2370, including growth rate (μ), cell volume, nitrate assimilation steps (i.e., uptake, reduction, and efflux) and corresponding isotope effects (i.e., ϵ_{in}, ϵ_{NR}, and ϵ_{out}), the medium and intracellular nitrate $\delta^{15}N$, and ϵ_{org}, in response to reducing pH and increasing pCO_2. The cell model refers to Bryan et al. (1983) and Granger et al. (2004). The upward and downward arrows indicate increases and decreases in variables that are shown in bold, respectively.

nitrate in *T. weissflogii* at low pH and/or high pCO_2 support our argument (Supporting Information Fig. S1). Furthermore, we find that $^{15}\varepsilon_{org}$ for *T. weissflogii* is tightly correlated with the $\delta^{15}N$ difference between the intercellular and medium nitrate under various culture conditions, suggesting a constant $^{15}\varepsilon_{NR}$ independent of environmental changes (Fig. 3). All eukaryotic NRs belong to the sulfite oxidase family of molybdoenzymes with similar nitrate reduction mechanisms (Campbell 1999). Previous studies have shown that the ε_{NR} values for the artificial reductant methyl viologen from *T. weissflogii*, *Pichia angusta*, and *Arabidopsis thaliana* are indistinguishable (27‰–28‰), as temperature and nitrate concentration change (Karsh et al. 2012; Treibergs and Granger 2017). Taken together, these findings demonstrate that ε_{NR} of eukaryotic phytoplankton is invariant in changing environmental conditions. In this context, we infer that possible changes in the relative rates between nitrate uptake and efflux and in their isotope effects under the low pH and high pCO_2 condition need to be taken into account.

Given a uniform ε_{NR} with high values, E/U for *T. weissflogii*, which is related to the $\delta^{15}N$ difference between intracellular and medium nitrate, largely determines the ε_{org} magnitude (Eqs. 4 and 5). Increases in ε_{org} under light limitation suggest increases in E/U and hence decreases in $\delta^{15}N$ difference between intracellular and medium nitrate (Fig. 3). By analogy, we observed that such $\delta^{15}N$ differences increased and $^{15}\varepsilon_{org}$ decreased at low pH and high pCO_2 in cultures of *T. weissflogii*, implying decreases in E/U (Fig. 3; Table 1). According to Eq. 4, we could estimate that E/U for *T. weissflogii* decreases by ~50%, from 0.23 to 0.12 under the low pH and high pCO_2 condition. On the other hand, the diatom cells may maintain intracellular nitrate homeostasis by balancing nitrate reduction and efflux with nitrate uptake. Based on the rate measurements we find that the calculated E/U for *T. weissflogii* reduces from 0.19 to 0.11 under the low pH and high pCO_2 condition, which is comparable to the isotope-based estimate (Table 1). We thus suggest that both low pH and high pCO_2 lead to decreases in ε_{org} for *T. weissflogii* by lowering E/U.

Isotope effects for nitrate uptake (ε_{in}) may make an important contribution to the low ε_{org}. The measured ε_{in} for *T. weissflogii* of ~2.0‰ (Karsh et al. 2014) can explain at least 25% of ε_{org} in this study (7.8‰; Fig. 1). Such a contribution may increase as ε_{org} decreases at low pH and high pCO_2. The ε_{in} could be largely determined by the intrinsic isotope effect associated with nitrate transport into the cell and its rate.

Table 1. N and O isotope effects of cellular nitrate reduction ($^{15}\varepsilon_{NR}$, $^{18}\varepsilon_{NR}$), the $\delta^{15}N$ differences between intracellular and medium nitrate, rates of nitrate uptake (NUR), nitrate reduction (NRR), and nitrate efflux (NER), and nitrate efflux/uptake ratios (E/U) for *T. weissflogii*. Nitrate reduction rate is determined by a linear regression of PN concentration vs. time. Nitrate efflux rate equals nitrate uptake rate minus nitrate reduction rate. All data are mean ± SD ($n = 3$). Numbers with different superscript letters indicate data significantly different among treatments ($p < 0.05$; One-way ANOVA followed by Turkey test), while numbers with the same superscript letter indicate data with no significant difference ($p \geq 0.05$). Values that are significantly different from ambient conditions (pH 8.1, pCO_2 400 μatm) are shown in bold.

pH-pCO_2 condition	$^{15}\varepsilon_{NR}$ (‰)	$^{18}\varepsilon_{NR}$ (‰)	$^{18}\varepsilon_{NR}$: $^{15}\varepsilon_{NR}$	$\delta^{15}N$ difference (‰)	NUR (pmol cell^{-1} d^{-1})	NRR (pmol cell^{-1} d^{-1})	NER (pmol cell^{-1} d^{-1})	E/U†	E/U‡
8.1–400	22.5 ± 0.6^{ab}	22.4 ± 0.9^{ab}	1.00 ± 0.04^{a}	14.5 ± 0.5^{a}	1.59 ± 0.20^{a}	1.29 ± 0.11^{a}	0.30 ± 0.09^{a}	0.19 ± 0.04^{a}	0.23 ± 0.02^{a}
8.1–800	22.7 ± 0.4^{ab}	21.8 ± 1.0^{a}	0.97 ± 0.03^{a}	**15.8 ± 0.9^{b}**	1.50 ± 0.19^{a}	1.25 ± 0.09^{a}	0.25 ± 0.13^{a}	0.16 ± 0.08^{a}	0.20 ± 0.02^{ab}
7.8–400	23.1 ± 0.7^{b}	23.3 ± 0.8^{b}	1.00 ± 0.04^{a}	**17.3 ± 0.7^{c}**	1.44 ± 0.12^{a}	1.20 ± 0.04^{a}	0.24 ± 0.09^{a}	0.16 ± 0.05^{a}	0.17 ± 0.05^{ab}
7.8–800	22.0 ± 0.7^{a}	21.5 ± 1.0^{a}	0.98 ± 0.03^{a}	**16.7 ± 0.7^{c}**	1.35 ± 0.16^{a}	1.19 ± 0.13^{a}	0.16 ± 0.04^{a}	0.11 ± 0.02^{a}	**0.12 ± 0.03^{b}**

†Values were calculated using nitrate efflux rate divided from nitrate uptake rate in this study.
‡Values were calculated according to Eq. 4 with variables (i.e., ε_{NR}, ε_{in}, and ε_{out}) from Karsh et al. (2014).

Dehydration occurring in this step breaks the hydrogen bonds formed between nitrate and surrounding water molecules, which may be a significant source of isotope fractionation. Reducing pH in the environment could change cytosolic pH and enhance hydrogen bonds, thus impacting nitrate transport into the cell (Miller et al. 2007; Shi et al. 2019). This is seemingly manifested by the slight drawdown of nitrate uptake rate in *T. weissflogii* under the low pH and high pCO_2 condition. We might infer that decreasing pH and increasing pCO_2 reduce ε_{in} in *T. weissflogii*. Similarly, low pH reduces the carbon isotope effect of marine diatom *S. costatum* by affecting the carbon uptake (Hinga et al. 1994).

While the evidence that supports the reduced E/U to largely cause decreases in ε_{org} for *T. weissflogii* under the low pH and high pCO_2 condition is convincing, there are some clues to suggest that decreases in $^{15}\varepsilon_{in}$ and/or $^{18}\varepsilon_{in}$ may also play a role. First, as E/U decreases and thus ε_{org} reduces, uptake would make a greater contribution to ε_{org} and in turn deviate $^{18}\varepsilon_{org} : {}^{15}\varepsilon_{org}$ from 1 (i.e., increase $^{18}\varepsilon_{org} : {}^{15}\varepsilon_{org}$), due to its $^{18}\varepsilon_{in} : {}^{15}\varepsilon_{in}$ of ~1.4 (Karsh et al. 2014). However, $^{18}\varepsilon_{org} : {}^{15}\varepsilon_{org}$ did not change at low pH and/or high pCO_2 (remained at ~ 1.1), as ε_{org} decreased (Supporting Information Table S3). A plausible interpretation is a concomitant decrease in ε_{in} induced by the effects of low pH and high pCO_2, such that the proportional influence of ε_{in} to ε_{org} is roughly unchanged. Alternatively, relative to $^{15}\varepsilon_{in}$, $^{18}\varepsilon_{in}$ may be susceptible to being affected by low pH because more O atoms of nitrate form hydrogen bonds with surrounding water molecules (Tongraar et al. 2006). Reducing pH could induce greater decreases in $^{18}\varepsilon_{in}$ and thus a progressive coupling of $^{18}\varepsilon_{in}$ and $^{15}\varepsilon_{in}$ (i.e., $^{18}\varepsilon_{in} : {}^{15}\varepsilon_{in}$ approaches ~ 1), as we observed. Our findings for *T. weissflogii* could be indicative of decreases in both E/U and ε_{in} causing lower ε_{org} under the influence of decreasing pH and increasing pCO_2 (Fig. 2a).

For prokaryotic Synechococcus

Unlike *T. weissflogii*, ε_{org} for *Synechococcus* did not change at low pH and/or high pCO_2. Contrasting responses of ε_{org} for two algae to changes in pH and pCO_2 could result from their physiological differences, the key point of which is that prokaryotes lacking vacuoles have extremely low intracellular nitrate concentrations and thus impose a stricter control between cellular nitrate uptake and efflux (Granger et al. 2010). Lower ε_{org} for *Synechococcus* may suggest limited nitrate efflux and minimal expression of its ε_{NR}, hence manifesting a rough similarity between nitrate uptake and N demand (Supporting Information Fig. S1; Table S3). In this regard, we could infer that E/U for *Synechococcus* is approximately 0 and its ε_{org} is mainly determined by ε_{in}. Lower $\delta^{15}N\text{-}NO_3^-{}_{in}$ values were found than $\delta^{15}N\text{-}NO_3^-{}_{ex}$ for the *Synechococcus* cultures, stretching the plausibility of our argument (Supporting Information Fig. S3). Although low pH promoted nitrate uptake rate and reduced NR activity in *Synechococcus*, changes in its ε_{NR} and E/U can be ignored under

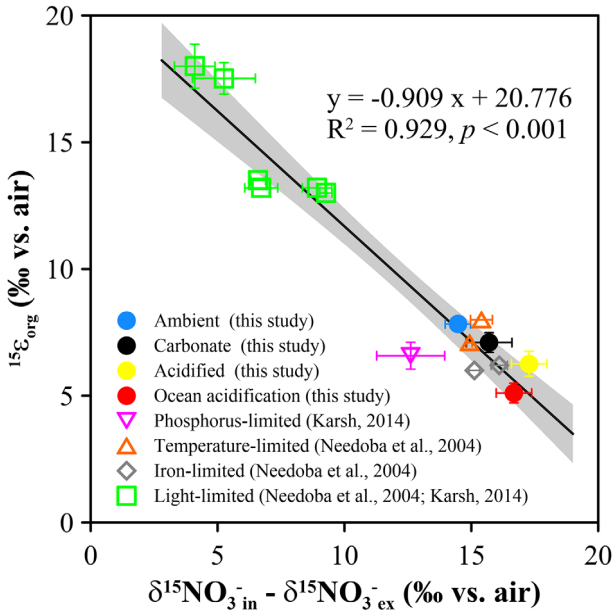

Fig. 3. Plot of the $^{15}\varepsilon_{org}$ vs. the $\delta^{15}N$ difference between intracellular and medium nitrate ($\delta^{15}NO_3^-{}_{in} - \delta^{15}NO_3^-{}_{ex}$) for *T. weissflogii* grown under different culture conditions. The solid line and gray shadow show the linear regression and the 95% confidence interval.

the low pH and high pCO_2 condition. However, further studies are needed to resolve whether ε_{NR} changes with environments since the reportedly prokaryotic nitrate reductase clade possesses high variability and expresses heterogeneous isotope fractionations (Granger et al. 2008; Frey et al. 2014).

While ε_{in} for prokaryotic phytoplankton is not yet measured, our observations for *Synechococcus* may imply its ε_{in} close to ε_{org} (~2‰) and $^{18}\varepsilon_{in} : {}^{15}\varepsilon_{in}$ of ~ 1. This is consistent with most field and laboratory studies showing that species with intracellular nitrate approaching 0 express ε_{org} near $^{15}\varepsilon_{in}$ (Karsh et al. 2014). Low pH and/or high pCO_2 seemed not to affect ε_{in} of *Synechococcus*, as its ε_{org} was invariant (Fig. 2). It is also noted that the presumable $^{18}\varepsilon_{in} : {}^{15}\varepsilon_{in}$ for *Synechococcus* is different from that for *T. weissflogii* (1.4; Karsh et al. 2014). Eukaryotic and prokaryotic phytoplankton possess different nitrate uptake systems, with the high-affinity NRT for *T. weissflogii* (part of NRT2 nitrate–nitrite porters family; Glibert et al. 2016) and the NrtP-type NRT for *Synechococcus* sp. CCMP2370 (part of the major facilitator protein superfamily; Su et al. 2006). In addition, these NRTs transport ions against the nitrate concentration gradient during nitrate uptake (Forde and Clarkson 1999), which is smaller for *Synechococcus* with lower intracellular nitrate concentration. We speculate that the differences in transporters and nitrate concentration gradients between the two algae may lead to differences in their $^{18}\varepsilon_{in} : {}^{15}\varepsilon_{in}$ and further induce their different changes at low pH and/or high pCO_2. However, the physiological mechanisms of how changes in pH and pCO_2 affect ε_{in} need to be further demonstrated.

Implications for understanding N isotope signals in acidified oceans

Knowledge of how ε_{org} responds to environmental changes is critical to better understanding the degree of nitrate consumption and nitrate N and O isotope distributions in the ocean. Evidence has shown that ocean acidification has rapidly been experienced in coastal seawaters and even in high-latitude oceans (Xue et al. 2018; Cai et al. 2021), leading to changes in nitrogen cycling processes (Wannicke et al. 2018). Our observations clearly suggest decreases in $^{15}\varepsilon_{org}$ for *T. weissflogii* at low pH and/or high pCO_2, providing new insight into the environmental controls on the magnitude of $^{15}\varepsilon_{org}$ for diatoms as the previous view shows that irradiance is the sole environmental driver. By comparison, reduced pH and/or elevated pCO_2 would not affect $^{15}\varepsilon_{org}$ for *Synechococcus*. It is noteworthy that our culturing results for diatoms are consistent with the field observations in the Southern Ocean. For example, $^{15}\varepsilon_{org}$ was found to decrease poleward from 8‰ to 9‰ in the Subantarctic Zone (40–52° S) with high pH to ~ 5‰ in the Polar Antarctic Zone (~ 66° S) with low pH (DiFiore et al. 2010; Xue et al. 2018). The field evidence also supports that besides light availability proposed previously (DiFiore et al. 2010), reduced pH may exert an influence on lowering $^{15}\varepsilon_{org}$. Eukaryotic phytoplankton, such as diatoms, play an important role in contributing to surface nitrate consumption and export production, especially in nutrient-rich oceans (Jin et al. 2006; Duret et al. 2020). Our findings of decreases in E/U for *T. weissflogii* at low pH and high pCO_2 may indicate enhanced efficiency of nitrate utilization by diatoms in future acidified oceans. Under projected future climate conditions, the warming of surface waters will cause shoaling of the surface mixed layer, leading to a decrease in nutrient supply from deep waters (Doney 2006). The enhancement of nitrate utilization efficiency by diatoms may facilitate their growth and contribution to primary production and export, as also reported in some modeling and experimental studies (Wu et al. 2014; Tréguer et al. 2018).

On the other hand, the higher degree of nitrate consumption leads to higher N isotope values in particulate N and sediments (Altabet and Francois 1994). However, decreases in $^{15}\varepsilon_{org}$ for diatoms observed at low pH and/or high pCO_2 would complicate the quantification of these changes, and thus require consideration in interpreting N isotope data in diatom-dominated, acidified oceans. It is also worth noting that the coherent effects of pCO_2 on $^{15}\varepsilon_{org}$ merit further investigation conducted on various algal species using a series of CO_2 concentrations, which would alleviate uncertainties in reconstructing past changes in nitrate utilization and thus the strength of the biological pump in high-latitude oceans during the glacial–interglacial CO_2 variations (e.g., Robinson et al. 2004; Studer et al. 2015) and the high-pCO_2 periods (e.g., the Plio-Pleistocene transition with pCO_2 of ~500 μatm; Bai et al. 2015).

While the combined effects of decreased pH and increased pCO_2 significantly lower the magnitude of $^{15}\varepsilon_{org}$ for *T. weissflogii*,

they may not change $^{18}\varepsilon_{org} : {}^{15}\varepsilon_{org}$ of ~1 for both *T. weissflogii* and *Synechococcus*. This suggests that changes in pH and pCO_2 would not modify the fundamental understanding of a uniform $^{18}\varepsilon_{org} : {}^{15}\varepsilon_{org}$ ratio during algal nitrate assimilation. Further research on other cultures of marine phytoplankton species and natural marine assemblages is needed to test the representativeness of our observations. This study also highlights the need for systematic studies to assess the effects of seawater pH and pCO_2 on the dual nitrate isotopes during other N cycle transformations (e.g., nitrate reduction and nitrate production), which could provide confidence in the broad field applications of coupled nitrate N and O isotopes to better understand the nitrate dynamics and N isotope biogeochemistry in modern and future acidified oceans.

Data Availability Statement

All data used in this manuscript are available in the repository Zenodo (https://zenodo.org/records/10208321).

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Conflict of Interest

None declared.

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