

NOTE

THE MECHANISM OF ISOTOPE FRACTIONATION DURING ALGAL NITRATE ASSIMILATION AS ILLUMINATED BY THE $^{15}\text{N}/^{14}\text{N}$ OF INTRACELLULAR NITRATE¹

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The $^{15}\text{N}/^{14}\text{N}$ of nitrate in the external medium and intracellular pool of the cultured marine diatom *Thalassiosira weissflogii* (Grun.) Fryxell et Hasle was measured during nitrate assimilation under low light, a 12:12-h light:dark cycle, low temperature, or low iron conditions. The $^{15}\text{N}/^{14}\text{N}$ of the nitrate in the medium and the particulate matter both followed the predicted Rayleigh fractionation model, and the intracellular nitrate always had a higher $^{15}\text{N}/^{14}\text{N}$ than did the medium nitrate. When the experiments were compared, the results showed a negative correlation between the isotope fractionation factor and the difference in the $^{15}\text{N}/^{14}\text{N}$ between the two pools of nitrate. These observations imply that the variations in the isotope effect result from variations in the degree to which the fractionation by nitrate reductase is expressed outside the cell, which is, in turn, controlled by the rate of nitrate efflux relative to nitrate reduction. The low iron and low temperature experiments showed relatively small isotope effects but a large intracellular-medium difference in nitrate $^{15}\text{N}/^{14}\text{N}$, consistent with a relative rate of efflux (compared with influx) that is small and similar to fast-growing cells. In contrast, large isotope effects and small intracellular-medium differences in nitrate $^{15}\text{N}/^{14}\text{N}$ were observed for low light and light:dark cycle grown cells and are explained by higher relative rates of nitrate efflux under these growth conditions.

Key index words: efflux; internal pool; isotope fractionation; iron; light; nitrate; phytoplankton; temperature; *Thalassiosira weissflogii*.

Abbreviations: LFe, low iron culture medium; LT, low temperature culture conditions; NR, nitrate reductase; PN, particulate organic nitrogen

Nitrogen isotope fractionation by phytoplankton follows the Rayleigh model (Mariotti et al. 1981) under batch culture conditions (Waser et al. 1998, Needoba et al. 2003), suggesting that the isotope fractionation at one step in the nitrogen assimilation process is responsible for the isotopic difference between the nitrate substrate and the biomass N. Possible steps during nitrate uptake and assimilation that could fractionate are (in order of occurrence) flux of NO_3^- across the diffusive boundary layer, active transport of NO_3^- across the plasma membrane (i.e. uptake step), intracellular reduction of NO_3^- to NO_2^- by nitrate reductase (NR), the reduction of NO_2^- to NH_4^+ , and the subsequent formation of amino acids and larger organic molecules. An important aspect of assimilation processes downstream of the uptake step is that any isotope fractionation that occurs will only be expressed if the ^{15}N enriched reactant pool is subsequently released from the cell (Wada and Hattori 1978).

Recent evidence suggests that isotope fractionation by phytoplankton occurs during the reduction of nitrate by NR and that efflux of isotopically “heavy” nitrate is ultimately responsible for the measured isotope effect (Shearer et al. 1991, Needoba and Harrison 2004). Here we provide support for this suggestion based on comparison of the $\delta^{15}\text{N}$ of nitrate inside versus outside cultured phytoplankton cells. We combine this with the $\delta^{15}\text{N}$ of the phytoplankton and Rayleigh-based estimates of the expressed isotope effect of nitrate assimilation to demonstrate how fractionation is expressed during phytoplankton growth.

Samples for $\delta^{15}\text{N}$ analysis of nitrate were obtained from a 3-L batch culture of *T. weissflogii* after acclimation to one of four different growth conditions (Table 1). In addition to the cultures grown for measurements of $\delta^{15}\text{N}$ of nitrate, three replicate cultures of *T. weissflogii* were grown in low temperature (LT) and three in low Fe (LFe) conditions to determine the isotope enrichment factor (ϵ). The LT experiment was conducted in an identical manner to the conditions for continuous

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TABLE 1. Summary of the four experimental culture conditions used in this study.

Culture condition	Culture medium	Irradiance ($\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	Temperature ($^{\circ}\text{C}$)	Iron (pFe)
L:D	ESAW	240	18	19
LL	ESAW	10–15	18	19
LT	ESAW	140	12	19
LFe	Aquil	140	18	20.5

L:D, 12:12-h light:dark cycle; LL, 24 h low light; LT, low temperature; LFe, low iron. LT and LFe were grown under continuous saturating light.

saturating light (Needoba and Harrison 2004), except the temperature was lowered to 12 $^{\circ}$ C. In the LFe experiment, standard trace metal techniques for growing phytoplankton in iron-limited cultures were used to limit the growth of *T. weissflogii* by iron. Briefly, 2-L polycarbonate culture vessels were acid cleaned and repeatedly rinsed with Milli-Q trace metal clean water (Millipore, USA). The culture medium was prepared using the protocol described for the artificial seawater medium Aquil (Morel et al. 1979, Price et al. 1989) and adding Fe-EDTA to achieve a pFe of 20.5 as calculated using the chemical equilibrium computer program MINEQL (Westall et al. 1976). For both the LT and LFe experiments, the phytoplankton cells were acclimated for at least eight generations before they were transferred to fresh medium to begin the experimental period.

To measure $\delta^{15}\text{N}$ of the nitrate in the medium, subsamples of the culture were removed and filtered onto combusted GF/F filters. The filtrate was collected and frozen for analysis of the nitrate concentration and its $\delta^{15}\text{N}$. The filter was oven dried at 60 $^{\circ}$ C and kept in a desiccator until $\delta^{15}\text{N}$ analysis of the phytoplankton cells. For the internal pool samples, 300–500 mL per

TABLE 2. Summary of the results of *Thalassiosira weissflogii* during growth in low temperature (LT) and low iron (LFe) growth conditions.

Parameter	LT	LFe
Growth rate (d^{-1})	0.34 ± 0.01	0.36 ± 0.03
Cell volume (μm^3)	650 ± 130	318 ± 30
PN/cell (pg)	18.5 ± 3.7	10.6 ± 1.5
Internal nitrate ($\text{mmol} \cdot \text{L}^{-1}$)	30 ± 16	28 ± 8
ϵ (‰)	6.6 ± 0.3	5.6 ± 0.6

sample were filtered onto 47-mm GF/F filters and extracted with boiling water (Thoresen et al. 1982). Isotopic analysis of the medium and internal pool nitrate used a recently developed method for nitrate isotopic analysis that uses denitrifying bacteria that lack an active N_2O reductase to convert nitrate (and nitrite) to N_2O , followed by isotopic analysis of the N_2O (Sigman et al. 2001, Casciotti et al. 2002). The “denitrifier” method has advantages relative to the ammonium-based methods (Liu et al. 1996, Sigman et al. 1997) that are critical to this study. First, a roughly 100-fold reduction in sample size requirement allows for cultures of small to moderate volume. Second, a lack of cross-contamination by dissolved organic N or NH_4^+ allows for accurate isotopic analysis of nitrate in the internal pool solutions, which can have very high concentrations of dissolved organic N.

Isotope ratios are reported using delta (δ) notation in units of per mil (‰):

$$\delta^{15}\text{N}_{\text{sample}} = \left(\frac{^{15}\text{N}/^{14}\text{N}}{^{15}\text{N}/^{14}\text{N}} \right)_{\text{sample}} / \left(\frac{^{15}\text{N}/^{14}\text{N}}{^{15}\text{N}/^{14}\text{N}} \right)_{\text{reference}} - 1$$

$$\times 1000\text{‰}$$

where the reference is N_2 in air, and values are based on comparison of sample analyses with analyses of potassium nitrate reference material IAEA-NO3, with

TABLE 3. Summary of the nitrogen concentration and $\delta^{15}\text{N}$ value for the particulate nitrogen (PN), nitrate in the medium, and intracellular nitrate (internal pool or IP) of the diatom *T. weissflogii* in four different growth conditions, and the calculated isotope enrichment factor (ϵ) using the Rayleigh model (Mariotti et al. 1981, initial $\text{NO}_3^- = 1.6\text{‰}$).

Culture condition	Sample	Notes	PN (μM)	$\delta^{15}\text{N}$ PN (‰)	NO_3^- in medium (μM)	$\delta^{15}\text{N}$ NO_3^- medium (‰)	[IP] (mmol/L cell volume)	$\delta^{15}\text{N}$ NO_3^- internal pool (‰)	ϵ	
									PN	NO_3^-
Low light	1		75	−8.11	107	9.86 ± 0.38	50.0	18.78 ± 0.2	13.5	13.2
	2	p.r.				9.73 ± 0.18	47.8	19.00 ± 0.06		13.0
	3		122	−5.62	47	20.77 ± 0.46	27.9	27.49 ± 0.19	16.2	13.2
	4	p.r.				21.13 ± 0.09	25.1	27.73 ± 0.20		13.5
	5	p.r.					22.9	27.88 ± 0.12		
Light/dark	1	11:30 AM	114	−4.47	61	14.85 ± 0.08	67.3	25.04 ± 0.26	11.6	11.2
	2	p.r.				14.96 ± 0.07	65.5	24.84 ± 0.08		11.3
	3	5:30 PM	132	−2.69	34	20.29 ± 0.62	13.2	36.08 ± 0.16	11.8	10.5
	4	p.r.				20.30 ± 0.41	13.2	35.47 ± 0.08		10.6
Low iron	1		85	−2.91	65	8.37 ± 0.19	23.8	23.49 ± 0.02	6.2	6.0
	2	p.r.				8.54 ± 0.18	21.1	24.64 ± 0.14		6.2
	3	p.r.				8.58 ± 0.21	23.8	24.76 ± 0.02		6.2
Low temp	1		123	−1.84	67	9.41	25.2	24.34 ± 0.1	8.6	7.1
	2		168	−2.49	52	12.31 ± 0.01	27.8	27.71 ± 0.42		8.0

$\delta^{15}\text{N}$ of nitrate measurements are the average of replicate measurements (± 1 SD) of the same sample. IP, internal pool; [IP] internal pool concentration; p.r., replicate measurements taken at the same time interval.

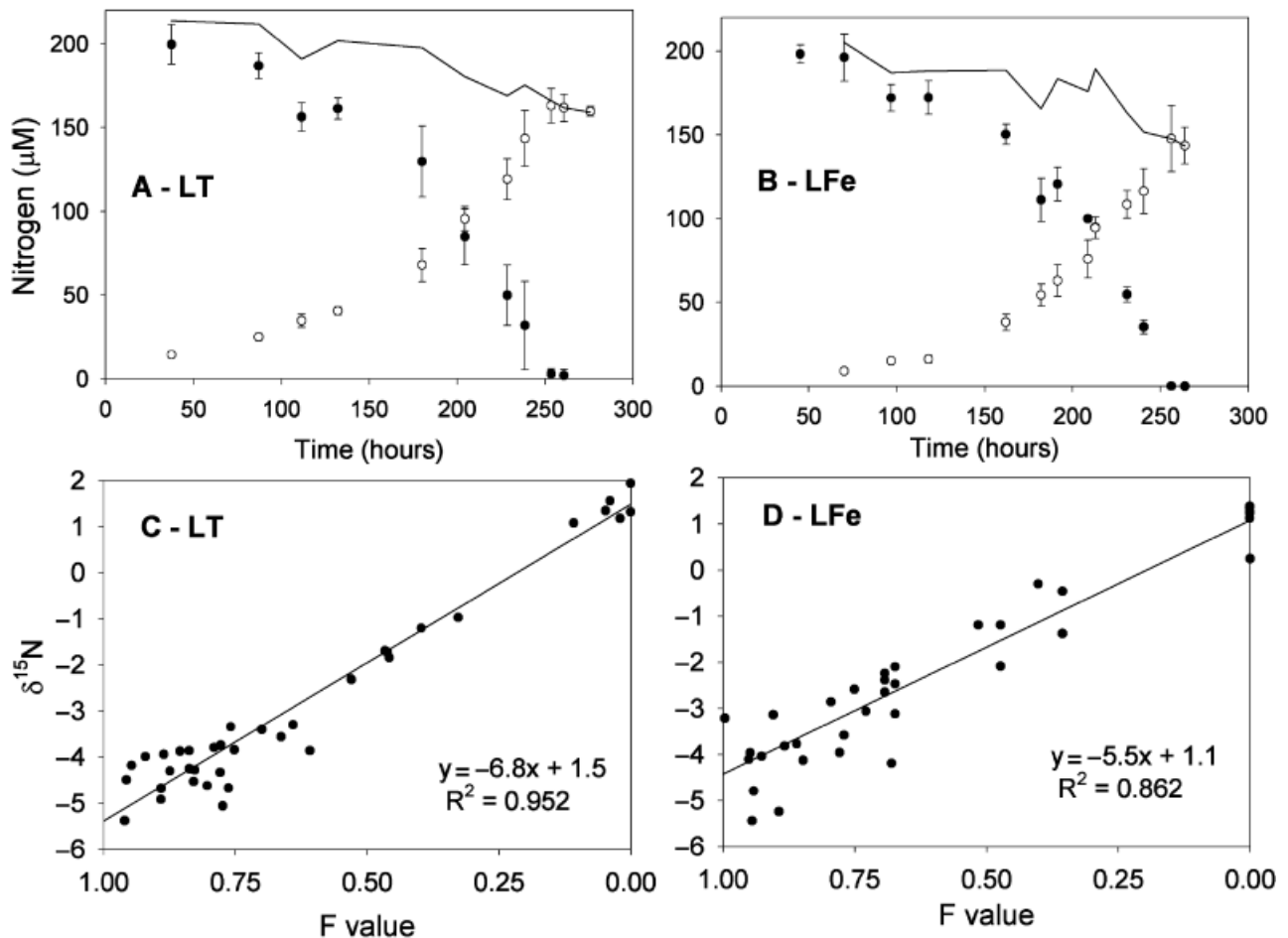


FIG. 1. Nitrogen mass balance and $\delta^{15}\text{N}$ as a function of the F value (see Needoba and Harrison 2004) of three replicate cultures of *Thalassiosira weissflogii* in low temperature (LT, A and C) and low iron (LFe, B and D) growth conditions. For A and B, open circles, particulate N; closed circles, NO_3^- ; solid line, mass balance of the two measurements.

an assigned $\delta^{15}\text{N}$ of +4.7‰. The isotope enrichment factor (ϵ) is defined as a function of the ratio of the assimilation rates (k^{14} and k^{15}) of nitrate containing the two isotopes, $\epsilon(\text{‰}) = (k^{14}/k^{15} - 1) \times 1000$, and was calculated following the methods described in Needoba and Harrison (2004).

Growth rates in the LT and LFe conditions were 0.34 and 0.36 d^{-1} respectively (Table 2). These growth rates are approximately 40% of the growth rate of nonlimiting conditions (Needoba et al. 2003). The average LT and LFe cell volumes were 650 and $316 \mu\text{m}^3$, respectively. Cell division and *in vivo* fluorescence increased exponentially and simultaneously (data not shown).

The cellular nitrogen content was higher in LT than in LFe, reflecting the difference in cell volume. However, when the particulate nitrogen (PN) was normalized to cell volume, the two culture conditions had similar N content. The internal nitrate concentration, which was about $30 \text{ mmol N} \cdot \text{L}^{-1}$ culture volume in both conditions, was similar to values obtained previously for continuous high light and high iron culture

conditions (Table 2). The change in $\delta^{15}\text{N}$ followed the Rayleigh model (Fig. 1A,B) and produced ϵ values for LT and LFe of $6.6 \pm 0.3\text{‰}$ and $5.6 \pm 0.6\text{‰}$, respectively (Fig. 1C,D). These values are statistically indistinguishable from the cultures grown in high light nonlimiting conditions, suggesting that the differences in growth rate or cell volume did not influence the isotope fractionation mechanism.

The concentration and $\delta^{15}\text{N}$ of PN, nitrate in the medium, and nitrate in the internal pool for a set of focused sampling are shown in Table 3. The low light culture was harvested twice, when nitrate was approximately 50% and 75% depleted. The light:dark (L:D) culture was harvested after 50% of the nitrate was depleted, two times during one L:D cycle (2.5 and 8.5 h after the light period began). The LFe culture was harvested once when nitrate was depleted by approximately 60%, and the culture grown in LT was harvested twice after the nitrate was depleted by 60%. The N isotopes in all four conditions displayed a similar pattern of low $\delta^{15}\text{N}$ of PN, intermediate $\delta^{15}\text{N}$ of the nitrate in the medium, and high $\delta^{15}\text{N}$ of the internal

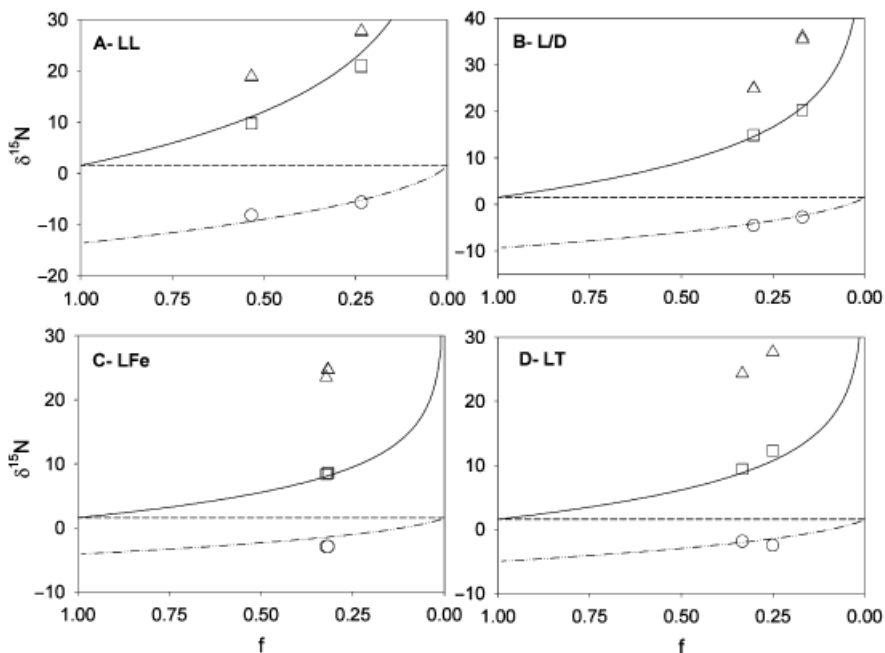


FIG. 2. $\delta^{15}\text{N}$ values of particulate nitrogen (circles), nitrate (squares), and internal nitrate pool (triangles) for *Thalassiosira weissflogii* grown in four different environmental conditions: (A) low light (LL), (B) 12:12-h light:dark cycle (L:D), (C) low iron medium (LFe), and (D) low temperature (LT). Also shown are the Rayleigh curves calculated from the measured ϵ values for *T. weissflogii*, as reported in Needoba and Harrison (2004) for LL (15.2‰) and L:D (10.9‰) and in this study for LFe (5.6‰) and LT (6.6‰). The solid line is the expected change of nitrate, the dotted-dashed line is the expected change for the particulate nitrogen, and the dashed line is the original $\delta^{15}\text{N}$ of the nitrate.

nitrate pool (Fig. 2). Also plotted in Figure 2 are the predicted Rayleigh curves, using the ϵ values for *T. weissflogii* presented above or in Needoba and Harrison (2004) using separate cultures. The close fit of the data for the $\delta^{15}\text{N}$ of PN and $\delta^{15}\text{N}$ of nitrate to the predicted values based on ϵ indicate that the N isotope dynamics of each culture followed the Rayleigh model. In addition, the fit of the nitrate $\delta^{15}\text{N}$ to the Rayleigh curve supports the assumption that nitrate in the medium reflects the fractionation process that determines the $\delta^{15}\text{N}$ of the phytoplankton, as proposed by Mariotti et al. (1981).

The calculated ϵ value and the difference between the $\delta^{15}\text{N}$ of the internal pool and the $\delta^{15}\text{N}$ of the nitrate in the medium for the continuous light cultures showed a significant inverse relationship (Fig. 3). The y-intercept of the relationship predicts that, for the case where there is no difference in nitrate $\delta^{15}\text{N}$ between the internal pool and medium, ϵ will be approximately 20‰ (Fig. 3). Note that the L/D experiment data was omitted from the regression analysis in Figure 3.

The results of the internal nitrate pool $\delta^{15}\text{N}$ measurements provide evidence to support the hypothesis that isotope fractionation arises from fractionation at NR, coupled with nitrate efflux from the cell. Our results show that the $\delta^{15}\text{N}$ of internal nitrate is always higher than the nitrate outside the cell. This observation requires that the ϵ of the reduction step is greater than the ϵ of the uptake step, since the inverse scenario requires that the $\delta^{15}\text{N}$ of the internal nitrate is lower than the medium. This supports previous circumstantial evidence, such as the low fractionation factor associated with nitrite utilization (Waser et al. 1998), that the diffusion or transport step is not a significant cause of fractionation.

We propose that the difference between the $\delta^{15}\text{N}$ of the internal pool and the $\delta^{15}\text{N}$ of the medium gives a measure of the efflux rate, such that when the difference is large, the efflux rate (compared with the influx rate) is small, and when the difference is small, the efflux rate is high and acts to raise the $\delta^{15}\text{N}$ of the medium towards the $\delta^{15}\text{N}$ of the intracellular pool. That is, efflux transports the isotopically enriched internal nitrate into the medium, lowering the difference

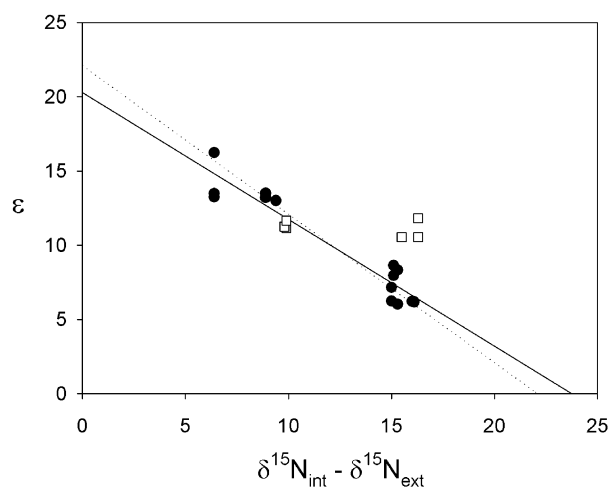


FIG. 3. Inverse relationship between the isotope fractionation factor (ϵ) and the $\delta^{15}\text{N}$ difference between the nitrate in the internal pool and the medium for *Thalassiosira weissflogii* in the four different environmental conditions: low light, 12:12-h light:dark cycle, low iron, and low temperature. The 12:12-h light:dark data (open squares) were omitted from the calculation of the regression line (solid line, $y = -0.9x + 20.3$, $R^2 = 0.916$, $p < 0.01$). Also shown is the estimated 1:1 line for the efflux mechanism (see text).

in $\delta^{15}\text{N}$ between internal and ambient nitrate and allowing the NR fractionation step to express itself in the $\delta^{15}\text{N}$ of both the medium nitrate and the phytoplankton biomass. If no efflux occurred, all nitrate taken up across the cell boundary would eventually be fixed into biomass, rendering the $\delta^{15}\text{N}$ of a whole phytoplankton cell identical to the $\delta^{15}\text{N}$ of the nitrate in the medium, despite fractionation steps within the cell and associated ^{15}N enrichment of the internal nitrate pool. If we assume that the fractionation during uptake is minimal (e.g. Shearer et al. 1991), then the 1:1 line that approximates relationships derived in Figure 3 predicts that the nitrate reductase enzyme in *T. weissflogii* is approximately 22‰ (y-axis intercept), which is consistent with other estimates (15–30‰) (Ledgard et al. 1985, Schmidt and Medina 1991). Working in the opposite direction, when ϵ is 0‰, then we predict that there is no efflux of nitrate from the cell, and the internal pool nitrate $\delta^{15}\text{N}$ has reached its greatest difference from the medium (x-axis is 22‰). If so, the efflux:influx ratio can be estimated from the x-axis of Figure 3. For example, the ϵ measured for low light (13.8‰) corresponds to a $\delta^{15}\text{N}$ difference of 8.3 between the inside and outside nitrate pools. The ratio of efflux:influx is then $(22 - 8.3)/22$, or 62%. Likewise, the LFe experiment had an efflux:influx ratio of $(22 - 15.4)/21$ or 30%. However, we cannot rule out fractionation at the uptake step, which will not alter the 1:1 slope of the efflux model as long as the same fractionation occurs during nitrate efflux, but will reduce the calculated efflux term. For example, if we assume that ϵ for nitrate reduction is equal to our largest measured $\delta^{15}\text{N}_{\text{int}} - \delta^{15}\text{N}_{\text{ext}}$ of 16.1‰, then fractionation during transport would have to account for the ϵ measured in this condition, $\sim 6\text{‰}$, and 16.1‰ on the x-axis of Fig. 3 would correspond to 0% efflux. In this case, efflux:influx for the LFe experiment is $(16.1 - 16.1)/16.1 = 0\%$ and the low light experiment is $(16.1 - 8.3)/16.1 = 48\%$. Complex models of this process have been applied to isotope fractionation in cyanobacteria (Shearer et al. 1991) and widely developed for higher plant models of isotope fractionation (Evans et al. 1996, Robinson et al. 1998, Comstock 2001). Additional data similar to that in Table 3 for other species are required to develop similar models for marine eukaryotic phytoplankton.

The efflux model can be used to explore some of the processes responsible for setting the ϵ values obtained here and previously reported (Needoba et al. 2003, Needoba and Harrison 2004). The fractionation factors between 2 and 6‰ that have been observed in many phytoplankton species (Needoba et al. 2003) suggest that efflux rates vary between 1% and 27%. This is not surprising for cultures that are growing in continuous light at a high specific growth rate, because efflux is expected to be low, as most of the nitrate entering the cell would be assimilated to account for the high growth demands. Efflux rates in the roots of vascular plants grown on nitrate (measured with the radioactive tracer ^{13}N) are routinely as high as 30% (Crawford and Glass 1998). On the other hand, low

growth rates could also be accompanied by low efflux (and therefore exhibit lower ϵ values) if the influx rate and the assimilation rate are similar, as suggested for cultures in balanced growth (Collos 1986). This explains the relatively small ϵ values of the LT and LFe experiments reported here. The low relative efflux rates are expected if cells acclimate to these low growth rate conditions by lowering their nitrate transport ability to meet the assimilation demands.

In contrast, unbalanced growth may lead to higher efflux and increased isotope fractionation. The reason for high efflux in low light conditions may be related to the evolutionary adaptations in the oceanic environment. The potential for rapid changes in light (due to water column mixing) could select for phytoplankton that can react quickly to such changes. This could result in cells retaining the ability to transport nitrate at high rates despite low growth conditions. Additionally, this implies that the ability to transport nitrate is linked to the potential for efflux. One possible scenario for this explanation is that the nitrate efflux is dominated by “leakage” from the cell via the nitrate uptake transport protein, as discussed below.

The variable effect of the L:D cycle on the nitrate efflux-to-influx ratio potentially explains the deviation of the L:D cycle samples from the general relationship between ϵ and internal pool/medium $\delta^{15}\text{N}$ difference shown in Figure 3. The 10.9‰ ϵ derived in Needoba and Harrison (2004) is probably an overestimate of fractionation expression during the light period (relative efflux is low) and an underestimate during the dark period (relative efflux is high). Over several days the average ϵ is a combination of the two different uptake and assimilation patterns. If an ϵ of 6‰ for the afternoon time point is used for the relationship in Figure 3, the data point falls on the regression line, close to the other data points corresponding to the lower efflux condition.

The trends reported here suggest that changes in cell volume and growth rate will only influence ϵ if they affect the efflux-to-influx ratio. For example, Raven (1986) argued that efflux (or leakage) would be higher in small cells, suggesting that the difference between ϵ of small diatoms such as *T. pseudonana* (5‰) and large diatoms such as *Ditylum brightwellii* (2‰) in similar culture conditions (Waser et al. 1998, Needoba et al. 2003) is the result of higher relative efflux by the smaller cell. The efflux model is analogous to the initial description of isotope fractionation during CO_2 assimilation (O’Leary 1981), where ^{12}C is preferentially assimilated by the enzyme RUBISCO and ^{13}C is lost from the cell by diffusion. However, nitrate uptake is exclusively by active transport; therefore, diffusion to the cell membrane is distinct from the diffusion processes within the cell. If growth rate increases or if the surface area-to-volume ratio decreases, diffusion limitation to the cell membrane will become more important and may result in a lowering of the influx rate (relative to nitrate assimilation rate). This would result in a lower efflux, because most of the nitrate would be

quickly incorporated (Waser et al. 1999). At present there are no accepted hypotheses for the mechanism of nitrate efflux from plant or algal cells. Because of the electrochemical gradient that is “downhill” for nitrate efflux (Boyd and Gradmann 1999), it is possible that marine phytoplankton either lose nitrate through back reactions at the transport protein (no energy required) or use the free energy gradient for a biochemical purpose, analogous to the coupled proton-solute transporters common in many other aquatic plant systems (Raven 1984).

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