

Nitrogen isotope fractionation during the uptake and assimilation of nitrate, nitrite, ammonium, and urea by a marine diatom

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Abstract

Knowledge of the fractionation of nitrogen isotopes by phytoplankton is a key requirement for the calibration of the new $\delta^{15}\text{N}$ paleotracer. An essential part of information required in this calibration concerns the magnitude of isotopic fractionation during the incorporation of N substrates by phytoplankton. To this end, the $\delta^{15}\text{N}$ of batch cultures of *Thalassiosira pseudonana* grown on nitrate, nitrite, ammonium, and urea was determined. This paper reports the first $\delta^{15}\text{N}$ study of phytoplankton growth on urea (e.g. organic N substrate). The $\delta^{15}\text{N}$ of the particulate nitrogen (PN) collected during the logarithmic growth phase, thus for N-sufficient cells, was lower than the $\delta^{15}\text{N}$ of the source due to kinetic isotope fractionation. With increasing drawdown of the N substrate, the $\delta^{15}\text{N}$ of the accumulating PN increased in accordance with the Rayleigh distillation model. Enrichment factors (ϵ) derived from a least-squares analysis of the accumulated $\delta^{15}\text{N}_{\text{PN}}$ data were $5.2 \pm 0.2\text{‰}$, $0.9 \pm 0.6\text{‰}$, $20 \pm 1\text{‰}$, and $0.8 \pm 0.6\text{‰}$ for NO_3^- , NO_2^- , NH_4^+ , and urea incorporation, respectively. Overall, ϵ values for nitrate incorporation were consistent with field estimates of $\sim 6\text{‰}$, and could be used to estimate past relative nitrate utilization as estimated from the $\delta^{15}\text{N}$ of bulk sedimentary organic matter.

Nitrogen isotopic fractionation associated with N uptake and assimilation (the overall process is defined as N incorporation) by phytoplankton is one of the most important fractionation processes in the biogeochemical cycle of N in the ocean. However, the mechanisms that control fractionation by algae are poorly understood (Handley and Raven 1992; Goericke et al. 1994). In nitrate- and ammonium-rich environments, fractionation during the growth of algae exerts a major control on particulate $\delta^{15}\text{N}$ in the surface ocean (Wada and Hattori 1976; Wada 1980; Cifuentes et al. 1989; Horrigan et al. 1990; Montoya et al. 1991; Altabet et al. 1991). This conclusion has been supported by culture studies of both marine and freshwater microorganisms (Wada and Hattori 1978; Macko et al. 1987; Hoch et al. 1992; Montoya and McCarthy 1995; Pennock et al. 1996) showing that, in most cases, ^{14}N is preferentially incorporated relative to ^{15}N .

Recently, there has been renewed interest in stable N isotopes with the discovery that the sedimentary $^{15}\text{N}:^{14}\text{N}$ ratio underlying nutrient-rich oceanic regimes could be used as a tracer of past relative nitrate utilization and under certain assumptions of past productivity (François et al. 1992; Calvert et al. 1992; Altabet and François 1994; Farrell et al. 1995). This new approach to problems in paleoceanography stems from the observation of a striking correspondence between low particulate $^{15}\text{N}:^{14}\text{N}$ ratios of bulk sedimentary organic matter and nitrate-rich waters and vice versa (Fran-

çois et al. 1992; Altabet and François 1994; Farrell et al. 1995). This pattern has been attributed to isotopic fractionation during the incorporation of nitrate by phytoplankton. A further application of sedimentary $\delta^{15}\text{N}$ has been the identification of changes in the intensity of denitrification, i.e. the major sink of N in the ocean (Altabet et al. 1995; Ganeshram et al. 1995). Such an analysis relies to a certain extent on the magnitude of fractionation, particularly if the record is located in an area where surface nitrate is always in excess.

In laboratory cultures, reported fractionation during growth of marine microorganisms on both nitrate and ammonium varies widely (Wada and Hattori 1978; Wada 1980; Montoya and McCarthy 1995; Pennock et al. 1996). Variations with light intensity, species, N substrate (millimolar concentrations), and culture conditions have been reported, with values of 0.7–23, 0.7, and –9.7–12‰ for nitrate-, nitrite-, and ammonium-grown algae, respectively (Wada and Hattori 1978; Wada 1980). Evidence for a growth rate effect (as controlled by light intensity) was found for growth of *Phaeodactylum tricorutum* on nitrate (Wada and Hattori 1978). Recently, growth of phytoplankton on micromolar N substrate in well-controlled continuous (Montoya and McCarthy 1995) and batch cultures (Pennock et al. 1996) provided data that are more representative of oceanic situations. Evidence for a species effect was shown for the first time when contrasting the growth of flagellates and diatoms on nitrate (Montoya and McCarthy 1995). These authors found lower fractionation factors for flagellates (1–3‰) compared with the diatoms *Skeletonema costatum* and *Thalassiosira weissflogii* (9–12‰). Pennock et al. (1996) found a factor of 9‰ for the growth of *S. costatum* on nitrate, in good agreement with the estimate of Montoya and McCarthy (1995). Furthermore, Pennock et al. (1996) found that frac-

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tionation was independent of the micromolar nitrate concentration. In contrast, when the diatoms were grown on ammonium, fractionation increased with concentration, from 8 to 25‰ for NH_4^+ concentrations of 5–20 to 50–100 μM , respectively.

The large variability in experimentally-determined fractionation may be due, in part, to the fact that few species of marine phytoplankton have been studied under well-controlled conditions. Furthermore, there have been many more studies of growth on nitrate than with any other N substrate, although ammonium and to a lesser extent urea and nitrite are very important N substrates for phytoplankton in many oceanic environments (Syrett 1981; Harrison 1992). In view of the lack of comprehensive studies regarding these aspects of N fractionation, we have conducted the first study of N isotope fractionation by a marine diatom grown on the four most important N sources for phytoplankters: NO_3^- , NO_2^- , NH_4^+ , and urea. A coastal diatom, *Thalassiosira pseudonana*, was grown on micromolar substrate concentrations in well-controlled conditions and in continuous light. Batch cultures were chosen because they are thought to best mimic the high productivity provinces where there is great interest in the use of ^{15}N as a paleotracer.

Materials and methods

Culture methods—*T. pseudonana* (Clone 3H) from the Northeast Pacific Culture Collection (Dept. Earth and Ocean Sciences, Univ. British Columbia) was grown on artificial seawater (ESAW) following a modified recipe of Harrison et al. (1980) and Price et al. (1987). Nitrate, nitrite, and ammonium concentrations in the medium ranged from 189 to 234 μM . Urea concentration was 95 μM since a molecule of $\text{CO}(\text{NH}_2)_2$ contains 2 atoms of N. The N:P and Si:N ratios in the medium were 4:1 and 2:1, respectively, to ensure that N was limiting biomass at stationary phase. Bicarbonate (NaHCO_3) was initially 2 mM, and 0.3 g was added daily to the 2-liter cultures to prevent C limitation. The pH of the medium was initially 7.5–8.0 and increased to 8.7–9.5 at stationary phase (the pH increase was mostly due to C uptake during growth). Culture medium was filter sterilized (0.22 μm Millipore) and transferred into 2-liter sterilized flat-bottom flasks, inoculated with the stock culture, and placed in a circulating water bath at $18.0 \pm 0.5^\circ\text{C}$. The cultures were stirred with a magnetic stir bar (60 rpm) continuously and gently bubbled with filtered (0.22 μm), N-free (NH_3 was removed with a 5% H_2SO_4 trap), and humidified (distilled water trap) air. In the case of urea, the cultures were not bubbled to minimize NH_3 gas exchange (NH_3 is leaked from cells due to intracellular urease) and avoid a small correction to be made on the magnitude of isotopic fractionation (see results ammonium-grown cultures). Normal procedures were used to minimize bacterial contamination (autoclaving and handling in flow hood). The cultures were continuously illuminated with 140–320 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

The cells were acclimated to the N substrate in the 2-liter flasks for at least 1 week, and when samples were first collected, the cells had been growing exponentially for a min-

imum of about 10 generations. For growth of *T. pseudonana* on urea, the experiment proceeded in two phases. In the first phase, the culture proceeded through a normal log and stationary phase. In the second phase, the culture was diluted with new medium, allowed to grow back to stationary phase, and starved for 24 h. At this point urea was added to give a culture concentration of 75 μM urea (or 150 $\mu\text{mol N liter}^{-1}$) and the culture was allowed to grow to a new stationary phase. Trace metals and macronutrients (Si, P, and C) were also added to the culture after 24 h starvation so that their concentrations were as in ESAW. The experiment was designed to contrast a normal exponential growth (i.e. phase 1) and growth following the addition of urea after a 24-h starvation period (i.e. phase 2). This was done to minimize the excretion of NH_4^+ that is known to occur during growth of *T. pseudonana* on urea (Price and Harrison 1988) and to minimize the fraction of NH_4^+ taken up by the diatom relative to urea in phase 2.

Biomass, particulate N, and nutrient analysis—Samples of nutrients, particulate matter, fluorescence, and cell density were mostly collected at relatively high cell densities because of the large sample size required for $\delta^{15}\text{N}$ analysis (min. of 1.5 $\mu\text{mol N}$). The specific growth rate μ was calculated from the following relationship: $N = N_0 e^{\mu t}$, where N was either cell density or in vivo fluorescence measured during log phase. Cell density and fluorescence were measured on a Coulter counter model TA II and Turner Designs model 10 fluorometer, respectively. Particulate nitrogen (PN) samples were collected by vacuum filtration at 0.5 atm on pre-combusted (450°C) glass-fiber filters (GF/F) and determinations were made on a Fisons automated CHN analyzer (model NA1500) online with the mass spectrometer. The precision of each PN analysis was 1–2%. N isotopes were analyzed as outlined below. The filtrate was kept for nutrient analysis. Nitrite, ammonium, and phosphate were determined by standard colorimetric methods (Parsons et al. 1984) and urea by the diacetyl monoxime method (Price and Harrison 1987). Nitrate was monitored by UV absorption (APHA 1980). UV absorption allowed quick and precise measurements without sample dilution from 200 μM down to 1–2 μM (detection limit). The precision was 3.3%. The accuracy was increased when we used early stationary phase medium as the blank for calculations of the concentrations. Overall, the accuracy of UV measurements ranged from 4% at 200 μM to 15% at 20 μM (due to the high blank), which corresponded to the range of concentration of most samples.

Nitrogen isotope analysis—N isotope abundance was determined with a VG PRISM mass spectrometer. Particulate samples were prepared by rolling one-half of a GF/F filter in tin foil and compressing the foil into small pellets. Pellets were then combusted in a stream of oxygen at an oven temperature of $1,020^\circ\text{C}$. Results are reported in the delta notation:

$$\delta^{15}\text{N} = \left(\frac{R_{\text{sample}}}{R_{\text{std}}} - 1 \right) \times 1,000, \quad (1)$$

where R is the $^{15}\text{N} : ^{14}\text{N}$ ratio and the standard (std) is N_2 gas (NBS-14). The $\delta^{15}\text{N}$ of N1 and N2 standards, i.e. $(\text{NH}_4)_2\text{SO}_4$,

was 1.34 and 20.85‰ relative to NBS-14, respectively, while it was 0.54 and 20.05‰ relative to air. Although the internationally accepted standard is air, we continue to report $\delta^{15}\text{N}$ relative to NBS-14 because absolute values are not critical for this study—the difference between the $\delta^{15}\text{N}$ of PN and the source is important, not the absolute value of either of them (*see below*). The precision of $\delta^{15}\text{N}$ measurements was 0.17‰ (\pm SD of replicate pairs, $n = 44$ pairs). The $\delta^{15}\text{N}$ of the N sources (mean \pm SE) were $3.82 \pm 0.18\text{‰}$ ($n = 12$) for NaNO_3 , $5.05 \pm 0.20\text{‰}$ ($n = 2$) for NaNO_2 , $-0.34 \pm 0.20\text{‰}$ ($n = 3$) for NH_4Cl , and $0.06 \pm 0.12\text{‰}$ ($n = 3$) for $\text{CO}(\text{NH}_2)_2$. We estimate that the correction for carryover of ^{15}N -enriched nitrate from the inoculum (0.5 mM NO_3^-) to the culture medium was small (i.e. $<0.2\text{‰}$) because of the small volume of inoculum used. A small ^{15}N enrichment was also potentially introduced upon culture dilution.

Calculations of the fractionation factor—The isotope fractionation factor α was calculated using the accumulated product equation (Mariotti et al. 1981). In this paper, note that contrary to the definition adopted by Mariotti et al. (1981), α is typically ≥ 1 and ϵ (i.e. the per mil enrichment factor of the substrate relative to the product) is typically ≥ 0 (with $\epsilon = (\alpha - 1) \times 1,000$). In a closed system, as in a batch culture, the $\delta^{15}\text{N}$ of the first accumulated product is given by

$$\delta^{15}\text{N}_{\text{PN}_0} = \delta^{15}\text{N}_{\text{DN}_0} - \epsilon, \quad (2)$$

where PN_0 and DN_0 are the initial particulate N and dissolved N, respectively. Assuming ϵ was constant during the consumption of the substrate, ϵ was then derived from the accumulated product equation

$$\delta^{15}\text{N}_{\text{PN}} = \delta^{15}\text{N}_{\text{DN}_0} - \epsilon \times \frac{-f}{1-f} \times \ln f, \quad (3)$$

where f is the fraction of unreacted substrate at any time during exponential growth. A curve of $\delta^{15}\text{N}_{\text{PN}}$ vs. F (F is defined as $[-f/(1-f)]\ln f$) was fitted with a linear function. The slope (i.e. ϵ) and the intercept (i.e. $\delta^{15}\text{N}$ of the source) were then determined from least-squares analysis using the Marquardt–Levenberg algorithm. When the $\delta^{15}\text{N}_{\text{PN}}$ could be approximated to that of the first product (i.e. $\delta^{15}\text{N}_{\text{PN}}$ corresponding to f and F close to 1), ϵ was also estimated from Eq. 1. Only data in log phase were analyzed for the calculation of ϵ because it was only during that phase that N incorporation could be approximated by a one-step unidirectional reaction (i. e. conversion of nitrate to PN) as needed in order for Eq. 2 to be applicable. Once nitrate has been exhausted, cell lysis will start, releasing various forms of N.

Results

Isotope fractionation during growth on nitrate—When *T. pseudonana* was grown on nitrate, fluorescence and PN increased exponentially until stationary phase, at which point they both reached a plateau (Fig. 1A). In log phase, both curves were fit to exponential functions yielding maximum growth rates (\pm SD) of $2.1 \pm 0.1 \text{ d}^{-1}$ and $1.6 \pm 0.2 \text{ d}^{-1}$ for fluorescence and PN, respectively. These estimates were in

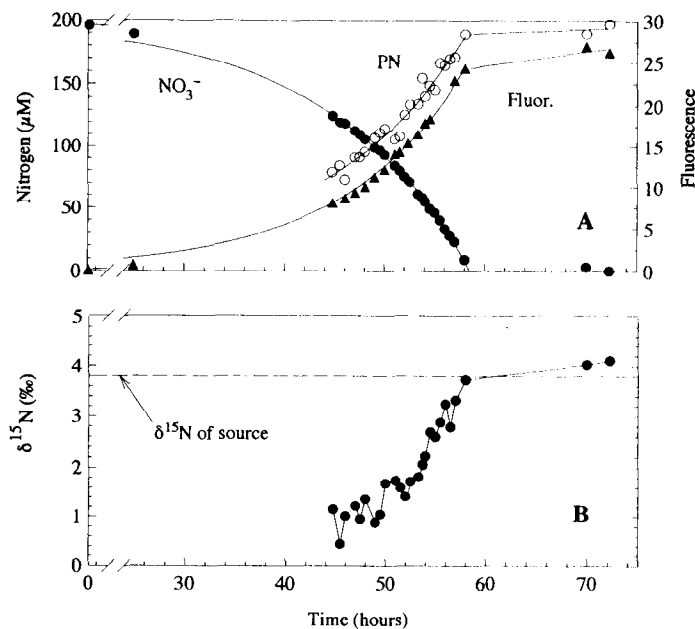


Fig. 1. *T. pseudonana* growth on nitrate during log and stationary phase at an initial NO_3^- concentration of $196 \mu\text{M}$. A. Time series of NO_3^- (●), PN (○), and biomass (▲) fluorescence. B. Time series of the $\delta^{15}\text{N}$ of the accumulated PN. The dashed line represents the $\delta^{15}\text{N}$ of the NaNO_3 source of 3.8‰. Nitrate, PN, and fluorescence curves were fit to exponential functions during the log phase (*see text* for details of the best fits) and smoothed during the stationary phase.

good agreement with the maximum specific growth rate of $1.8 \pm 0.1 \text{ d}^{-1}$ determined from cell density measurements, as would be expected for balanced growth. Cell density increased from 5.8×10^5 to $1.1 \times 10^6 \text{ cells ml}^{-1}$ during the 47–57-h period. N cell quota averaged $2.2 \text{ pg N cell}^{-1}$ during that period. Coinciding with the increase in biomass, nitrate decreased from an initial concentration of $196 \mu\text{M}$ to below detection limit at the onset of the stationary phase, at which point nitrate was limiting the biomass yield (Fig. 1A). At stationary phase, PN concentration was similar to initial nitrate, showing a good mass balance for total N. In log phase, the nitrate curve was fit with an exponential function with three parameters (i.e. the difference between a constant and an exponential function). The exponent of the best fit was $1.7 \pm 0.2 \text{ d}^{-1}$, again in very good agreement with the exponents of fluorescence and PN best fits.

The $\delta^{15}\text{N}$ of the accumulated PN was lower than the $\delta^{15}\text{N}$ of the source during the log phase owing to discrimination against ^{15}N as described by Eq. 2 (Fig. 1B). Coincident with the exhaustion of nitrate, the $\delta^{15}\text{N}_{\text{PN}}$ increased to $4.1 \pm 0.12\text{‰}$, indistinguishable from the $\delta^{15}\text{N}$ of the source (i.e. $3.8 \pm 0.2\text{‰}$), showing a good isotope mass balance.

Isotope fractionation during growth on ammonium—In ammonium-grown cultures, NH_4^+ , PN, and fluorescence curves were fit as in the previous culture experiments (Fig. 2A). Growth rates (\pm SD) calculated from fluorescence, PN, and NH_4^+ were 1.79 ± 0.07 , 1.58 ± 0.07 , and $1.3 \pm 0.1 \text{ d}^{-1}$, respectively. Cell density increased from 1.2×10^5 to

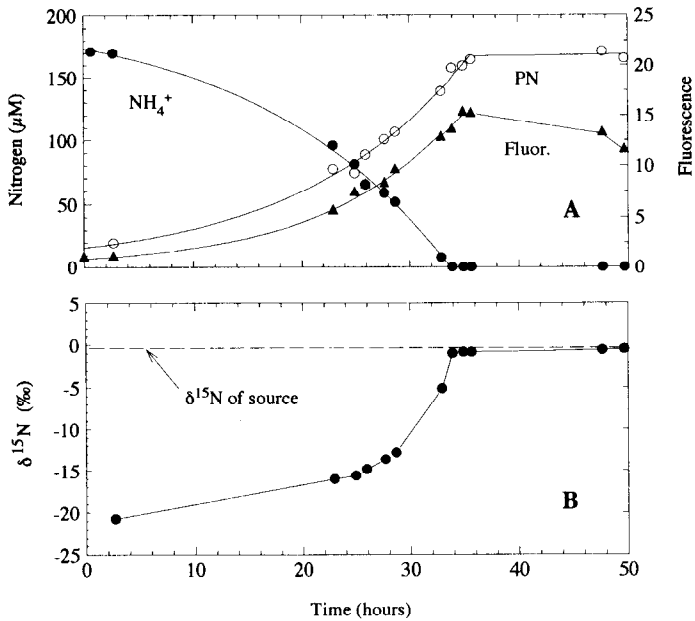


Fig. 2. Growth of *T. pseudonana* on ammonium during log and stationary phase at initial NH_4^+ concentration of $189 \mu\text{M}$. A. Time series of NH_4^+ (●), PN (○), and fluorescence (▲). B. Time series of the $\delta^{15}\text{N}_{\text{PN}}$ (the dashed line represents the $\delta^{15}\text{N}$ of the NH_4Cl source of -0.3‰). NH_4^+ , PN, and fluorescence curves were fit to exponential functions during the log phase (see text for details of the best fits), whereas the remaining data points were connected with straight lines.

$2.2 \times 10^6 \text{ cells ml}^{-1}$ during the 0–34-h period. N cell quota decreased from 1.4 to $1.0 \text{ pg N cell}^{-1}$ during that period. The $\delta^{15}\text{N}_{\text{PN}}$ increased dramatically from -19.9‰ (i.e. f of 0.94) to -0.2‰ at stationary phase (Fig. 2B). Assuming negligible increase in the $\delta^{15}\text{N}$ of NH_4^+ between f of 1 and f of 0.94, Eq. 1 allowed a rough estimate of ϵ of -19.6‰ to be made (see end of results for a more accurate estimate of ϵ). This is not quite true of course, since ambient NH_4^+ decreased by $\sim 20 \mu\text{M}$ during that time. This decrease has to be accompanied by an increase in $\delta^{15}\text{N}$ of ambient NH_4^+ estimated to be 1.2‰ (i.e. using the equation for the substrate, $\delta^{15}\text{N}_s = \delta^{15}\text{N}_{s_0} - \epsilon \ln f$, with f of 0.94, $\delta^{15}\text{N}_{s_0}$ of -0.34‰ , and ϵ of 19.6‰). The N mass balance showed the loss of $\sim 14\%$ of total N during growth. This loss was probably due to loss of NH_3 via gas exchange. The equilibrium fractionation factor for $\text{NH}_3(\text{aq})$ exchange with $\text{NH}_3(\text{g})$ has been estimated at $\sim 5\text{‰}$ (Kirshenbaum et al. 1947), with the $\text{NH}_3(\text{aq})$ being enriched in ^{15}N by 5‰ relative to $\text{NH}_3(\text{g})$. In addition to this process, there is a 20‰ thermodynamic isotope effect between $\text{NH}_3(\text{aq})$ and $\text{NH}_4^+(\text{aq})$, with $\text{NH}_4^+(\text{aq})$ being enriched in ^{15}N by 20‰ relative to $\text{NH}_3(\text{aq})$ (Hermes et al. 1985). A 14% loss of N as NH_3 would imply an enrichment of ambient NH_4^+ of $\sim 2\text{‰}$. Thus, it was estimated that the correction to be made for gas exchange was relatively small, $\sim 2\text{‰}$, consistent with the fact that we found a good isotopic mass balance with $\delta^{15}\text{N}_{\text{PN}}$ of -0.3 to -1‰ (close to the $\delta^{15}\text{N}$ of the source).

Isotope fractionation during growth on urea—Growth rates ($\pm\text{SD}$) calculated from fluorescence, PN, and urea were

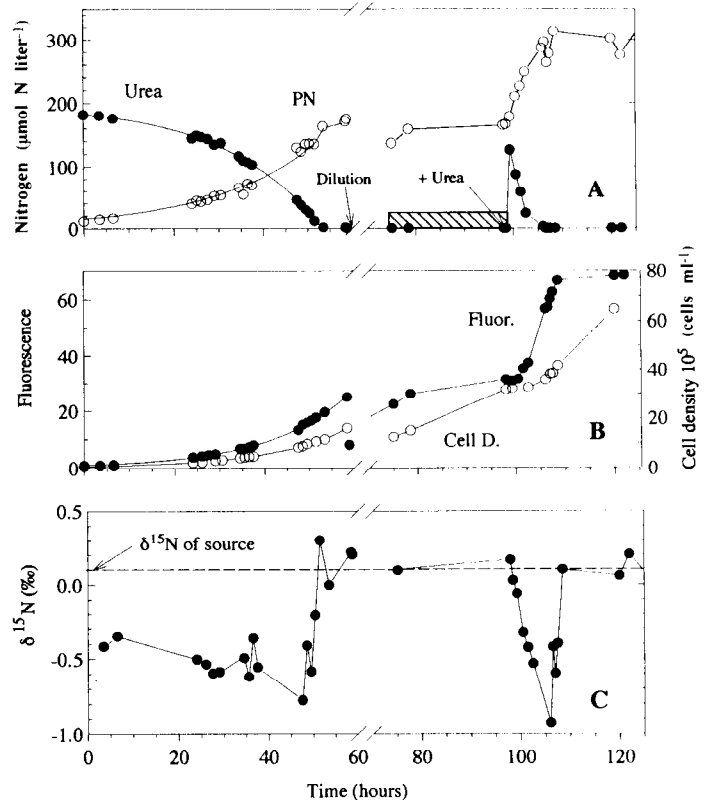


Fig. 3. Growth of *T. pseudonana* on urea. A. Time series of urea (●) and PN (○) expressed in $\mu\text{mol N liter}^{-1}$ with an initial urea concentration of $190 \mu\text{mol N liter}^{-1}$. B. Time series of fluorescence (●) and cell density (○). C. Time series of the $\delta^{15}\text{N}_{\text{PN}}$ (the dashed line represents the $\delta^{15}\text{N}$ of the urea source of 0.1‰). In the first log phase, urea, PN, and fluorescence were fit to exponential functions, whereas the remaining data points were connected with straight lines. The first and second arrow represent, respectively, times when the culture was diluted with new medium and when urea was added to the N-starved culture (at 99 h). The shaded area in panel A represents, within 2 h, the time during which the cells were starved of N.

1.33 ± 0.02 , 1.03 ± 0.04 , and $1.14 \pm 0.05 \text{ d}^{-1}$, respectively (Fig. 3A,B). N cell quota decreased from 2.6 to $2.0 \text{ pg N cell}^{-1}$ in the 0–53-h period. NH_4^+ concentrations were $<0.5 \mu\text{M}$ during phase 1. The $\delta^{15}\text{N}_{\text{PN}}$ increased with urea draw-down to $\sim 0.1\text{‰}$, a value similar to the source (Fig. 3C). During the starvation period (from $\sim 75 \text{ h}$ to 99 h), the $\delta^{15}\text{N}_{\text{PN}}$ was again similar to the source $\delta^{15}\text{N}$ of 0.1‰ , showing a good isotopic mass balance. Coinciding with the rapid uptake of urea after its addition at 99 h, PN and fluorescence increased rapidly until 110 h (Fig. 3A,B). In contrast, cell density increased at a slower rate of 0.6 d^{-1} , showing that cell division was no longer coupled with N incorporation and biomass increase. Associated with these changes, the $\delta^{15}\text{N}_{\text{PN}}$ showed a decrease due to discrimination during urea incorporation (Fig. 3C). Interestingly, the $\delta^{15}\text{N}_{\text{PN}}$ remained $<0.1\text{‰}$ until $\sim 108 \text{ h}$, when it reached 0.1‰ again. This pattern was surprising because from the rapid decrease in ambient urea (Fig. 3A), presumably urea was completely consumed at $\sim 103 \text{ h}$, and one expected the $\delta^{15}\text{N}_{\text{PN}}$ to equal

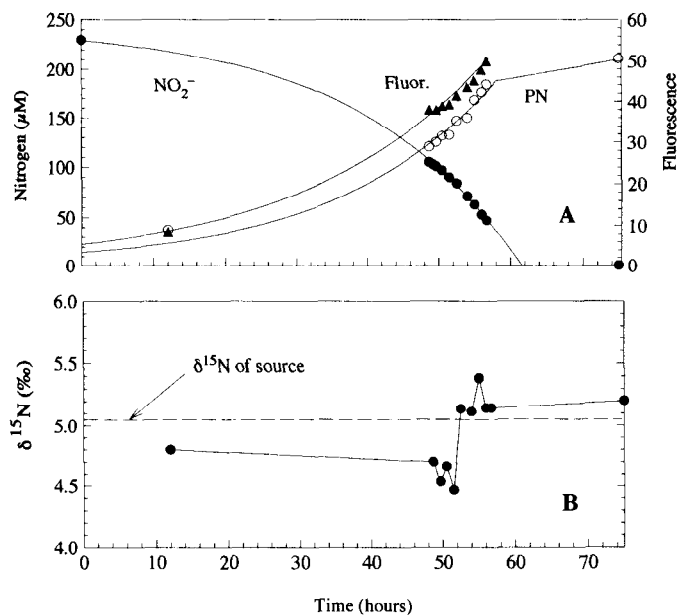


Fig. 4. *T. pseudonana* growth on nitrite during log phase. A. Time series of nitrite (●), PN (○), and fluorescence (▲), with an initial nitrite concentration of 234 μM . B. Time series of the $\delta^{15}\text{N}$ of accumulated PN. The dashed line represents the $\delta^{15}\text{N}$ of the NaNO_2 source of 5.05‰. Nitrite, PN, and fluorescence were fit to exponential functions.

0.1‰ at that time. Instead, the $\delta^{15}\text{N}_{\text{PN}}$ remained $<0.1\text{‰}$ for ~ 5 more hours. Therefore, more than one N substrate was involved in phase 2. Ammonium was measured immediately following the addition of urea, and was 0.25 μM at 99.3 h. This decreased to 0.03 μM at 101.5 h. Dissolved organic nitrogen (DON) may also have been released. Whatever the nature of the N substrate, it was completely consumed at ~ 108 h, as shown by the good N mass balance (Fig. 3A).

Isotope fractionation during growth on nitrite—For nitrite-grown cultures, growth rates ($\pm\text{SD}$) calculated from fluorescence, PN, and nitrite were 0.91 ± 0.05 , 1.1 ± 0.1 , and 1.07 ± 0.04 d^{-1} , respectively (Fig. 4A). Cell density increased from 3.5×10^5 to 2.2×10^6 cells ml^{-1} during the 12–56-h period. N cell quota decreased from 1.5 to 1.0 pg N cell $^{-1}$ during that period. The $\delta^{15}\text{N}$ showed only a very slight change with nitrite drawdown, indicating a small overall fractionation factor (Fig. 4B). In stationary phase, the $\delta^{15}\text{N}$ of 5.2‰ was close to $5.1 \pm 0.2\text{‰}$ (i.e. nitrite source), indicating a good isotopic mass balance. In addition, during log growth $[\text{NO}_2^-]_0 - [\text{NO}_2^-]$ was similar to the measured PN, indicating no loss of N from the medium other than the production of PN.

Estimates of ϵ for growth on nitrate, nitrite, ammonium, and urea—The results of a precise calculation of the enrichment factor ϵ are presented in Fig. 5A–D for the growth of *T. pseudonana* on nitrate, nitrite, ammonium, and urea. The values of ϵ and the intercept, as well as the results of duplicate experiments, are reported in Table 1. Two nitrate-grown cultures with an initial N:P ratio of 14:1 instead of 4:1 show similar ϵ values (Table 1). The calculated average

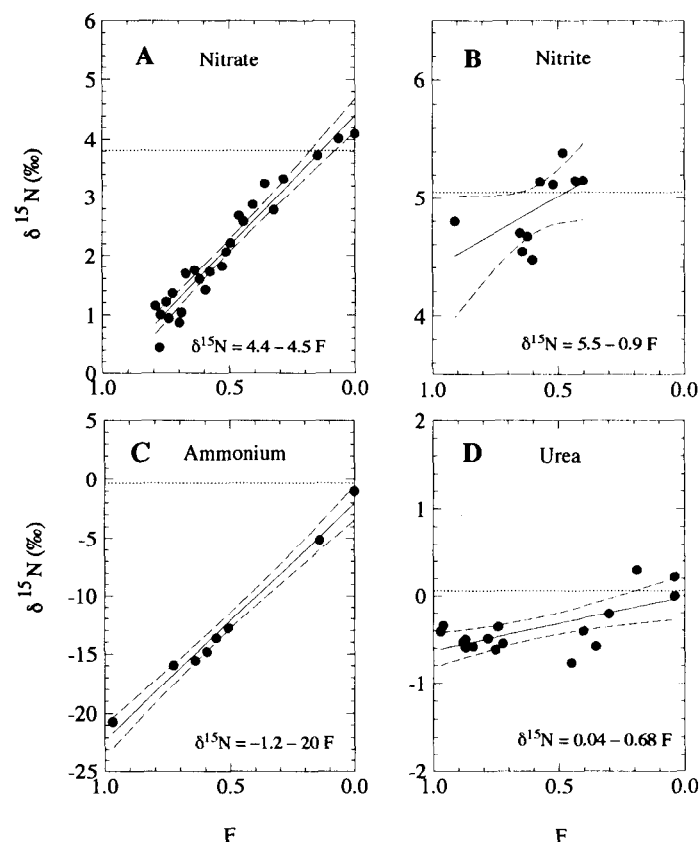


Fig. 5. Kinetic isotope fractionation during log phase for *T. pseudonana* growth on nitrate (data from Fig. 1), nitrite, ammonium, and urea (data from the first log phase, i.e. from 0 to 55 h, presented in Fig. 3). F is related to the fraction of unconsumed dissolved N, f , by the relationship $F = [-f/(1-f)] \ln f$. The solid line represents the least-squares fit of the $\delta^{15}\text{N}_{\text{PN}}$, the dashed lines are the 95% confidence intervals, and the dotted lines are the $\delta^{15}\text{N}$ of the initial N source. The regression is shown for each substrate.

Table 1. Nitrogen isotopic fractionation by *T. pseudonana* during the uptake and assimilation of nitrate, nitrite, ammonium, and urea. Standard deviations are indicated for each computed intercept and ϵ values.

| Ex-periment | Sub-strate | μ^{\dagger} (d^{-1}) | Intercept (‰) | ϵ (‰) | r^2 | α |
|-------------|-----------------|--|------------------|-------------------|-------|----------|
| 1 | NO_3^- | 2.1 | 4.4 ± 0.1 | 4.5 ± 0.2 | 0.94 | 1.0045 |
| 1† | NO_3^- | 2.1 | 5.2 ± 0.2 | 6.3 ± 0.3 | 0.90 | 1.0063 |
| 1† | NO_3^- | 2.2 | 5.0 ± 0.2 | 4.5 ± 0.2 | 0.81 | 1.0045 |
| 2‡ | NO_3^- | 1.9 | 4.7 ± 0.2 | 5.1 ± 0.4 | 0.90 | 1.0051 |
| 2†,‡ | NO_3^- | 1.9 | 4.9 ± 0.2 | 5.9 ± 0.3 | 0.96 | 1.0059 |
| 3 | NO_2^- | 0.9 | 5.5 ± 0.3 | 0.9 ± 0.6 | 0.25 | 1.0009 |
| 4 | NH_4^+ | 1.9 | -1.2 ± 0.6 | 20 ± 1 | 0.98 | 1.0200 |
| 4† | NH_4^+ | 1.8 | -1.1 ± 0.8 | 19 ± 1 | 0.90 | 1.0190 |
| 5§ | Urea | 1.3 | 0.0 ± 0.1 | 0.7 ± 0.2 | 0.55 | 1.0007 |
| 5†,§ | Urea | 1.4 | -0.2 ± 0.2 | 1.0 ± 0.3 | 0.52 | 1.0010 |

* Maximum specific growth rates were derived from in vivo fluorescence.

† Duplicate cultures not shown in the paper.

‡ Cultures similar to Exp. 1 except that initial N:P was 14:1 instead of 4:1.

§ Data from phase 1 only.

in vivo enrichment factors (means \pm SE) were $5.2 \pm 0.2\%$ ($n = 5$) for nitrate, $0.9 \pm 0.6\%$ for nitrite, $20 \pm 1\%$ ($n = 2$) for ammonium, and $0.8 \pm 0.6\%$ ($n = 2$) for urea incorporation (calculation was made for phase 1 only) (see Table 1). For nitrite and urea, the small changes in the $\delta^{15}\text{N}_{\text{PN}}$ relative to the 1σ of each measurement (i.e. 0.17%) is responsible for the lower r^2 . For growth on nitrate, the slope is constant over the entire range of F values, showing that the approximation of a one-step unidirectional reaction was valid. The intercepts averaged $4.8 \pm 0.2\%$ ($n = 5$), a little higher than the source $\delta^{15}\text{N}$ (i.e. $3.8 \pm 0.2\%$). Carryover of ^{15}N -enriched NO_3^- during the inoculation and dilution of the culture with new medium might account for a small enrichment of 0.2–0.3% each but is unlikely to account for all of it. Therefore, the remaining 0.4–0.6% enrichment has to be attributed to some other process. For nitrate-grown cultures, the total N mass balances were very good (within $10 \mu\text{mol N liter}^{-1}$), but we cannot rule out that a small fraction of total N (<5%) might not have been accounted for. Excretion of DON, NO_2^- , and(or) NH_4^+ with low $\delta^{15}\text{N}$ would produce a small ^{15}N enrichment in PN, which might explain the occurrence of slightly higher intercepts.

For growth on ammonium, ϵ agreed well with the estimate of 19.6% made from first product calculation, showing that the accumulated product equation applied well to the data. This conclusion was further corroborated by the constant slope over the entire range of F values, showing that the assumption of a one-step unidirectional reaction was valid (Fig. 5C).

Discussion

Biogeochemical implications—Oceanic estimates of ϵ for nitrate incorporation are rare because of lack of data on the $\delta^{15}\text{N}$ of nitrate as well as difficulties in sampling events such as blooms. Nonetheless, estimates ranging from 4 to 9% have been derived using various approaches (Table 2). Interestingly, our estimate of ϵ of 5.2% for nitrate incorporation by *T. pseudonana* falls close to an average oceanic value of ~6%. Environmental variables such as light intensity, temperature, substrate concentration, and other variables such as taxa may be responsible for the range of values observed in the ocean (Wada and Hattori 1978; Montoya and McCarthy 1995; Pennock et al. 1996). Laboratory cultures have shown that diatoms have a higher ϵ value than do flagellates (Montoya and McCarthy 1995). The present estimate of 5.2% for *T. pseudonana* (we determined an ϵ of 5–6% for growth of *T. weissflogii* on 200 μM of nitrate; unpubl. results) is lower than the estimates of 9 and 12% for *S. costatum* and *T. weissflogii*, respectively (Montoya and McCarthy 1995; Pennock et al. 1996).

For NH_4^+ -grown phytoplankton, the present estimate of 20% is substantially higher than the field estimates of 6.5–9% determined for ammonium-rich conditions in estuaries (Table 2). The discrepancy might be due in part to differences in the NH_4^+ concentrations in estuaries (i.e. generally <20 μM) vs. laboratory cultures (Hoch et al. 1992; Pennock et al. 1996). Alternatively, two N substrates might be taken up simultaneously by phytoplankton (i.e. ammonium and

Table 2. Estimates of the “per mil enrichment factor” ϵ in various oceanic regions.

| Location | ϵ (‰) | Reference |
|--|-------------------|------------------------------|
| NO_3^--based productivity | | |
| Pacific | | |
| North Pacific Ocean (44°N, 154°E) | 5 | Wada 1980 |
| Subarctic Pacific Ocean (50°N) | 5–6 | Wu et al. 1997 |
| Atlantic | | |
| North Atlantic Ocean (47°N, 20°W) | 8–9 | Altabet et al. 1991 |
| Warm-core ring | 4 | Altabet and McCarthy 1985 |
| Southern Ocean | | |
| Weddell Sea (60–70°S) | 6 | Biggs et al. 1988 |
| Southern Ocean | 5–6 | Sigman et al. 1996 |
| Coastal Environment | | |
| Auke Bay, Alaska (58°N) | 4 | Goering et al. 1990 |
| Chesapeake Bay | 7 | Horrigan et al. 1990 |
| NH_4^+-based productivity | | |
| Delaware Estuary | 9* | Cifuentes et al. 1989 |
| Chesapeake Bay | 6.5–8 | Montoya et al. 1991 |

* The dominant species was *Skeletonema costatum*.

urea), thus producing a lower apparent isotope fractionation (Waser unpubl. results). In the oligotrophic Atlantic and Pacific Oceans, where NH_4^+ is the major N substrate for phytoplankton, there is no evidence to support a large isotope fractionation. In nutrient-poor environments, the $\delta^{15}\text{N}$ of suspended particulate matter is generally quite low, i.e. -2 – 2% (Altabet 1988; Villareal et al. 1993), compared to 4–10% found in more eutrophic areas (Wada and Hattori 1976; Altabet and McCarthy 1985; Waser et al. 1997), suggesting the use of a substrate other than nitrate. Nevertheless, it is not low enough to suggest a 20% fractionation. This is not entirely surprising. It may mean that fractionation during N incorporation at nanomolar levels of NH_4^+ is close to zero because N uptake may become rate limiting. Thus, in this situation, all the NH_4^+ that is transported into the cell is likely to be assimilated with little or no N efflux (of whatever N species is leaking out of the cell). The recent findings of Pennock et al. (1996) of a lower fractionation of 8% at 5–20 μM NH_4^+ relative to the higher fractionation of 25–27% at higher NH_4^+ concentrations support the idea of a change in fractionation with NH_4^+ concentrations. Field studies have shown that the kinetics of NH_4^+ uptake are saturated well below 1 μM NH_4^+ in the oligotrophic ocean (Harrison et al. 1996), suggesting that a change in fractionation may occur at nanomolar NH_4^+ concentrations. Alternatively, the absence of evidence for a large fractionation in the oligotrophic ocean does not necessarily mean that fractionation is small. Fractionation may be larger than zero but due to the efficient and quick recycling of N in the surface layer (i.e. low f ratios and low new production, see Harrison et al. 1996), the $\delta^{15}\text{N}_{\text{PN}}$ may tend to always reflect the $\delta^{15}\text{N}$

Table 3. Relevant kinetic and thermodynamic N isotope fractionation for the incorporation of nitrate and ammonium (Glu, glutamate; Gln, glutamine; Ox, oxaloacetic acid; Asp, aspartic acid; α , α -ketoglutaric acid).

| Reaction (enzyme) | β (‰) | Material | Reference |
|--|-----------------|--------------------------------------|--|
| Kinetic isotope effects | | | |
| $\text{NO}_3^- \rightarrow \text{NO}_2^-$ (Nitrate reductase) | 15 30 | Spinach <i>Chlorella vulgaris</i> | Ledgard et al. 1985 Schmidt and Medina 1991 |
| $\text{NH}_3 + \text{Glu} \rightarrow \text{Gln}$ (Glutamine synthetase) | 16.5 8–12.5* | Spinach <i>E. coli</i> | Yoneyama et al. 1993 Hoch et al. 1992 |
| $\text{Glu} + \text{Ox} \rightarrow \text{Asp} + \alpha$ (Transaminase) | 8.3 | Porcine heart | Macko et al. 1986 |
| $\text{Asp} + \alpha \rightarrow \text{Glu} + \text{Ox}$ (Transaminase) | 1.7 | Porcine heart | Macko et al. 1986 |
| $\text{CO}(\text{NH}_2)_2 \rightarrow 2\text{NH}_3 + \text{CO}_2$ (Urease) | 8.6 | | Schmidt and Medina 1991 |
| Thermodynamic effects | | | |
| $\text{NH}_4^+(\text{aq}) \leftrightarrow \text{NH}_3(\text{aq}) + \text{H}^+$ | 19 | | Hermes et al. 1985 |
| $\text{NH}_3(\text{aq}) \leftrightarrow \text{NH}_3(\text{g})$ | 5 | | Kirshenbaum et al. 1947 |

* Corresponding to pH of 7.1–8.6.

of NH_4^+ , which has been estimated at -2.1 , -1.5% (Altabet 1988).

Fractionation during growth of diatoms on nitrate—Part of the difference between our present estimate of 5.2% and the recent estimates of 9 – 12% (Pennock et al. 1996; Montoya and McCarthy 1995) is possibly related to a difference in the balance between the rate of N influx and N efflux across the plasmalemma membrane; the greater the N efflux, the closer the in vivo isotope fractionation would be to the intrinsic isotope fractionation of the rate-limiting enzymatic step (Wada and Hattori 1978; Handley and Raven 1992). In turn, the rate of N efflux among phytoplankton species may be related to the size of the intracellular N pools. The N isotope studies are consistent with a study by Dortch et al. (1984), who reported that *S. costatum* grown on nitrate (in batch cultures with initial nitrate concentration of $100 \mu\text{M}$) accumulated a larger internal pool of nitrate than did *T. pseudonana* grown in the same conditions.

In earlier culture work, Wada and Hattori (1978) argued that the high ϵ values of 13 – 16% observed for the growth of *P. tricornutum* at low light (i.e. 500 lux or $8 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) were due to the breakage of the N–O covalent bond by nitrate reductase (NR). This assimilation step is thought to become rate limiting at low light, allowing the full expression of the intrinsic isotope fractionation (β) associated with NR of perhaps 15 – 30% (Table 3). A high ϵ value of 18% has also been determined in higher plant studies when NR activity was lowest and thus possibly rate limiting (Mariotti et al. 1982). Furthermore, Yoneyama and Kaneko (1989) found high ^{15}N enrichment of intracellular nitrate relative to other internal pools as well as extracellular nitrate and concluded that isotopic fractionation occurred during reduction of nitrate to nitrite. However, in most higher plants studies, ϵ is $<5\%$ and most often zero (Mariotti et al. 1980; Kohl and Shearer 1980; Mariotti et al. 1982). This

small fractionation has been attributed to fractionation during the transport of nitrate across the plasmalemma membrane. In marine diatoms and other phytoplankton, the mechanism of N fractionation is not known. In *T. pseudonana*, nitrate is actively taken up and accumulates inside the cells (i.e. cytoplasm and/or vacuoles) to concentrations that are much higher (usually mM per cell volume) than ambient nitrate (Dortch et al. 1984). This is consistent with the relatively large (relative to oceanic nitrate concentrations) K_m of 0.047 mM for NR of *T. pseudonana* (Berges and Harrison 1995). There is still great debate as to which step is rate limiting in the incorporation of N, i.e. transport across the membrane or reduction of nitrate to nitrite (Berges 1993). A simultaneous study of N-stable isotope determinations in extra- and intracellular nitrate, nitrite, ammonium, and organic N pools could provide insight into this issue.

For nitrite, the estimate of ϵ is in good agreement with the only other estimate of 0.7% for *P. tricornutum* (Wada and Hattori 1978). Both studies suggest that the intrinsic isotope fractionation by nitrite reductase is either small or, alternatively, it is not expressed because transport across the membrane is rate limiting. Note that growth on nitrite is only possible if the cells have been acclimated to nitrate prior to growth on nitrite (or if some nitrate is added to the medium).

Fractionation during diatom growth on ammonium—Fractionation during the growth of *T. pseudonana* on ammonium found in the present study is similar to the estimate of Pennock et al. (1996) for *S. costatum* grown in batch cultures at NH_4^+ concentrations of 20 – $100 \mu\text{M}$. Contrary to the findings of these authors, we do not observe a concentration dependence at low concentrations (i.e. 5 – $20 \mu\text{M}$) owing to the fact that the high initial NH_4^+ concentration makes the accumulated product approach used in this paper to calculate ϵ insensitive at low NH_4^+ concentrations. Previous determinations of ϵ for NH_4^+ incorporation have ranged

widely, i.e. from inverse discrimination (ϵ of -9.7%) to normal positive discrimination of up to 25% (Wada and Hattori 1978; Wada 1980; Pennock et al. 1996). A high ϵ value of 13.6% was also observed for a freshwater alga (Macko et al. 1987). Results from higher plants also show substantial discrimination, with ϵ as high as 12.6% for whole rice plants (Yoneyama et al. 1991). The values of ϵ for growth on micromolar levels of NH_4^+ appear to be higher than for growth on millimolar levels of NH_4^+ . Part of this difference may be due to a difference in the primary assimilation pathway (e.g. at low and high NH_4^+ concentrations). This hypothesis is supported by a study of a marine bacterium where a switch in pathway was suggested to account for the dramatic change in ϵ with NH_4^+ concentration (Hoch et al. 1992).

The presence of an acid-base equilibrium between NH_4^+ and NH_3 and the possibility that both species may be taken up (Syrett 1981) are possibly in part responsible for the great variability of ϵ . As the pH of the culture increased from an initial value of 7.5 to 8.7 at stationary phase and using a pK of 9.58 at 18°C (Whitfield 1978), NH_3 was estimated to increase from 0.8 to 10% of dissolved inorganic N (DIN) (the pH effect is somewhat counterbalanced by the decrease in DIN). Furthermore, at pH of 8–8.6 in log phase, NH_3 comprised between 3 and 10% (1–10 μM) of total N in our culture experiment. In contrast to the behavior of NH_4^+ , NH_3 is very permeable in lipid microlayers and can thus diffuse passively across the plasmalemma membrane depending on the chemical gradient (Kleiner 1981). With a permeability of $1.8 \times 10^{-5} \text{ m s}^{-1}$ (Hoch et al. 1992), an internal pH of 7, a cell surface area of $64 \mu\text{m}^2$, and a NH_3 gradient of 10 μM across the membrane (assuming no intracellular NH_3), an estimated flux of $10^{-5} \text{ pmol N s}^{-1} \text{ cell}^{-1}$ was determined. An incorporation rate of $2 \times 10^{-6} \text{ pmol N s}^{-1} \text{ cell}^{-1}$ was calculated from a cell quota of $1.2 \text{ pg N cell}^{-1}$ and a specific growth rate of 1.9 d^{-1} . Thus, passive diffusion of NH_3 is on the same order of magnitude as the observed N incorporation rate. Nevertheless, we suspect that the gradient of NH_3 may be reversed because of active uptake of NH_4^+ . In a batch culture study of *T. pseudonana* grown on 100 μM NH_4^+ , Dortch et al. (1984) showed that the intracellular pool of NH_4^+ may be large, perhaps as large as 36 mM (per cell volume) for N-sufficient cells. Thus, NH_3 leaking out of the cells would supply ^{15}N -depleted NH_3 to the medium relative to NH_4^+ in the cells (Table 3). The NH_3 leak, if important, would tend to produce an inverse discrimination. This process may be in part responsible for values of α lower than zero found in *Chaetoceros* spp. grown on high NH_4^+ (Wada and Hattori 1978). In the present study this is clearly not the case, suggesting that the leak may be relatively small.

How is the large and rather constant 20% enrichment observed for *T. pseudonana* grown on 190 μM of NH_4^+ to be explained? Potential candidates for an explanation include the primary enzymes for NH_4^+ assimilation, i.e. glutamine synthetase (GS) and transaminases. The transaminases have intrinsic isotope fractionations that are lower than the observed in vivo isotope fractionation and are thus unlikely to be responsible for the observed 20% (Table 3). In a recent study of spinach leaves, Yoneyama et al. (1993) determined that β was 17% for GS and suggested that if NH_3 was the substrate for GS (Kleiner 1981), then essentially all the frac-

tionation may be due to the equilibrium between NH_3 and NH_4^+ . Furthermore, if the observed 20% in vivo isotope fractionation for *T. pseudonana* grown on ammonium is due mostly to the intrinsic isotope fractionation of GS, then a significant N efflux must exist. NH_4^+ would be the most likely N species to efflux since we have argued that NH_3 efflux must be small.

Fractionation during growth of diatoms on urea—This is the first study on urea fractionation by phytoplankton. The overall fractionation of 0.9% is surprisingly small. We had anticipated that a large discrimination between ^{14}N and ^{15}N would take place in view of the large fractionation observed during NH_4^+ incorporation and previous knowledge of NH_3 efflux (passive and unavoidable diffusion across the membrane) and NH_4^+ influx (via active uptake) during growth of *T. pseudonana* on urea (Price and Harrison 1988). We observed a small fractionation of 0.9% and low concentrations of NH_4^+ throughout the growth phases. Thus, if ammonium was excreted during growth on urea, it must have been taken up immediately and never mixed with the medium outside the unstirred boundary layer. This futile cycle would explain the absence of a large fractionation during both phase 1 and 2. The involvement of DON (possibly free amino acids) during phase 2 following the addition of urea is likely to be the reason for the prolonged occurrence of low $\delta^{15}\text{N}_{\text{PN}}$, even at times when the added urea was totally utilized. Price and Harrison (1988) reached similar conclusions when 85% of the urea transported into the cells of *T. pseudonana* in nitrate-sufficient cultures could not be accounted for.

This study has shown that under nonlimiting nitrate concentrations, fractionation during the incorporation of nitrate by a marine diatom is in good agreement with field estimates made in oceanic regions where nitrate is in excess (i.e. Equatorial Pacific and Southern Ocean). In these regions, the quantification of past changes in the excess of nitrate at the ocean surface has been made using a constant fractionation of 5% (Farrell et al. 1995). The relatively high $\delta^{15}\text{N}$ of bulk sedimentary organic matter suggests that fractionation during NO_3^- incorporation is the dominant process relative to NO_2^- , NH_4^+ , and urea incorporation. The present study based on one diatom species grown in micromolar levels of nitrate supports, perhaps fortuitously, the use of an average oceanic fractionation of 5% for past excess nitrate reconstruction. Further studies will be required to predict the extent to which discrimination changes with temperature, light intensity, species composition, and substrate concentration.

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