Dilution of $^{15}$N in *Dunaliella tertiolecta* by uptake of an unidentified compound following nitrate exhaustion

**Abstract** — Nitrate (about 20 μM) was added as $^{15}$NO$_3^{-}$ to a nitrate-limited continuous culture of *Dunaliella tertiolecta* at steady-state. Nitrate uptake was then estimated from the decrease in nitrate in the medium, the incorporation of $^{15}$N into cells, and the increase in cellular nitrogen. Although the overall nitrogen budget over 5 h was balanced, there were large differences in estimates (up to a factor of five) of nitrate assimilation by the three methods on shorter time scale. After nitrate was exhausted from the medium, cellular nitrogen continued to increase while the $^{15}$N content of the particulate matter decreased over the next 1.5 h. This indicated that an unidentified, unlabelled nitrogen form, which was neither nitrite, ammonium nor dissolved free amino acids, was being taken up by the cells, at rates comparable to those of nitrate. This phenomenon leads to an underestimation of new biomass production when assessed through $^{15}$N incorporation into cells. (© Académie des sciences / Elsevier, Paris.)

---

Note communicated by Michel Thellier

*Correspondence and reprints
E-mail: collos@hydrobio.univ-montp2.fr

1. Introduction

Wide discrepancies in estimates of new production by different methods [1-3] have revealed a need for a detailed understanding of the underlying mechanisms of nitrate assimilation by phytoplankton. During this process, there are several steps during which nitrogen can be lost from the cells. For example, nitrite excretion may occur in significant amounts relative to nitrate uptake [4-6]. Dissolved organic nitrogen has also been shown to be produced [7, 8] in amounts which could seriously affect estimates of nitrate flux through the phytoplankton compartment. In order to improve interpretations of such measurements, we chose to study an unialgal culture, as a simple system, where the number of influencing variables is reduced relative to a complex food web. In this paper, we examine several aspects of nitrate assimilation in a nitrate-limited culture of a marine flagellate.

2. Methods

Dunaliella tertiolecta was grown in nitrate-limited continuous cultures [6, 9] under 417 μE.m⁻².s⁻¹ and a 8-h L-20-h D cycle. Temperature was 18 °C, and dilution rate 0.5 day⁻¹. The input medium was enriched seawater [10] with a nitrate concentration set at 46 μM. The culture was not axenic. However, the biovolume associated with small particles (about 1 μm) was about a thousand times lower than that associated with Dunaliella. Thus, we do not expect bacterial activity to have any significant effect on our results. At time zero (around 10 h 15 min, about 2 h after the beginning of the light period), the feed pump was stopped and sodium nitrate (9.5 % enrichment in ¹⁵N) was added to give a final concentration of about 20 μM. Samples were then taken every 30 min for the next 5 h 30 min and the following analyses were carried out. Nitrate and nitrite in the medium were measured immediately by colorimetric methods [11], ammonium was fixed immediately by addition of reagents [12], dissolved free amino acids by HPLC [13]. Dissolved inorganic nitrogen (DIN) is defined as the sum of nitrate, nitrite and ammonium, with a precision of 1 %. Cells are collected on Whatman GF/C glass fibre filters under reduced vacuum. Cellular nitrogen and incorporation of ¹⁵N are measured with a Roboprep/TracerMass instrument [14]. Precision is 1.4 % for PN and 1.5 % for the isotopic ratio. Net nitrate uptake rates (Rho) were calculated as follows [15]:

\[
\text{RhoNO}_3 = \frac{\text{PNf(Cp - Co)}/(Cd - Co)}{dt}
\]

where \(\text{PNf} = \text{final cellular nitrogen}\), \(\text{Cp} - \text{Co} = ¹⁵\text{N}\) enrichment of cells, \(\text{Cd} - \text{Co} = ¹⁵\text{N}\) enrichment of nitrate and \(dt = \text{incubation duration}\).
The concentrations of N compounds in the medium and in the cells were assayed following a pulse of 20 μM NO₃. The changes in cell total N (ΔPN) were estimated from chemical assays of cell samples. NO₃ is the NO₃ concentration in the medium and DFAA that of the dissolved free amino acids. Net nitrate uptake was estimated from disappearance of nitrate measured by chemical means (ΔNO₃) and ¹⁵N accumulation in cells (Δ¹⁵NO₃).

### 3. Results

The changes in nitrate, cellular nitrogen (PN) and dissolved free amino acids (DFAA) are shown in figure 1. The increase in PN, rather than the absolute values, is shown in order to be directly comparable to the decrease in nitrate concentration. Immediately following the addition of labelled substrate, nitrate began to decrease in the medium. In contrast, there was a 1-h lag in PN increase. Nitrite ranged from 0.31 to 0.01 μM and showed a decreasing trend in concentrations with time, without evidence of nitrite excretion. Ammonium remained at low levels throughout the experiment. The DFAA pool exhibited a small pulse in the first 30 min following the addition of nitrate, as well as another one once nitrate was exhausted. The sum of nitrate, nitrite, ammonium, DFAA and PN was 63.7 μM at time zero and 65.5 μM at t = 5 h, indicating essentially no changes in system nitrogen over the duration of measurements. Over shorter time scales, however, this sum exhibited significant variations (figure 2), going through a minimum at T=3.5 h, which corresponded to exhaustion of nitrate. Afterwards, this sum increased back to the initial value.

Disappearance of nitrate from the medium and the incorporation of nitrate in cells, estimated by the ¹⁵N tracer method, were in good agreement (figure 1). Comparisons of those two estimates with changes in cellular nitrogen also indicated good agreement over a time scale of 5 h (17.6 and 17.8 μM, respectively). However, on shorter time scales, there was a clear imbalance, which is apparent in figure 1 where PN increases showed a lag phase of about 1 h. During the next half hour, the ratio between estimates of net nitrate uptake (disappearance of nitrate in the medium and incorporation of nitrate in cells estimated by the ¹⁵N method) and increases in cell nitrogen was about five. At t = 3 h (figure 1), the trend was reversed, with more accumulation of N in the cells than could be accounted for by the decrease in nitrate in the medium or the incorporation of ¹⁵N. The ¹⁵N enrichment of cells reached a maximum when nitrate was exhausted (at 3.5 h). Then, it decreased significantly (by about 5 %,

---

**Figure 1.** Nitrogen use during NO₃-triggered growth of D. tertiolecta suspension.

**Figure 2.** Changes in system N (sum of cellular nitrogen (PN), dissolved inorganic nitrogen (DIN) and dissolved free amino acids (DFAA)) with time following the nitrate pulse.
from 2.72 to 2.59 atom% excess) during the next 1.5 h when the measurements were terminated. Over the same time period, nitrate uptake estimated from both substrate disappearance and $^{15}$N incorporation was equal to zero, but cell nitrogen kept on increasing (figure 1), by about 4 µM over the next 1.5 h.

4. Discussion

The patterns outlined in figure 1 have already been observed in several data sets [16]. While the overall long-term N budget is balanced, there are wide imbalances over shorter time scales, and these do not appear to be due to analytical errors or lack of precision in measurements. The first part of the incubation (until $T = 3$ h) could be interpreted as due to release of nitrogen compounds such as dissolved organic nitrogen (DON). This is similar to what Flynn and Davidson [17] called loss of 'system N' (i.e. the sum of DIN + PN + DFAA) which amounted to about 20% in their control culture of Isochrysis galbana. The magnitude of this loss is consistent with known rates of DON release during nitrate uptake [7, 18]. In our study and that of [16], the only indication about the nature of the DON released is that DFAA can be ruled out. The levels of DFAA are similar to those measured in a previous study [19], indicating that Dunaliella tertiolecta released or used very little DFAA.

The patterns outlined in our 'system N' (figure 2) are also remarkably similar to those shown in a culture of Skeletonema costatum following a nitrate pulse [20]. In this study, while the system N (NO$_3$ + NH$_4$ + PN) at time zero is equal to that 4 days later, it exhibits a 35% drop in the meantime, and goes through a minimum corresponding to nitrate exhaustion. Thereafter, the PN increases by about 20 µM over the next 24 h (a 40% increase over the minimum value). Although part of this increase could be explained by uptake of previously excreted nitrite, which was not measured [20], most of it has to be attributed to another N compound because $S$. costatum does not excrete such high levels of nitrite [3, 21, 22].

The second part of our incubation (3.5–5 h in figure 1) exhibited exactly the same pattern as discussed above: increase of PN in absence of DIN, as well as constant DFAA levels. In addition, the striking contrast between the simultaneous increase in PN and decrease in $^{15}$N enrichment of the particulate matter once nitrate is exhausted indicates that $^{15}$N is diluted inside the cells by an unidentified N compound which is taken up actively. Moreover, this compound does not seem to be DFAA, nitrite or ammonium. Regular checks on these compounds can rule out this possibility. The lack of nitrite excretion, at least during the light phase, confirms previous studies on this species [23]. Note that the dilution of the $^{15}$N (by about 5%) in the cells following DIN exhaustion is consistent with the PN increase over the same time interval (about 7%).

Except for urea, very little quantitative work has been carried out on uptake of DON other than DFAA, but it is known that a large number of organic N compounds can be used by microalgae [24, 25]. However, in contrast to these reviews which point out low DON uptake rates, it appears from the PN increase after nitrate exhaustion (figure 1) that the uptake rate of the unidentified compound is high, and as high as that of nitrate in the first part of the incubation. Our results are consistent with more recent studies [26, 27] showing that DON uptake can be as high as DIN uptake.

5. Conclusion

The comparison of net nitrate uptake and N accumulation in cells allows us to define three different phases. First (from $t = 0$ to 1 h 30 mins), a release of the unidentified compound (X) from a pre-existing unlabelled pool, which is triggered by the nitrate pulse (initial lag phase in PN accumulation). A second phase of stability (until $t = 3$ h) indicates that the net uptake of X is negligible as long as nitrate is present in the medium. Finally, X is reabsorbed following nitrate exhaustion from the medium.

The internal consistency of the data on isotopic dilution and PN increase following nitrate exhaustion allows us to conclude that the compound taken up is not labelled with $^{15}$N. If this compound has been previously excreted, then isotopic equilibrium between source and product was not reached within the time scale of our experiment. It remains, however, that the dilution of the internal $^{15}$N leads to underestimate nitrate uptake and new production as estimated by the $^{15}$N isotopic tracer method.

Acknowledgment: This paper is a contribution from the APPA group (Dyfamed/France JGOFS program).

6. Références


