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

Dilution de ^{15}N dans Dunaliella tertiolecta *par absorption d'un composé non identifié après épuisement du nitrate*

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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.)

nitrogen budget / nitrate uptake / new production / ^{15}N dilution / Dunaliella

Résumé – L'absorption nette de nitrate par une culture continue de *Dunaliella tertiolecta* est suivie à partir de la diminution de nitrate dans le milieu ainsi que par l'incorporation dans les cellules d'azote 15 ajouté sous forme de nitrate. Bien que le bilan d'azote soit équilibré sur la totalité de l'expérience (5 h), il apparaît, à plus court terme, de grandes différences (jusqu'à un facteur de cinq) entre les estimations de prise de nitrate par les deux méthodes. À partir du moment où le milieu est épuisé en nitrate, l'azote des cellules continue de croître et son enrichissement en azote 15 diminue. Cette dilution isotopique indique qu'une forme d'azote non identifiée, qui n'est ni le nitrite, ni l'ammonium ni les acides aminés libres dissous, est assimilée par les cellules, à des taux comparables à ceux du nitrate. Ce phénomène entraîne une sous estimation de la production nouvelle (production primaire soutenue par le nitrate). (© Académie des sciences / Elsevier, Paris.)

bilan d'azote / absorption nette de nitrate / production nouvelle / dilution isotopique / Dunaliella

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Note communicated by Michel Thellier

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Version abrégée

Une controverse concerne actuellement les différentes méthodes d'estimation de la production primaire de biomasse océanique basée sur le budget azoté du phytoplancton. Cela révèle la nécessité d'une compréhension détaillée des mécanismes de l'assimilation du nitrate par le phytoplancton marin. Au cours de ce processus, plusieurs étapes entrent en jeu, durant lesquelles de l'azote peut être perdu par les cellules. Afin d'améliorer l'interprétation de telles mesures, nous avons utilisé un système simple, une culture unialgale de flagellé marin, où le nombre de variables est réduit par rapport à un réseau trophique naturel. L'expérience est réalisée à l'aide d'une culture continue de *Dunaliella tertiolecta* dont la croissance est limitée par le nitrate. Une fois la culture maintenue à l'équilibre, on effectue une addition de nitrate (20 µM) marqué à l'azote 15. On suit la diminution de nitrate dans le milieu ainsi que l'incorporation de l'azote 15 dans les cellules. En fin d'expérience, la somme des composés azotés mesurés (nitrate, nitrite, ammonium, acides aminés libres dissous, azote particulaire) est identique à celle de départ. Néanmoins, cette somme passe par un minimum à $t = 3{,}5$ h qui correspond à l'épuisement en nitrate du milieu. La comparaison entre les deux méthodes d'estimation de la prise de nitrate et l'augmentation de l'azote cellulaire montre également un déséquilibre à court terme et leur différence un minimum marqué au même moment. L'enrichissement en ^{15}N des cellules atteint un maximum également à ce moment, puis diminue ensuite de manière significative (2,72 à 2,59 atome% excès) pendant l'heure suivante. Pendant le même laps de temps, la prise de nitrate estimée par voie chimique et isotopique est égale à zéro. Néanmoins, l'azote des cellules continue d'augmenter.

La comparaison des cinétiques d'absorption nette de nitrate (calculée d'après la diminution dans le milieu) et d'accumulation de N dans les cellules montre que le phénomène étudié comprend trois phases distinctes. Une première phase qui consiste en une exsorption du composé inconnu (X) à partir d'un pool préexistant non marqué, déclenchée par l'apport de nitrate (latence initiale de l'accumulation de N). Cette phase se termine à 1 h 30. Une phase de stabilité s'ensuit pendant l'heure et demi suivante (jusqu'à $t = 3$ h). Le parallélisme raisonnable des deux courbes (diminution de nitrate et accumulation de N dans les cellules) indique que l'absorption nette de X est négligeable tant que le nitrate est présent dans le milieu. Enfin, une phase de réabsorption de X qui suit l'épuisement du nitrate du milieu. S'il s'agit d'un phénomène de réabsorption d'un composé préalablement excrété, il n'y a pas eu équilibre isotopique entre source et produit à l'échelle de temps utilisée pour réaliser cette expérience. Néanmoins, la dilution isotopique entraîne une sous-estimation de la prise de nitrate et de la production nouvelle estimée par la méthode à l'azote 15.

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 $\mu E.m^{-2}.s^{-1}$ 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 ^{15}N) 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 ^{15}N are measured with a Roboprep/TracerMass instrumentation [14]. Precision is 1.4 % for PN and 1.5 % for the isotopic ratio. Net nitrate uptake rates (Rho) were calculated as follows [15]:

$$\mathrm{RhoNO_3 = PNf(Cp - Co)/(Cd - Co)(dt)}$$

where PNf = final cellular nitrogen, Cp – Co = ^{15}N enrichment of cells, Cd – Co = ^{15}N enrichment of nitrate and dt = incubation duration

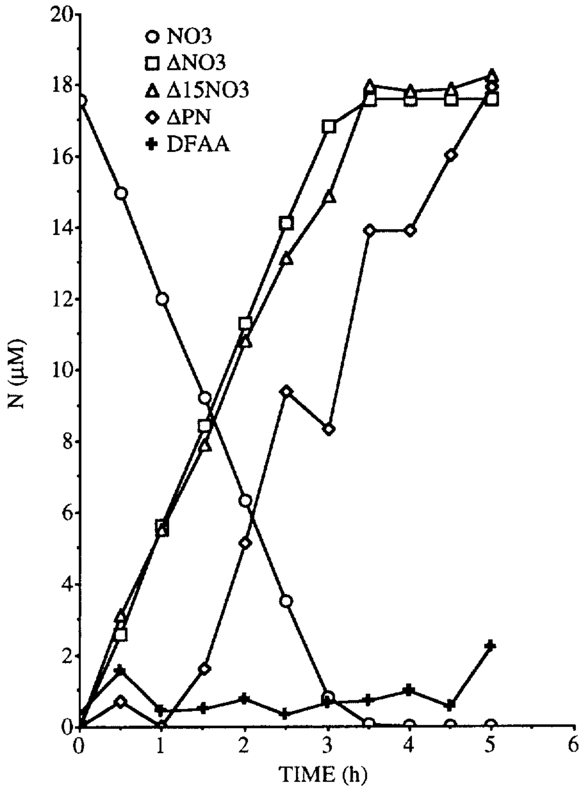

Figure 1. Nitrogen use during NO$_3$-triggered growth of *D. tertiolecta* suspension.

The concentrations of N compounds in the medium and in the cells were assayed following a pulse of 20 µM NO$_3$. The changes in cell total N (ΔPN) were estimated from chemical assays of cell samples. NO$_3$ is the NO$_3$ 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 (ΔNO$_3$) and ^{15}N accumulation in cells (Δ^{15}NO$_3$).

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 ^{15}N 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 ^{15}N 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 ^{15}N. The ^{15}N enrichment of cells reached a maximum when nitrate was exhausted (at 3.5 h). Then, it decreased significantly (by about 5 %,

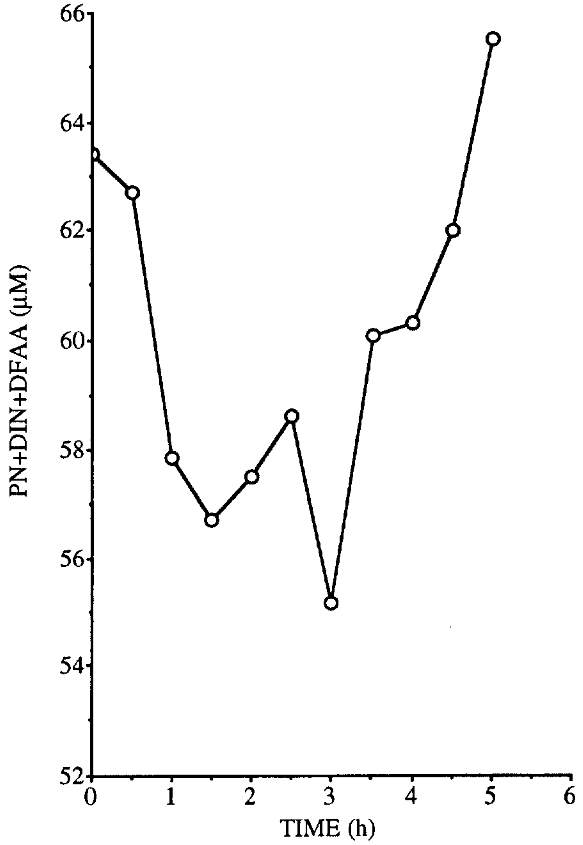

Figure 2. Changes in system N (sum of cellular nitrogen (PN), dissolved inorganic nitrogen (DIN) and dissolved free amino acids (DFAA)) with time following the nitrate pulse.

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 [5, 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.

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6. Références

[1] Iawyk G., Raimbault P., Gentilhomme V., On the discrepancies between a colorimetric and isotopic method for measuring nitrate utilization in nutrient-depleted waters: implications for the design of experimental protocols in new production studies, Hydrobiol. 207 (1990) 333–339.

[2] Eppley R.W., Renger E.H., Nitrate utilization by plankton in the Equatorial Pacific March 1988 along 150°W, J. Geophys. Res. 97 (1992) 663–668.

[3] Wilkerson F.P., Dugdale R.C., Measurement of nitrogen productivity in the equatorial Pacific, J. Geophys. Res. 97 (1992) 669–679.

[4] Kiefer D.A., Olson R.J., Holm-Hansen O., Another look at the nitrite and chlorophyll maxima in the central North Pacific, Deep-Sea Res. 23 (1976) 1199–1208.

[5] Collos Y., Transient situations in nitrate assimilation by marine diatoms. 2. Changes in nitrate and nitrite following a nitrate perturbation, Limnol. Oceanogr. 27 (1982) 528–535.

[6] Sciandra A., Amara R., Effects of nitrogen limitation on growth and nitrite excretion rates of the dinoflagellate *Prorocentrum minimum*, Mar. Ecol. Prog. Ser. 105 (1994) 301–309.

[7] Collos Y., Dohler G., Biermann I., Production of dissolved organic nitrogen during uptake of nitrate by *Synedra planctonica*: implications for estimates of new production in the oceans, J. Plankton Res. 14 (1992) 1025–1029.

[8] Bronk D.A., Glibert P.M., Ward B.B., Nitrogen uptake, dissolved organic nitrogen release, and new production, Science 265 (1994) 1843–1846.

[9] Malara G., Sciandra A., A multiparameter phytoplanktonic culture system driven by microcomputer, J. Appl. Phycol. 3 (1991) 235–241.

[10] Guillard R.R.L., Ryther J.H., Studies of marine planktonic diatoms. 1. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran, Can. J. Microbiol. 8 (1962) 229–239.

[11] Tréguer P., Le Corre P., Manuel d'analyse des sels nutritifs dans l'eau de mer, Lab. océanogr. chim., univ. Bretagne Occident., Brest, 1975, 110 p.

[12] Koroleff F., Determination of ammonia, in: Grasshoff K. (ed.), Methods of Seawater Analysis, Verlag Chemie, Weinheim, 1983, 150–157.

[13] Lindroth P., Mopper K., High performance liquid chromatography determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with O-phtaldialdehyde, Analyt. Chem. 51 (1979) 1667–1674.

[14] Owens N.J.P., Rees A.P., Determination of nitrogen 15 at submicrogram levels of nitrogen using automated continuous-flow isotope ratio mass spectrometry, Analyst 114 (1989) 1655–1657.

[15] Collos Y., Calculations of ^{15}N uptake rates by phytoplankton assimilating one or several nitrogen sources, Appl. Radiat. Isot. 38 (1987) 275–282.

[16] Collos Y., Nitrogen budgets and dissolved organic matter cycling, Mar. Ecol. Prog. Ser. 90 (1992) 201–206.

[17] Flynn K.J., Davidson K., Predator-prey interactions between *Isochrysis galbana* and *Oxyrrhis marina*. II. Release of non-protein amines and faeces during predation of *Isochrysis*, J. Plankton Res. 15 (1993) 893–905.

[18] Bronk D.A., Glibert P.M., A ^{15}N tracer method for the measurement of dissolved organic nitrogen release by phytoplankton, Mar. Ecol. Prog. Ser. 77 (1991) 171–182.

[19] Martin-Jézéquel V., Poulet S.A., Harris R.P., Moal J., Samain J.F, Interspecific and intraspecific composition and variation of free amino acids in marine phytoplankton, Mar. Ecol. Prog. Ser. 44 (1988) 303–313.

[20] DeManche J.M., Curl H.C. Jr., Lundy D.W., Donaghay P.L., The rapid response of the marine diatom *Skeletonema costatum* to changes in external and internal nutrient concentration, Mar. Biol. 53 (1979) 323–333.

[21] Serra J.L., Llama M.J., Cadenas E., Nitrate utilization by the diatom *Skeletonema costatum*. I. Kinetics of nitrate uptake, Plant Physiol. 62 (1978) 987–990.

[22] Martinez R., Transient nitrate uptake and assimilation in *Skeletonema costatum* cultures subject to nitrate starvation under low irradiance, J. Plankton Res. 13 (1991) 499–512.

[23] Laws E.A., Wong D.C.L., Studies of carbon and nitrogen metabolism by three marine phytoplankton species in nitrate-limited continuous culture, J. Phycol. 14 (1978) 406-416.

[24] Paul J.H., Uptake of organic nitrogen, in: Carpenter E.J., Capone D.G. (eds.), Nitrogen in the Marine Environment, Academic Press, New York, 1983, 275–308.

[25] Antia N.J., Harrison P.J., Oliveira L., The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology, Phycologia 30 (1991) 1–89.

[26] Wilkerson F.P., Grunseich G., Formation of blooms by the symbiotic ciliate *Mesodinium rubrum*: the significance of nitrogen uptake, J. Plankton Res. 12 (1990) 973–989.

[27] Bronk D.A., Glibert P.M., Application of a ^{15}N tracer method to the study of dissolved organic nitrogen uptake during spring and summer in Chesapeake Bay, Mar. Biol. 115 (1993) 501–508.