Vol. 187: 283–287, 1999 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published October 14

NOTE

Nitrate transport and ammonium-nitrate interactions at high nitrate concentrations and low temperature

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ABSTRACT: The implications of the operation of biphasic nitrate transport and the effects of low temperature incubations on phytoplankton N-physiology are discussed. The inclusion of 2 nitrate transport systems (low rate, high affinity; high rate, low affinity) with different regulatory thresholds and temperature-dependent steps can be included readily in the structure of existing mechanistic models of N-assimilation. These models can then reproduce the lack of complete repression of nitrate transport by ammonium at high nitrate concentrations documented for some species, and the enhancement of nitrate assimilation in low temperature incubations. Further research is required to clarify the temporal dynamics of these interactions in order to demonstrate their ecological significance and to validate the performance of the models.

KEY WORDS: Ammonium · Interaction · Nitrate · Model · Phytoplankton · Temperature

To enable the application of concepts and hypotheses generated by studies of individual species of phytoplankton to phytoplankton populations in general, we need to identify the more common and important themes. One approach is to attempt the construction of mechanistic models, containing biochemically identifiable components, which are capable of simulating these themes in different groups of phytoplankton. The ammonium-nitrate interaction model (ANIM) of Flynn et al. (1997) is such a model, developed in part to act as a dynamic review of our knowledge of phytoplankton physiology. If a model such as ANIM is found to be incapable of being readily modified to match the behaviour of major groups of phytoplankton, then we need to reconsider its structure and perhaps our understanding of the physiology on which it was based.

The purpose of this note is to consider the underlying physiology and simulation of aspects of nitrate assimilation at high concentrations of nitrate reported for some species. These aspects are the near-linear kinetics of nitrate transport (Watt et al. 1992, Collos et al. 1992, 1997, Lomas & Glibert 1999b), and the diminished repression (so called 'inhibition') of nitrate assimilation by ammonium at low temperature (Lomas & Glibert 1999a,b). This subject is important in certain marine systems, especially coastal lagoons and polar waters, which may often have high nitrate levels.

Kinetics of nitrate transport and incomplete ammonium depression of nitrate assimilation. The kinetics of nitrate transport are not always best described by a simple single Michaelis-Menten function, but may appear biphasic or near linear at high nitrate concentrations. Collos et al. (1997) and Lomas & Glibert (1999b) consider the involvement of diffusion of nitrate at high extracellular nitrate concentrations. However, nitrate is essentially lipid-insoluble and cannot pass through the plasma membrane unaided at any physiologically useful rate. In addition, an anion such as nitrate cannot be accumulated across a negatively charged membrane without the input of energy; an active (primary or secondary) transporter is required (see Raven 1980). Indeed, the equilibrium state is actually of a lesser concentration within the cell than outside (Raven 1980). The biphasic form of nitrate transport could not therefore be explained by enhanced diffusion into the cell (Collos et al. 1997). Even the occurrence of 'slippage' (Eddy 1982) through the nitrate transporter could only contribute to transport if the external nutrient concentration exceeded the internal. This is not likely in marine systems, though it may have been a contributing feature in the work of Watt et al. (1992), who employed up to mM nitrate concentrations.

Some leakage of nutrient via the transporter through which it entered the cell occurs with all transport systems, irrespective of any other process (Eddy 1982). Flynn & Berry (1999) used this argument in consideration of the loss of $DO^{15}N$ from phytoplankton. It is possible that high nitrate concentrations could counter the

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inevitable loss of nitrate out of the cell back through the transporter, down the electrochemical and concentration gradients. However, when attempting to model the system (with a leakage term as used by Flynn & Flynn 1998) to achieve the observed nitrate transport kinetics, a gross nitrate transport very much higher (by an order of magnitude) than the net transport is required. Such a system would appear to be extremely inefficient and not very plausible.

There is a precedent in higher plants and some algae for the operation of high rate, low affinity transporters for nitrate and other nutrients in addition to the low rate, high affinity transporter usually considered (reviewed by Nissen 1991). Multi-phasic or multiple transport systems are also common for various nutrients in bacteria and yeast (e.g. Kaback 1972, Eddy 1982). This perhaps offers a more likely explanation for the nitrate transport kinetics into at least some phytoplankton.

Linked to the multi-phasic nature of nitrate transport is the possible differential effect of ammonium on the kinetics of nitrate assimilation. Growth in the presence of ammonium concentrations >1 µM usually represses the use of nitrate completely, or nearly so (Dortch 1990, Flynn et al. 1997). If there is to be a significant use of nitrate under these conditions, it follows that repression cannot be complete, that nitrate is transported at a significant rate and that the enzymes of nitrate assimilation are synthesised and operable. Maximum transport rates for ammonium and nitrate vary with the N-status of cells, typically increasing with moderate N-stress and then declining with extreme N-stress. In ANIM these relationships are described by mathematical functions relating maximum transport rates to cellular N:C (Flynn et al. 1999). In microalgae that attain higher growth rates and N:C when growing on ammonium, there is little or no residual capacity to use nitrate. On the other hand, for there to be a potential for cells to use nitrate when supplied with ammonium at >1 µM, the difference between the maximum rates of nitrate and ammonium transport cannot be too dissimilar during N-replete growth at high N:C (discussed in Flynn et al. 1999). Further, the significance of nitrate-nutrition under such conditions will be enhanced if the capacity to transport nitrate is elevated at high nitrate concentrations by the presence of bi- or multiphasic transporters.

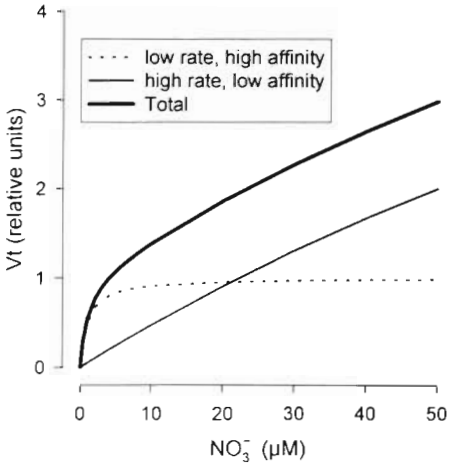

Fig. 1. Net nitrate transport kinetics for the operation of a low rate, high affinity system with a half-saturation constant (K_t) of 1 µM and maximum rate (Vt_{max}) of 1 d^{-1}, together with a high rate, low affinity system with K_t of 200 µM, and Vt_{max} of 10 d^{-1}

It would be logical for such multiple transport systems for nitrate to have different control thresholds, different levels of control metabolites (in this instance presumably early products of ammonium assimilation) being required to halt nitrate transport. This could allow, in the presence of ammonium, the continued use of nitrate supplied at high concentration entering via the low affinity system, but not of nitrate entering through the high affinity system. The latter might be expected to be more costly to construct or operate (perhaps being a primary rather than secondary active transporter, Falkowski 1975a,b, Raven 1980) and thus subject to tighter regulation.

Whatever the physiological basis for the duality of the nitrate transport, the process is simplest to include within models such as ANIM and the short-form version, SHANIM (Flynn & Fasham 1997), as a dual transport system. Collos et al. (1992) show data for the variability of biphasic nitrate transport with nutrient status, another indication that the transport must be mediated rather than diffusive. Further experimental studies are required to determine the relationship between the operation of the dual transport system and the N:C status, and to measure Vt_{max} and K_t (maximum rate and half-saturation constant for transport, respectively) for the transporters. Feedback control may be expected to occur rapidly for the high rate nitrate transporter, generating problems similar to those encountered in studies of ammonium transport kinetic (Flynn 1998). However, values of Vt_{max} an order of magnitude greater, and K_t 2 orders of magnitude greater than for the high affinity transporter give combined transport kinetics (low plus high affinity systems, Fig. 1) similar to those in Lomas & Glibert (1999b). These have been used in the simulations presented here; alternative strategies or values of Vt_{max} and K_t from other data sets could be substituted readily.

When ANIM is equipped with a dual nitrate transport system (Fig. 1), with differential regulation of transport via these porters by internal glutamine (see legend to Fig. 2), it can reproduce data sets showing different degrees of ammonium repression of nitrate

assimilation, like those shown in Lomas & Glibert 1999a. There is a problem with the model, however, in that the status shown in the experimental data after a few hours of incubation with ammonium is not readily achieved by the model in less than 24 h. The solution is not yet clear; the interpretation of the dynamics of ammonium-nitrate interactions is complicated by the time scale of the processes (Flynn 1998) as well as by simplifications within the model. An obvious simplification that would influence the time scale of feedback is the lack of internal compartments in the model. Hence all cellular glutamine (GLN used in ANIM as an early product of N-assimilation regulating transport) is considered together, while in reality transport would be affected only by cytoplasmic metabolites present at lower cellular concentrations and thus responding more rapidly to nutrient assimilation.

Effect of low temperature. Lomas & Glibert (1999a,b) reported that over the short term (hours) especially at low temperature, diatoms continue high rates of nitrate assimilation even in the presence of high ammonium. They hypothesised that nitrate reduction is a drain for surplus photoreductant generated at low temperature, because of the relatively greater sensitivity of rubisco to low temperature in comparison with nitrate reductase (NR). A concurrent release of nitrite or organic N was also predicted. However, NR is normally considered to be cytoplasmic, with nitrite reductase (NiR) in the chloroplast at the site of the generation of photoreductant (reviewed by Solomonson & Barber 1990). It is thus not clear why or how nitrate reduction, especially NR rather than NiR activity, should act as a sink for photoreductant. Photoinhibition is enhanced at high light and low temperature (e.g. Oquist et al. 1993). This is because reaction centres close due to low temperature constraints on photosynthesis, and the D1 repair cycle responsible for rebuilding photodamaged reaction centres is temperature sensitive while the photochemistry causing damage is relatively temperature insensitive (Oquist et al. 1993, Chaumont et al. 1995, Huner et al. 1998). Although we have developed a mechanistic model describing photodamage (Marshall, Geider & Flynn unpubl.), the experimental observations of N-assimilation may, in any case, be simulated within the existing ANIM structure by employing temperature response curves.

ANIM contains 3 points that can be manipulated to alter the ammonium-nitrate interaction in addition to the relationship between transport rates and N-status mentioned above. These points are the synthesis and level of NR activity, and the concentration of GLN that represses nitrate transport by either or both of the transporters suggested above to explain biphasic transport. Any of these could also legitimately be candidates for temperature-sensitive operation, though in

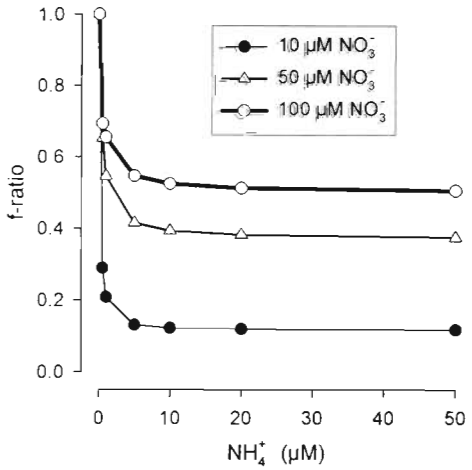

Fig. 2. Simulated f-ratios for cells grown with the indicated nitrate concentrations, after 24 h incubations with ammonium. The f-ratio is here defined as $V_N/(V_N + V_A)$ where V_N and V_A are the rates of assimilation of nitrate and ammonium respectively. Nutrient concentrations were constant for the duration of each incubation. Over longer periods the depression of nitrate transport may increase to 100% at higher ammonium concentrations depending on model parameters. For the simulations shown, the value of the constant NO3mGLN in ANIM (the size of the glutamine pool terminating nitrate transport, Flynn et al. 1997) was set at 0.003 (g N g^{-1} C) for the high affinity transporter, and 0.005 for the low affinity system. If both were set at 0.003, or if the high rate, low affinity system was not present, then the f-ratio plots declined more rapidly with increasing ammonium; nitrate transport from 10 µM was then close to zero with ammonium >1 µM

reality the regulatory system is understood poorly and is no doubt far more complex. The simplified form of ANIM, SHANIM (Flynn & Fasham 1997), does not have internal pools of inorganic N or a state variable describing the level of nitrate and nitrite reductases (NNiR in the ANIM). In this instance the only modification available is to the level of GLN that halts nitrate transport.

In order to simulate the effects of temperature on ammonium and nitrate assimilations, temperature functions were added to ANIM to alter the value of μ_{max} (maximum theoretical growth rate) and the activity of nitrate/nitrite reductases (NNiR). Normally all rate functions in ANIM are normalised to μ_{max}, allowing ready modification of model output with species specific growth rates at different temperatures. Here, NNiR was regulated separately according to a temperature-activity curve adapted from that for NR in the diatom *Skeletonema costatum* (Kristiansen 1983). The variation of μ_{max} with temperature for this diatom was modified after Jørgensen (1968). The temperature curves used in the model are shown in Fig. 3; the value (rate) of μ_{max} and NNiR in ANIM were simply multi-

plied by the rate factor given by the curves at the required temperature.

Output from simulations of temperature shift experiments (similar to Lomas & Glibert 1999b) are shown in Fig. 4. At low temperature the production of ammonium from nitrate is enhanced in the simulations while restricting the production of GLN, which would otherwise repress nitrate transport. This results in the depression of ammonium uptake as well, similar in operation to the nitrate repression of ammonium transport (Dortch & Conway 1984) which ANIM is capable of simulating (Flynn et al. 1997). If NiR cannot match NR activity, then nitrite may be released (Flynn & Flynn 1998). If the use of amino acids for protein synthesis and growth in general were affected more than the synthesis of amino acids, then DON levels would rise and be more likely to leak (simulated in Flynn & Berry 1999).

It is notable in the data of Lomas & Glibert (1999b) that the total N uptake did not increase with increasing temperature during the incubation as one may expect, and as the model shows (Fig. 4). This suggests that some other time and temperature-dependant process occurred within the organisms sampled by Lomas & Glibert (1999b) that is not being simulated adequately, or simply that the temperature response curves used do not match closely enough to those of the organisms tested in reality. One factor that partly explains the model output is that at higher temperatures ammonium transport from 1 µM was inadequate to match the growth rate possible at that temperature. As a consequence, levels of glutamine were lower and nitrate transport was derepressed, being faster at higher temperatures. For the simulations shown in Fig. 4 the maximum transport rates were not made a function of temperature. When they were, the total N uptake curve more closely resembled the growth-temperature curve in Fig. 3, as would be expected to occur in the longer-term for real cells. Raimbault (1984) specifically suggests the use of low temperature incubations to aid the measurement of transport kinetics by minimising the subsequent N-assimilation with its attendant formation of organic regulatory products. It is not clear whether the enhanced *in vivo* NR activity implied by the results of Lomas & Glibert (1999b) is only a transitory event. One would expect that, on prolonged low temperature incubation, the level of enzymes will be optimised, hence retaining the close relationship between NR activity and nitrate assimilation rates (Berges & Harrison 1995). More information on the short- and long-term temperature response curves for transport, nitrate reduction, N-assimilation and growth would be of use to improve both our understanding of the physiology and the construction/performance of the model.

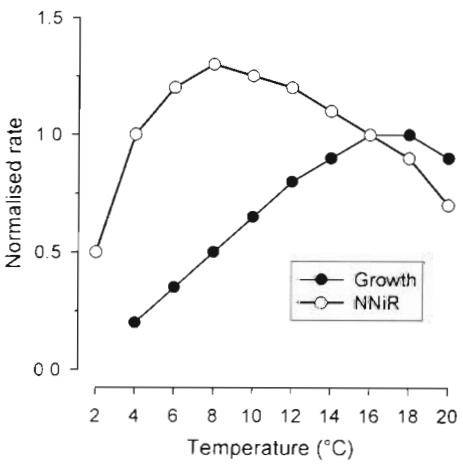

Fig. 3. Relationships between growth and NNiR activity with temperature adapted from the data for *Skeletonema costatum* of Jørgensen (1968) and Kristiansen (1983) respectively. The curves have been normalised to give a value of 1 at 16°C for placement in the model

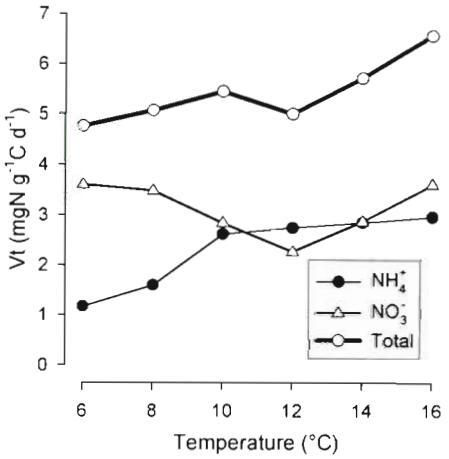

Fig. 4. Simulated transport rates (Vt) after 1 h of incubation at the indicated temperatures, into cells originally acclimated to 12°C. Throughout the acclimation and temperature shift period the nitrate concentration was 20 µM, with ammonium at 1 µM

Conclusion. The data shown in the works of Collos et al. (1992, 1997) and Lomas & Glibert (1999a,b) are interesting and important. They should promote further research both into the multiphasic nature of nitrate transport and interaction with ammonium, ideally with models acting as a focus. It would be useful to have further data sets including steady-state solutions as well as time courses for nutrient-shift and temperature-shift incubations. Does the operation of the high transport rate system for nitrate enable higher growth rates? Does ammonium ever completely halt nitrate assimilation at low temperature or at high nitrate concentrations for these diatoms? It would also be interesting to measure levels of GLN and other metabolites,

and NR and NiR activities over different temporal scales, in cells incubated or grown at different temperatures pulsed with nitrate and ammonium.

As Collos et al. (1997) point out, traditional methods of modelling the ammonium-nitrate interaction are inadequate for simulating situations where biphasic kinetics are important, and hence for simulating the competition between species that may exhibit such kinetics. In contrast, existing mechanistic models of ammonium-nitrate interaction appear capable, with minor modifications, of simulating the behaviour of phytoplankton in these situations. There is no need to resort to using inhibition constants to describe the interaction except in steady state situations. The 'constants' are not constant and the 'inhibition' is not an inhibition.

Acknowledgements. This work was supported by the Natural Environment Research Council (UK). Suggestions made by anonymous referees are acknowledged gratefully.

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Editorial responsibility: Otto Kinne (Editor), Oldendorf/Luhe, Germany

Submitted: June 17, 1999; Accepted: August 9, 1999
Proofs received from author(s): September 23, 1999