

Miniview:

Algal nitrate reductases

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The past decade has seen substantial breakthroughs in understanding the biochemistry, molecular biology and regulation of nitrate reductases (NR) in higher plants and green algae. In contrast, although there has been considerable interest in using various measurements of NR to provide ecophysiological information, comparable knowledge of NR is largely lacking in algal groups other than chlorophytes. Applying information about NR from chlorophytes and higher plants to other algae may be difficult. There is evidence that non-chlorophyte forms of NR are diverse and distinct in terms of biochemical characteristics and regulatory features. Key areas to be pursued in non-chlorophyte algae include the identification and adoption of model organisms for NR research in different algal groups; the creation of selected and engineered mutants; the purification, biochemical characterization and production of antibodies to different algal forms of NR; the identification of NR genes; and the undertaking of coordinated research on nitrate uptake proteins and other enzymes in pathways of nitrate assimilation.

Key words: biochemistry, Chlorophyta, enzyme activity, marine phytoplankton, molecular biology, Plantae

Purpose and scope of the review

Within living organisms, there are several distinct proteins that reduce nitrate to nitrite and are thus termed nitrate reductases (NR). This review is concerned with assimilatory forms of NR, which are structurally distinct from dissimilatory NR in organisms that use nitrate as an alternative electron acceptor to O₂ (see Guerrero *et al.*, 1981). Although dissimilatory nitrate use occurs predominantly in prokaryotes (Hochstein & Tomlinson, 1988), it is also a feature of eukaryotic metabolism (Zvyagil'skaya *et al.*, 1996). I will confine the review to eukaryotic algae because assimilatory NR in cyanobacteria is a quite different enzyme (Andriesse *et al.*, 1989), though comparisons with NR research in cyanobacteria will be made. Three eukaryotic assimilatory NR forms are recognized, two of which occur in eukaryotic algae and higher plants (EC 1.6.6.1, specific for NADH, and EC 1.6.6.2, using either NADH or NADPH) and one of which is found only in fungi (EC 1.6.6.3, specific for NADPH).

There is an enormous spectrum of interest in NR. At one extreme, NR is a useful model for exploring the reaction mechanisms of multi-component redox enzymes (e.g. Kay *et al.*, 1991; Dwivedi *et al.*, 1994). At the other, there is interest in using NR in an ecological context to predict rates of nitrate incorporation that are otherwise difficult to determine (e.g. Eppley *et al.*, 1969; Blasco *et al.*, 1984; Berges & Harrison, 1995). The use of NR in an ecological context has been particularly advocated for

marine environments where nitrogen is often limiting and rates of nitrate uptake may provide information about the biogeochemical fate of carbon fixed by phytoplankton (Howarth, 1988; Platt *et al.*, 1992). NR is also one of the very few reasonably well characterized inducible/repressible enzyme systems in photosynthetic organisms, and it shows many regulatory features that make it of interest to physiologists (see Solomonson & Barber 1990; Campbell 1996). Furthermore, as evidence presented below will show, the characteristics of NR differ among algal groups and this diversity may reveal evolutionary links or patterns of adaptation.

Research on assimilatory NR has been going on for more than half a century. It falls into three broad categories, each of which has come to focus on particular groups of organisms: (1) research concerned with the biochemistry and molecular biology of the enzyme itself, an area pursued almost solely using green algae and higher plants; (2) research centred on the physiological regulation and role of NR in nitrogen metabolism, the considerable majority of which, again, has been conducted with chlorophytes and higher plants; and (3) research directed towards using NR measurements to gain information about ecological processes, most of which has been carried out with non-chlorophyte algae, often in natural assemblages.

My goals in writing this Miniview are threefold. (1) I will briefly point out current understanding of NR structure, regulation and roles in nitrogen metabolism, based on higher plant and green algal research. (2) I will provide evidence to suggest that NR might be substan-

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tially different in other algal groups, and indicate where data are lacking. (3) I will point out research directions that have proven useful in higher plants and green algae and which should be pursued in other algal groups.

Terminology and semantics

A great deal of confusion is caused by differing terminology. Consider the statement: 'nitrate reductase is rate limiting for nitrogen assimilation'. There are three key questions: (1) What is meant by 'nitrate reductase', i.e. protein quantity, protein activity? (2) What does 'rate limiting' mean? (3) What is meant by 'nitrogen assimilation'? Each of these concepts is complex, and the same terms have been used in different ways by different authors. The following paragraphs will explicitly define terms as I will use them in this Miniview. In so doing, I recognize that each definition has particular advantages and disadvantages and will be controversial; clarity and utility are the reasons I have chosen them.

A definition of 'nitrate reductase' should distinguish between the quantity of NR protein and the activity displayed by that protein. One can further distinguish between the activity of NR measured in an *in vitro* assay, and the activity of the enzyme *in vivo*. The first distinction is relatively straightforward: NR protein can be quantified using an immunoassay (e.g. Smarrelli & Campbell, 1981; Campbell & Remmler, 1986). Activity measurements are more difficult to define. By definition, *in vitro* assays require fixing conditions such as pH, temperature and substrate concentration. In most cases, activity is measured with saturating substrates, resulting in what is termed a V_{\max} assay. Under these conditions, activity is used as a surrogate for enzyme quantity (Rossomando, 1990); post-translational modifications such as phosphorylation state may or may not be preserved. In this review I will use NR activity to mean the activity measured in a V_{\max} assay. Confusingly, ecologically oriented researchers have sometimes defined this activity as 'potential activity', a term which suggests that it represents some theoretical maximum for the *in vivo* activity of the enzyme; this may or may not be so. Even more confusing is the use of so-called *in vivo* assays. In this case the enzyme is not extracted but left inside cells, which are permeabilized to allow release of reaction products. In general, such methods are more loosely controlled and more difficult to reproduce because substrates may be present in non-saturating quantities. Since its application normally requires permeabilization of cell membranes and results in a decidedly non-physiological situation, it has been suggested that the assay is better termed *in situ* (Corzo & Niell, 1991). There are several assumptions necessary and shortcomings inherent when using *in situ* assays (Thomas & Harrison, 1988; Brinkhuis *et al.*, 1989; Hurd *et al.*, 1995). Currently, direct determination of an *in vivo* rate of NR activity is not possible, but if experiments are well designed, the NR activity necessary to support the observed fluxes of nitrogen into the cell may be calculated (e.g. Berges & Harrison, 1995). Whether a V_{\max} assay bears any re-

lationship to *in vivo* enzyme activity is difficult to predict, but there are many cases when V_{\max} assays do provide valuable information (Newsholme & Crabtree, 1986).

'Rate limitation' is a concept that can be defined clearly in terms of metabolic control theories. An enzyme is said to be rate limiting if a change in enzyme quantity leads to a proportional change in flux through a metabolic pathway (Crabtree & Newsholme, 1985; Kacser & Porteous, 1987). The pathway must be specified; thus NR could be rate limiting for reduction of nitrate to nitrite within the cell but might not be limiting the rate of reduction of nitrate to ammonium, for example. Such a definition has very seldom been critically applied to work with NR (but see Ingemarsson, 1987; Dortch *et al.*, 1979; Berges & Harrison, 1995). Rate limiting enzymes have several characteristics, including equilibrium constants (k_{eq}) greater than 5 (indicating the tendency for the reaction to proceed in one direction rather than the other), a complex structure, and post-translational regulatory mechanisms (Newsholme & Crabtree, 1986).

Finally, consistent definitions are needed for the processes of nitrogen acquisition. I suggest, following Wheeler (1983), that *uptake* be defined as removal of nitrate from the environment and physical transport into the alga (in macroalgae and higher plants a further distinction between transport into intercellular spaces and transport into cells themselves may be useful; see discussion in Redinbaugh & Campbell, 1991); *assimilation* be defined as the processes by which nitrate is used to form small organic molecules, such as amino acids; and *incorporation* be defined as the processes by which nitrogen-containing organic molecules are combined to form macromolecules, such as proteins and nucleic acids. These definitions are functional and largely pragmatic; for example, disappearance of nitrate from the medium can be easily measured, and a separation of small organic and inorganic molecules is relatively straightforward. Note, however, that the components of each process are not specified and that some problems remain in applying these definitions.

Current views of nitrate reductases

Structure and characteristics of nitrate reductases

The past 10 years have seen numerous reviews of NR dealing with the biochemistry and molecular biology of the enzymes (e.g. Campbell & Kinghorn, 1990; Wray & Fido, 1990; Campbell, 1996). It is generally accepted that NR is a homodimer, with each subunit composed of a polypeptide of approximately 100 kDa associated with molybdenum, molybdopterin, iron, haeme and flavin adenine dinucleotide (FAD). Three regions of the enzyme are recognized: an NAD(P)H domain, a cytochrome *b* domain, and an FAD domain. Each domain can function independently under certain conditions, resulting in several partial activities of the enzyme (Wray & Fido, 1990). The equilibrium is very strongly toward nitrite formation (k_{eq} of 10^{25} to 10^{40} : Hewitt *et al.*, 1976). Recent evidence provides an explanation for the differences

between NADH- and NADPH-specific forms, based on the specific amino acid residues in the active site (Campbell, 1996), but the reason why some forms of NR can use both NADH and NADPH remains unclear. NR has traditionally been viewed as a cytoplasmic enzyme, but there is growing evidence of plasmalemma-bound forms in green algae and higher plants (e.g. Fernandez-Lopez *et al.*, 1996).

Regulation of nitrate reductases

Many recent reviews have been devoted to the regulation of NR (e.g. Solomonson & Barber, 1990; Lillo, 1994; Crawford, 1995), and there are several recurring ideas. NR is largely regulated by synthesis and degradation of the protein, with synthesis being induced by the presence of nitrate, and degradation by absence of nitrate or presence of other more reduced nitrogen forms. Little is known about the degradative process. NR activity is influenced by irradiance. The mechanisms are as yet unknown, but may be due to indirect effects on nitrate uptake and hence availability of substrate to the enzyme, direct effects of light on the NR protein (i.e. post-translational effects), or effects on transcription or translation of NR (Ninneman, 1987). Light effects appear to involve both a red-light receptor (possibly a phytochrome) and a blue-light receptor which may be a flavoprotein (see Rudiger & Lopez-Figueroa, 1992). Both the activity of NR and the abundance of NR protein show a diel cycle, which is possibly circadian (Lillo, 1983; Deng *et al.*, 1991). NR is high in light periods (and shows a midday peak) and low in darkness. Post-translational regulatory mechanisms identified include phosphorylation (Huber *et al.*, 1992) involving a specific protein kinase, protein phosphatase and an inactivator protein (MacKintosh *et al.*, 1995; Glaab & Kaiser, 1996). A mechanism has been proposed whereby intracellular cyanate could inactivate the enzyme (Pistorius *et al.*, 1976). The inactivation can be reversed by blue light, flavins and, in a non-physiological manner, by mild oxidation with ferricyanide (Franco *et al.*, 1987).

Role of nitrate reductases in nitrogen metabolism

Bearing in mind the difficulty in terminology, the majority of reviews claim a rate-limiting role for NR. As noted above, NR fits many criteria of a rate-limiting enzyme (see Newsholme & Crabtree, 1986), but appropriate experiments to test this idea are mostly lacking. Alternatively, a growing number of plant physiologists have suggested that nitrate incorporation is limited by nitrate uptake (e.g. Tischener, 1990), or by enzymatic steps downstream of NR, such as that catalysed by nitrite reductase (NiR) (see Wray, 1993).

Algal nitrate reductases

Too few NR forms from non-chlorophyte algae have been characterized to allow broad generalizations about the enzyme structure. NR from marine diatoms appears to be close in denatured molecular weight to that of higher

plants, in the range 95–120 kDa (Gao *et al.*, 1993; J. J. Vergara, J. A. Berges & P. G. Falkowski, unpublished), but NR subunits from dinoflagellates are considerably smaller at around 52 kDa (Ramalho *et al.*, 1995). Antisera against NR from marine diatoms react poorly with higher plant NR and, within the algae, only relatively closely related taxa cross-react. Gao *et al.* (1993) reported no cross-reaction of NR antisera raised against *Skeletonema costatum* with the chlorophyte *Dunaliella tertiolecta*, the chromophytes *Emiliania huxleyi* or *Isochrysis galbana*, or the dinoflagellate *Amphidinium carterae*. Antiserum against NR from *Thalassiosira weissflogii* cross-reacts well with congeners such as *T. pseudonana*, weakly with other diatoms such as *S. costatum* within the same family (Thalassiosiraceae), and very poorly or not at all with other diatoms tested (J. J. Vergara, J. A. Berges & P. G. Falkowski, unpublished).

Optimal activity assay conditions (which include extractability of the enzyme) provide more evidence of diversity in characteristics of NR. FAD appears to be required to achieve highest activity in assays of some species of marine algae but not in others (Vennesland & Solomonson, 1972; Everest *et al.*, 1984; Berges & Harrison, 1995), suggesting that the FAD factor may be bound to the enzyme to varying degrees. With respect to half-saturation constants for NADH and nitrate, and inhibitory effects of these substrates, there is wide variation (Berges & Harrison, 1995). Based on use of different substrates, there is also evidence of diversity in forms of NR; NADPH use is apparently confined to the green algae (Syrett, 1981; Berges & Harrison, 1995).

Regulatory features of algal NR forms also diverge from those found in higher plants. Nitrate may not be necessary for NR synthesis: in green algae and diatoms, transfer of cells growing in media with ammonium as the sole nitrogen source to nitrogen-free media resulted in the appearance of NR activity (Kessler & Osterheld, 1970; Amy & Garrett, 1974). The phenomenon of ferricyanide activation has not been found in the algae other than the chlorophytes (Serra *et al.*, 1978; Hochman, 1982; Berges & Harrison, 1995). Phosphorylation of NR has yet to be established in algae; characteristic inhibition of activity of phosphorylated NR by magnesium has not been found in marine diatoms (Gao *et al.*, 1993; Berges, unpublished). The inhibitory effect of ammonium on NR activity is established in many algal groups, though there is controversy as to whether it acts on NR indirectly by eliminating nitrate transport or directly on the enzyme at the levels of synthesis or post-translational modification (Syrett, 1981; Larsson *et al.*, 1985; Collos, 1989; Flynn, 1991). It is also worth pointing out that Dortch (1990) has cautioned that effects on nitrate uptake should distinguish between true inhibitory effects of ammonium on nitrate uptake from preference for ammonium over nitrate. There are also cases where NR is not inhibited by ammonium, particularly in the dinoflagellates (Harrison, 1976; Collos & Slawyk, 1980; Hochman, 1982). It may be meaningful in this context that NR from dinoflagellates has been

localized in the chloroplast, in contrast to other groups of algae where the enzyme is usually considered to be cytoplasmic (Fritz *et al.*, 1996).

Diel patterns in NR activity differ among green algae (Velasco *et al.*, 1988), cyanobacteria (Lara *et al.*, 1993), and diatoms and other chromophytes (Eppley *et al.*, 1971; Berges *et al.*, 1995). The importance of light in promoting NR synthesis is probably indirect and linked to carbon metabolism. It has long been known that *Chlorella* will assimilate nitrate in darkness, if provided with a source of carbon (Cramer & Myers, 1948). Furthermore, induction of NR has been demonstrated in a marine chlorophyte in darkness (Delrio *et al.*, 1994), and a 'predawn' increase in NR activity is a well-established feature of marine diatoms (see Berges *et al.*, 1995).

The assumption that NR activity is rate limiting for nitrate incorporation appears to be justified in some diatom species (Berges & Harrison, 1995; Berges *et al.*, 1995), but control at other steps of the incorporation pathways has also been identified. Nitrate uptake can be limiting (Ingemarsson, 1987; Watt *et al.*, 1992), but accumulation of pools of nitrate in certain diatoms is evidence that this is not always so (see discussion in Dortch, 1982). In particular cases, accumulation and excretion of nitrite suggest that NiR may be limiting (Collos & Slawyk, 1980; Martinez, 1991), while glutamine synthase (GS) could be another point of control (Clayton & Ahmed, 1987; Sequineau *et al.*, 1989). In fact, the concept of a single rate-limiting step may be an oversimplification; arguments have been made that because of constraints on total protein within a cell, all enzymes in a pathway will tend to change in response to changes in flux through that pathway (Brown, 1991).

Issues and future directions

I suggest that future research might profitably be directed to the following areas:

(1) Relevant model organisms in diverse algal taxa, including diatoms, dinoflagellates and red algae, should be identified and adopted for research. It is clear if one examines the literature that the vast majority of NR work in chlorophytes has been done with *Chlorella* and *Chlamydomonas* species. The advantage of this is that studies using different techniques or considering different aspects of nitrogen metabolism can be integrated. In contrast, those working with non-chlorophyte algae have used a very large number of different organisms, or have considered assemblages of organisms in natural environments. It is debatable whether any consensus can be reached on what constitutes a 'model' organism, and it is unclear how general acceptance of particular species can be achieved. I submit that this is nonetheless a worthwhile objective. Important elements of a model organism would include ready availability of stock cultures, ease of maintenance of cultures and reproducibility of growth characteristics. Furthermore, the species should be generally reflective of the characteristics of the group it represents. I would suggest that components such as abundance in

natural ecosystems (i.e. 'ecological relevance') and the amount of previous work done on a given species be considered of secondary importance as selection criteria. Simply as an example, in selecting a model diatom, one would have to assess whether the considerable fluctuations in cell size and growth rate often seen in cultures of *Skeletonema costatum*, or the dramatic morphological variation and silica requirements of *Phaeodactylum tri-cornutum*, make either of the organisms less desirable. Of course, research using other organisms must proceed, but application of new methods and new experimental approaches will benefit vastly from including a 'reference' species in such studies. Work on NR in higher plants has profited by focusing on spinach, but this has clearly not prevented work being done in corn or barley, for example.

(2) Effort should be concentrated on isolation, purification and biochemical characterization of NR, and production of antibodies against NR from different algae. This has begun in some groups such as rhodophyte macroalgae (Nakamura *et al.*, 1994), diatoms (Smith *et al.*, 1992; Gao *et al.*, 1993) and dinoflagellates (Ramalho *et al.*, 1995), and must be encouraged as more than strictly a biochemical exercise.

(3) Research on the identification of NR genes from different algae must proceed, since it has significantly advanced research efforts in higher plants and green algae (see Campbell, 1996; Dawson *et al.*, 1996). Recent technical advances, such as development of transformation systems in diatoms (e.g. Dunahay *et al.*, 1995), are making this approach feasible.

(4) Attempts should be made to provide selected or engineered mutants to NR and nitrate incorporation pathways in various algal groups. Results obtained using mutants have been decisive in resolving issues concerning NR for green algae and higher plants (e.g. Hoff *et al.*, 1994; Ferrario *et al.*, 1995), but mutants are virtually unknown in other taxa.

(5) Finally, coordinated research should be undertaken that includes nitrate uptake proteins and other components of nitrate incorporation pathways. Research on nitrate transporters is advanced in cyanobacteria and higher plants (Lara *et al.*, 1993; Crawford, 1995; Omata, 1995) but far behind in groups such as the diatoms (Falkowski, 1975). Work involving NiR (Eppley & Rogers, 1970) and GS (Clayton & Ahmed, 1987) begun more than a decade ago has not (with some exceptions, e.g. Robertson & Alberte, 1996) been followed up.

In concluding this Miniview, a general comment on the state of NR research in non-chlorophyte algal groups is perhaps in order. As noted above, research in these groups has focused almost solely on NR activity assays performed in physiological and ecological contexts. More than a decade ago it was pointed out that: 'the potential uses of enzyme assays in ecological studies have been both overstated by ecologically-oriented biochemists and under utilized by nonbiochemically-oriented ecologists' (Falkowski, 1983). This remains true today because research in this area has proceeded with a relatively poor

biochemical context and relied heavily on generalizations from higher plants and green algae. Too little emphasis has been placed on the importance of ensuring proper extraction and assay of NR (see Berges & Harrison, 1995). Assay temperatures used are sometimes up to 20 °C higher than those in which the organisms live, despite clear indications that assays performed at higher temperatures may not be related to those conducted at *in situ* temperatures (see Kristiansen, 1983). Numerous experiments have been conducted using loosely controlled *in situ* assays for NR. Furthermore, laboratory studies with non-chlorophyte algae have usually focused on experiments that attempt to simulate field conditions or transient states before testing methods using carefully controlled steady-state conditions. How can one determine whether NR measurements are providing a good index of processes related to nitrogen metabolism by performing experiments in which such processes cannot be independently measured? In evaluating the literature for non-chlorophyte algae, it is little wonder that many researchers have decided that NR assays do not provide interpretable ecophysiological information. In my opinion, such a conclusion is premature.

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References

- AMY, N. K. & GARRETT, R. H. (1974). Purification and characterization of the nitrate reductase from the diatom *Thalassiosira pseudonana*. *Plant Physiol.*, **54**: 629–637.
- ANDRIESE, A. J., WEISBEEK, P. J. & VAN ARKEL, G. A. (1989). Biochemistry, regulatory aspects, and genetics of nitrate assimilation in cyanobacteria. In *Molecular and Genetic Aspects of Nitrate Assimilation* (Wray, J. L. & Kinghorn, J. R., editors), 40–50. Oxford University Press, Oxford.
- BERGES, J. A. & HARRISON, P. J. (1995). Nitrate reductase activity quantitatively predicts the rate of nitrate incorporation under steady state light limitation: a revised assay and characterization of the enzyme in three species of marine phytoplankton. *Limnol. Oceanogr.*, **40**: 82–93.
- BERGES, J. A., COCHLAN, W. P. & HARRISON, P. J. (1995). Laboratory and field responses of algal nitrate reductase to diel periodicity in irradiance, nitrate exhaustion, and the presence of ammonium. *Mar. Ecol. Prog. Ser.*, **124**: 259–269.
- BLASCO, D., MACISAAC, J. J., PACKARD, T. T. & DUGDALE, R. C. (1984). Relationship between nitrate reductase and nitrate uptake in phytoplankton in the Peru upwelling region. *Limnol. Oceanogr.*, **29**: 275–286.
- BRINKHUIS, B. H., RENZHI, L., CHAOYUAN, W. & XUN-SEN, J. (1989). Nitrite uptake transients and consequences for *in vivo* algal nitrate reductase assays. *J. Phycol.*, **25**: 539–545.
- BROWN, G. C. (1991). Total cell protein concentration as an evolutionary constraint on the metabolic control distribution in cells. *J. Theor. Biol.*, **153**: 195–203.
- CAMPBELL, W. H. (1990). Purification, characterization and immunochemistry of higher plant nitrate reductase. In *Nitrogen in Higher Plants* (Abrol, Y. P., editor), 65–91. Wiley, New York.
- CAMPBELL, W. H. (1996). Nitrate reductase biochemistry comes of age. *Plant Physiol.*, **111**: 355–361.
- CAMPBELL, W. H. & KINGHORN, J. R. (1990). Functional domains of assimilatory nitrate reductases and nitrite reductases. *Trends Biochem. Sci.*, **15**: 315–319.
- CAMPBELL, W. H. & REMMLER, J. L. (1986). Regulation of corn leaf nitrate reductase. I. Immunochemical methods for analysis of the enzyme's protein component. *Plant Physiol.*, **80**: 435–441.
- CLAYTON, J. R. & AHMED, S. I. (1987). Modified assay procedure for enhanced sensitivity of *in vitro* glutamine synthetase (GS) activity measurements in marine phytoplankton. *Mar. Ecol. Prog. Ser.*, **36**: 177–180.
- COLLOS, Y. (1989). A linear model of external interactions during uptake of different forms of inorganic nitrogen by microalgae. *J. Plankton Res.*, **11**: 521–533.
- COLLOS, Y. & SLAWYK, G. (1980). Nitrogen uptake and assimilation by marine phytoplankton. In *Primary Productivity in the Sea* (Falkowski, P. G., editor), 195–211. Plenum Press, New York.
- CORZO, A. & NIELL, F. X. (1991). Determination of nitrate reductase activity in *Ulva rigida* C. Agardh by the *in situ* method. *J. Exp. Mar. Biol. Ecol.*, **146**: 181–191.
- CRABTREE, B. & NEWSHOLME, E. A. (1985). A quantitative approach to metabolic control. *Curr. Top. Cell. Regul.*, **25**: 21–76.
- CRAMER, H. & MYERS, J. (1948). Nitrate reduction and assimilation in *Chlorella*. *J. Gen. Physiol.*, **32**: 93–102.
- CRAWFORD, N. M. (1995). Nitrate: nutrient and signal for plant growth. *Plant Cell*, **7**: 859–868.
- DAWSON, H. N., PENDLETON, L. C., SOLOMONSON, L. P. & CANNONS, A. C. (1996). Cloning and characterization of the nitrate reductase-encoding gene from *Chlorella vulgaris*: structure and identification of transcription start points and initiator sequences. *Gene*, **171**: 139–145.
- DELRIO, M. J., RAMAZANOV, Z. & GARCIA-REINA, G. (1994). Dark induction of nitrate reductase in the halophilic alga *Dunaliella salina*. *Planta*, **192**: 40–45.
- DENG, M. D., MOUREAUX, T., CHEREL, I., BOUTIN, J. P. & CABOCHE, M. (1991). Effects of nitrogen metabolites on the regulation and circadian expression of tobacco nitrate reductase. *Plant Physiol. Biochem.*, **29**: 239–247.
- DORTCH, Q. (1982). Effect of growth conditions on accumulation of internal nitrate, ammonium, amino acids and protein in three marine diatoms. *J. Exp. Mar. Biol. Ecol.*, **61**: 243–264.
- DORTCH, Q. (1990). The interaction between ammonium and nitrate uptake in phytoplankton. *Mar. Ecol. Prog. Ser.*, **61**: 183–201.
- DORTCH, Q., AHMED, S. I. & PACKARD, T. T. (1979). Nitrate reductase and glutamate dehydrogenase activities in *Skeletonema costatum* as measures of nitrogen assimilation rates. *J. Plankton Res.*, **1**: 169–185.
- DUNAHAY, T. G., JARVIS, E. E. & ROESSLER, P. G. (1995). Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophila*. *J. Phycol.*, **31**: 1004–1012.
- DWIVEDI, U. N., SHIRAIISHI, N. & CAMPBELL, W. H. (1994). Identification of an essential cysteine of nitrate reductase via mutagenesis of its recombinant cytochrome *b* reductase domain. *J. Biol. Chem.*, **269**: 13785–13791.
- EPPLEY, R. E. & ROGERS, J. N. (1970). Inorganic nitrogen assimilation of *Ditylum brightwellii*, a marine plankton diatom. *J. Phycol.*, **6**: 344–351.
- EPPLEY, R. W., COATSWORTH, J. L. & SOLORZANO, L. (1969). Studies of nitrate reductase in marine phytoplankton. *Limnol. Oceanogr.*, **14**: 194–205.
- EPPLEY, R. W., ROGERS, J. N., MCCARTHY, J. J. & SOURNIA, A. (1971). Light/dark periodicity in nitrogen assimilation of the marine phytoplankters *Skeletonema costatum* and *Coccolithus huxleyi* in N-limited chemostat culture. *J. Phycol.*, **7**: 150–154.
- EVEREST, S. A., HIPKIN, C. R. & SYRETT, P. J. (1984). The effect of phosphate and flavin adenine dinucleotide on nitrate reductase activity of some unicellular marine algae. *J. Exp. Mar. Biol. Ecol.*, **76**: 263–275.
- FALKOWSKI, P. G. (1975). Nitrate uptake in marine phytoplankton: (nitrate, chloride)-activated adenosine triphosphatase from *Skeletonema costatum* (Bacillariophyceae). *J. Phycol.*, **11**: 323–326.
- FALKOWSKI, P. G. (1983). Enzymology of nitrogen assimilation. In *Nitrogen in the Marine Environment* (Carpenter, E. J. & Capone, D. G., editors), 839–868. Academic Press, New York.
- FERNANDEZ-LOPEZ, M., OLIVARES, J. & BEDMAR, E. J. (1996). Purification and characterization of the membrane bound nitrate reductase. *FEBS Lett.*, **392**: 1–5.
- FERRARIO, S., VALADIER, M. H., MOROT-GAUDRY, J. F. & FOYER, C. H. (1995). Effects of constitutive expression of nitrate reductase in transgenic

- Nicotiana plumbaginifolia* L. in response to varying nitrogen supply. *Planta*, **196**: 288–294.
- FLYNN, K. J. (1991). Algal carbon–nitrogen metabolism: a biochemical basis for modelling the interactions between nitrate and ammonium uptake. *J. Plankton Res.*, **13**: 373–387.
- FRANCO, A. R., CARDENAS, J. & FERNANDEZ, E. (1987). Involvement of reversible inactivation in the regulation of nitrate reductase enzyme levels in *Chlamydomonas reinhardtii*. *Plant Physiol.*, **84**: 665–669.
- FRITZ, L., STRINGHER, C. G. & COLEPICOLO, P. (1996). Immunolocalization of nitrate reductase in the marine dinoflagellate *Gonyaulax polyedra* (Pyrrophyta). *J. Phycol.*, **32**: 632–637.
- GAO, Y., SMITH, G. J. & ALBERTE, R. S. (1993). Nitrate reductase from the marine diatom *Skeletonema costatum*: biochemical and immunological characterization. *Plant Physiol.*, **103**: 1437–1445.
- GLAAB, J. & KAISER, W. M. (1996). The protein kinase, protein phosphatase and inhibitor protein of nitrate reductase are ubiquitous in higher plants and independent of nitrate reductase expression and turnover. *Planta*, **199**: 57–63.
- GUERRERO, M. G., VEGA, J. M. & LOSADA, M. (1981). The assimilatory nitrate-reducing system and its regulation. *Annu. Rev. Plant Physiol.*, **32**: 169–204.
- HARRISON, W. G. (1976). Nitrate metabolism of the red tide dinoflagellate *Gonyaulax polyedra* Stein. *J. Exp. Mar. Biol. Ecol.*, **21**: 199–209.
- HEWITT, E. J., HUCKLESBY, D. P. & NOTTON, B. A. (1976). Nitrate metabolism. In *Plant Biochemistry* (Bonner, J. & Varner, J. E., editors), 633–681. Academic Press, New York.
- HOCHMAN, A. (1982). Studies of nitrate reductase in the fresh water dinoflagellate *Peridinium cinctum*. *Arch. Microbiol.*, **133**: 62–65.
- HOCHSTEIN, L. I. & TOMLINSON, G. A. (1988). The enzymes associated with denitrification. *Annu. Rev. Microbiol.*, **42**: 231–261.
- HOFF, T., TRUONG, H. N. & CABOCHE, M. (1994). The use of mutants and transgenic plants to study nitrate assimilation. *Plant Cell Environ.*, **17**: 489–506.
- HOWARTH, R. W. (1988). Nutrient limitation of net primary production in marine ecosystems. *Annu. Rev. Ecol. Syst.*, **19**: 89–110.
- HUBER, J. L., HUBER, S. C., CAMPBELL, W. H. & REDINBAUGH, M. G. (1992). Reversible light/dark modulation of spinach leaf nitrate reductase activity involves protein phosphorylation. *Arch. Biochem. Biophys.*, **296**: 58–65.
- HURD, C. L., BERGES, J. A., OSBORNE, J. & HARRISON, P. J. (1995). An *in vitro* nitrate reductase assay for marine macroalgae: optimization and characterization of the enzyme for *Fucus gardneri* (Phaeophyta). *J. Phycol.*, **31**: 835–843.
- INGEMARSSON, B. (1987). Nitrogen utilization in *Lemna*. I. Relations between net nitrate flux, nitrate reduction, and *in vitro* activity and stability of nitrate reductase. *Plant Physiol.*, **85**: 856–859.
- KACSER, H. & PORTEOUS, J. W. (1987). Control of metabolism: what do we have to measure? *Trends Biochem. Sci.*, **12**: 5–17.
- KAY, C. J., SOLOMONSON, L. P. & BARBER, M. J. (1991). Electrochemical and kinetic analysis of electron transfer reactions of *Chlorella* nitrate reductase. *Biochemistry*, **30**: 11445–11450.
- KESSLER, E. & OSTERHELD, H. (1970). Nitrification and induction of nitrate reductase in nitrogen-deficient algae. *Nature*, **228**: 287–288.
- KRISTIANSEN, S. (1983). The temperature optimum of the nitrate reductase assay for marine phytoplankton. *Limnol. Oceanogr.*, **28**: 776–780.
- LARA, C., RODRIGUEZ, R. & GUERRERO, M. G. (1993). Sodium-dependent nitrate transport and energetics of cyanobacteria. *J. Phycol.*, **29**: 389–395.
- LARSSON, C. M., LARSSON, M. & GUERRERO, M. G. (1985). Photosynthetic nitrogen metabolism in high and low CO₂ adapted *Scenedesmus*. *J. Exp. Bot.*, **36**: 1387–1395.
- LILLO, C. (1983). Studies of diurnal variations of nitrate reductase activity in barley leaves using various assay methods. *Physiol. Plant.*, **57**: 357–362.
- LILLO, C. (1994). Light regulation of nitrate reductase in green leaves of higher plants. *Physiol. Plant.*, **90**: 616–620.
- MACKINTOSH, C., DOUGLAS, P. & LILLO, C. (1995). Identification of a protein that inhibits the phosphorylated form of nitrate reductase from spinach (*Spinacia oleracea*) leaves. *Plant Physiol.*, **107**: 451–457.
- MARTINEZ, R. (1991). Transient nitrate uptake and assimilation in *Skeletonema costatum* cultures subject to nitrate starvation under low irradiance. *J. Plankton Res.*, **13**: 499–512.
- NAKAMURA, Y., SAJI, H., KONDO, N. & IKAWA, T. (1994). Preparation of monoclonal antibodies against NADH-nitrate reductase from the red alga *Porphyra yezoensis*. *Plant Cell Physiol.*, **35**: 1185–1198.
- NEWSHOLME, E. A. & CRABTREE, B. (1986). Maximum catalytic activity of some key enzymes in provision of physiologically useful information about metabolic fluxes. *J. Exp. Zool.*, **239**: 159–167.
- NINNMANN, H. (1987). Photoregulation of eukaryotic nitrate reductase. In *Blue Light Responses: Phenomena and Occurrence in Plants and Microorganisms* (Senger, H., editor), 17–29. CRC Press, Boca Raton, FL.
- OMATA, T. (1995). Structure, function and regulation of the nitrate transport system of the cyanobacterium *Synechococcus* sp. PCC7942. *Plant Cell Physiol.*, **36**: 207–213.
- PISTORIUS, E. K., GEWITZ, H. S., VOSS, H. & VENNESLAND, B. (1976). Reversible inactivation of nitrate reductase in *Chlorella vulgaris* *in vivo*. *Planta*, **128**: 73–80.
- PLATT, T., JAUHARI, P. & SATHYENDRANATH, S. (1992). The importance and measurement of new production. In *Primary Productivity and Biogeochemical Cycles in the Sea* (Falkowski, P. G. & Woodhead, A. D., editors), 273–284. Plenum Press, New York.
- RAMALHO, C. B., HASTINGS, J. W. & COLEPICOLO, P. (1995). Circadian oscillation of nitrate reductase activity in *Gonyaulax polyedra*. *Plant Physiol.*, **107**: 225–231.
- REDINBAUGH, M. G. & CAMPBELL, W. H. (1991). Higher plant responses to environmental nitrate. *Physiol. Plant.*, **82**: 640–650.
- ROBERTSON, D. L. & ALBERTE, R. S. (1996). Isolation and characterization of glutamine synthetase from the marine diatom *Skeletonema costatum*. *Plant Physiol.*, **111**: 1169–1175.
- ROSSOMANDO, E. F. (1990). Measurement of enzyme activity. *Methods Enzymol.*, **182**: 38–49.
- RUDIGER, W. & LOPEZ-FIGUEROA, F. (1992). Photoreceptors in algae. *Photochem. Photobiol.*, **55**: 949–954.
- SEGUINEAU, C., BATREL, Y. & LE GAL, Y. (1989). Glutamine synthetase of *Dunaliella primolecta*: partial characterization and possible adenylation control in relation to nitrogen nutrient levels. *Biochem. Syst. Ecol.*, **17**: 503–508.
- SERRA, J. L., LLAMA, M. J. & CADENAS, E. (1978). Characterization of the nitrate reductase enzyme activity in the diatom *Skeletonema costatum*. *Plant Sci. Lett.*, **13**: 41–48.
- SMARRELLI, J., JR & CAMPBELL, W. H. (1981). Immunological approach to structural comparisons of assimilatory nitrate reductases. *Plant Physiol.*, **68**: 1226–1230.
- SMITH, G. J., ZIMMERMAN, R. C. & ALBERTE, R. S. (1992). Molecular and physiological responses of diatoms to variable levels of irradiance and nitrogen availability: growth of *Skeletonema costatum* in simulated upwelling conditions. *Limnol. Oceanogr.*, **37**: 989–1007.
- SOLOMONSON, L. P. & BARBER, M. J. (1990). Assimilatory nitrate reductase: functional properties and regulation. *Annu. Rev. Plant Physiol. Plant Molec. Biol.*, **41**: 225–253.
- SYRETT, P. J. (1981). Nitrogen metabolism of microalgae. *Can. Bull. Fish. Aquat. Sci.*, **210**: 182–210.
- THOMAS, T. E. & HARRISON, P. J. (1988). A comparison of *in vitro* and *in vivo* nitrate reductase assays in three intertidal seaweeds. *Bot. Mar.*, **31**: 101–107.
- TISCHNER, R. (1990). New regulatory steps in nitrate assimilation of lower and higher plants. In *Inorganic Nitrogen in Plants and Microorganisms* (Ulrich, W. R., Rigano, C., Fuggi, A. & Aparicio, P. J., editors), 51–53. Springer-Verlag, Berlin.
- VELASCO, P. J., TISCHNER, R., HUFFAKER, R. C. & WHITAKER, J. R. (1988). Synthesis and degradation of nitrate reductase during the cell cycle of *Chlorella sorokiniana*. *Plant Physiol.*, **89**: 220–224.
- VENNESLAND, B. & SOLOMONSON, L. P. (1972). The nitrate reductase of *Chlorella*. Species or strain differences? *Plant Physiol.*, **49**: 1029–1031.
- WATT, D. A., AMORY, A. M. & CRESSWELL, C. F. (1992). Effect of nitrogen supply on the kinetics and regulation of nitrate assimilation in *Chlamydomonas reinhardtii* Dangeard. *J. Exp. Bot.*, **43**: 605–615.
- WHEELER, P. A. (1983). Phytoplankton nitrogen metabolism. In *Nitrogen in the Marine Environment* (Carpenter, E. J. & Capone, D. G., editors), 309–346. Academic Press, New York.
- WRAY, J. L. (1993). Molecular biology, genetics and regulation of nitrite reduction in higher plants. *Physiol. Plant.*, **89**: 607–612.
- WRAY, J. L. & FIDO, R. J. (1990). Nitrate reductase and nitrite reductase. In *Enzymes of Primary Metabolism* (Lea, P. J., editor), 241–256. Academic Press, London.
- ZVYAGIL'SKAYA, R. A., VARTAPETYAN, B. B. & LVOV, N. P. (1996). Nitrate dissimilation in eukaryotes. *Appl. Biochem. Microbiol.*, **32**: 165–169.