*J. Phycol.* **34**, 952–961 (1998)

# DIEL PERIODICITY OF NITRATE REDUCTASE ACTIVITY AND PROTEIN LEVELS IN THE MARINE DIATOM *THALASSIOSIRA WEISSFLOGII* (BACILLARIOPHYCEAE)[1]

*Juan J. Vergara,*[2] *John A. Berges,*[3] *and Paul G. Falkowski*[4]

Environmental Biophysics and Molecular Biology Program, Oceanographic and Atmospheric Sciences Division, Brookhaven National Laboratory, Upton, New York 11973

ABSTRACT

*The diel variation and regulation of the enzyme nitrate reductase (NR) were examined in the diatom* Thalassiosira weissflogii *(Gru.) Fryxell et Hasle. NR was purified, and polyclonal antibodies were raised to a 98-kD polypeptide. The antibodies cross-reacted only with proteins from closely related diatom species, suggesting significant epitopic variation of this enzyme within algal divisions. Neither NR enzymatic activity nor protein was detected in cells grown with ammonium as the sole nitrogen source; the addition of ammonium to cells growing on nitrate decreased both protein levels and enzyme activity by 40% within 2 h. In cells grown on a 12:12 h LD cycle, NR activity and NR protein levels were highly correlated, with a peak at midday, a decrease toward the end of the photoperiod, and an increase in activity beginning near the end of the dark period. The addition of actinomycin D (an inhibitor of RNA synthesis) and cycloheximide (an inhibitor of protein synthesis) affected NR activity and NR protein levels identically, strongly suggesting that this nuclear-encoded protein is regulated primarily at a transcriptional level. The diel pattern of NR protein and activity ceased immediately following transfer to continuous light, indicating that the periodicity is not directly controlled by a circadian rhythm. Time-lagged cross-correlation analysis revealed a 6-h phased difference between the minimum enzyme activity or protein levels and the maximum cellular carbon pool. On the basis of the experimental results, we develop a model proposing that (1) NR activity is regulated primarily by transcriptional regulation of NR synthesis and that (2) the level of expression of the enzyme during a given day is correlated with the integrated pool of organic carbon accumulated during the preceding photoperiod.*

*Key index words: diatoms; diel cycle; nitrate reductase; transcriptional regulation*

*Abbreviations: DCMU, 3′-(3,4-dichlorophenyl)-1′1-dimethylurea; DMSO, dimethylsulfoxide; MSX, methionine sulfoximine; NR, nitrate reductase*

In photoautotrophs, the reduction of nitrate to nitrite is catalyzed by assimilatory nitrate reductase (EC 1.6.6.1, specific for NADH, or EC 1.6.6.2, a bispecific NAD[P]H form) and is often rate-limiting in the overall assimilation of inorganic nitrogen (Solomonson and Barber 1990). Although these nuclear-encoded proteins are found in virtually all oxygenic photosynthetic organisms, several characteristics of NR in unicellular algae in general and in chromophytes in particular differ from those in higher plants (Berges 1997). For example, in unicellular algae, but not in higher plants, the activity of the enzyme is rapidly repressed by ammonium (Eppley et al. 1969, Syrett 1981); the exact mechanism for the repression is unknown. In chlorophytes and higher plants, NR is bispecific for NAD(P)H, whereas in chromophytes the enzyme uses only NADH as a reductant (Syrett 1981, Berges and Harrison 1995a). In chlorophytes and higher plants, maximal NR activity occurs around midday and is low and constant in darkness (Deng et al. 1991, Lillo 1994). However, in chromophytic algae, more complex patterns emerge in which the activity of the enzyme often increases a few hours before the end of the dark period (Eppley et al. 1971, Packard and Blasco 1974, Berges et al. 1995). The processes responsible for the regulation and feedback of NR expression and activity are complex and poorly understood.

In higher plants, the level of expression of NR is regulated at both transcriptional and translational levels (Sherman and Funkhouser 1989), and the catalytic activity of the enzyme is post-translationally modified by redox and allosteric interactions (Kaiser and Brendle-Behnisch 1991) as well as by protein phosphorylation (Huber et al. 1992, Kaiser and Huber 1994, Glaab and Kaiser 1996). Although not conclusively established in eucaryotic algae, some form of post-translational modification of NR is implied by the influence on enzyme activity of such factors as irradiance (Gao et al. 1992), light quality (Ninnemann 1987), interactions at the uptake step (Collos 1989), and intracellular carbon and nitrogen compounds (Flynn 1991). However, despite the wide variety of regulatory mechanisms possible, *in vitro* NR assays of enzyme activity are highly correlated with nitrate incorporation rates in diatoms, even during LD cycles and in the presence of ammonium (Berges and Harrison 1995a, b, Berges et al. 1995). The correlation between *in vitro* assays and nitrate incorporation might simply be fortuitous; however, it suggests that NR expression and activity

[1] Received 16 September 1997. Accepted 17 July 1998.

[2] Present address: Departamento de Biología Animal, Vegetal y Ecología, Facultad de Ciencais del Mar, Universidad de Cádiz, Apartado 40, 11510 Puerto Real-Cadiz, Spain.

[3] Present address: School of Biology and Biochemistry, The Queens University of Belfast, Belfast, BT9 7BL, Northern Ireland, United Kingdom.

[4] Corresponding author. Present address: Environmental Biophysics and Molecular Biology Program, Institute of Marine and Coastal Sciences, Rutgers University, 71 Dudley Road, New Brunswick, New Jersey 08901; e-mail falko@imcs.rutgers.edu.

are coupled to growth and, by extension, photosynthetic processes (Falkowski and Raven, 1997).

The strongest natural oscillator of photosynthesis is the diel cycle. Diel variations in photosynthetic activity can be driven either by intrinsic circadian clocks or by metabolic feedbacks. To distinguish between these two phenomena and investigate potential feedback processes influencing NR activity, we studied the diel regulation of NR in a chromophyte, the marine diatom *Thalassiosira weissflogii*. Using a polyclonal antibody raised against NR purified from this organism, we demonstrate with simultaneous western blots and measurements of enzyme activity that the diel periodicity in NR is regulated primarily at the level of transcription. Although there is evidence of post-translational modification, the latter basically plays a minor role by "tuning" the enzyme activity. NR expression appears to be modulated by feedback signals from products of photosynthetic carbon metabolism and does not appear to be under the direct control of a circadian clock.

## MATERIALS AND METHODS

*Culture conditions.* The diatom *Thalassiosira weissflogii* (Gru.) Fryxell et Hasle (clone T-VIC) was grown in semicontinuous batch culture in an artificial seawater medium with f/2 nutrients and nitrate as the sole inorganic nitrogen source, as previously described (Berges and Falkowski 1996). Cells were maintained at 20° C and under 200 μmol quanta $m^{-2}$ $s^{-1}$ irradiance, provided either continuously or on a 12:12 h LD cycle.

*Sampling and general analyses.* All samples were taken from cultures growing in early to mid-logarithmic growth phase. Cell counts and cell volumes were determined on samples preserved in Lugol's iodine using a model TAII Coulter Counter following the procedures described by Montagnes et al. (1994). Culture growth and status were monitored by variable fluorescence measurements made with a Turner Designs model 10 fluorometer and DCMU (Geider et al. 1993). Carbon and nitrogen content were measured on samples collected on precombusted 13-mm Gelman A/E filters, using a Perkin Elmer Model 2400 Series II CHNS/O analyzer (Norwalk, Connecticut).

*NR activity.* NR activity was assayed at 25 EC by measuring $NO_2^-$ produced in a 10-min incubation, according to the basic methods described by Evans and Nason (1953) and modified by Berges and Harrison (1995a). Twenty-five milliliters of culture was filtered onto 13-mm Gelman-type A/E glass fiber filters and homogenized using a glass/Teflon tissue grinder on ice. Assays always included 20-μM flavin adenine dinucleotide. NR activity was expressed in units of activity (U), where 1 unit is the amount of NR catalyzing the conversion of 1 μmol of nitrate to nitrite per minute.

*Protein electrophoresis, blotting, and detection methods.* Cells were harvested by centrifugation, sonicated for two cycles of 20 s in a Kontes Ultrasonic Cell Disrupter (Vineland, New Jersey) in ice-cold TCA in acetone (10% w/v). Proteins were precipitated for 1 h at −20° C and collected by centrifugation. Pellets were washed in 100% acetone and air-dried. Protein was measured according to the bicinchoninic acid method (Smith et al. 1985). Samples were prepared for SDS-PAGE following Greene et al. (1991) and loaded on an equal protein basis. Total proteins were separated at a constant voltage of 150 V for 1 h, using either precast 4%–20% gradient (Biorad 161–0903) or 7.5% Tris-glycine polyacrylamide gels. Following electrophoresis, the proteins were transferred to nitrocellulose membranes (Greene et al. 1991) and detected by chemiluminescence (ECL method, Amersham) following reaction with specific antibodies (Shanklin et al. 1995). The abundance of specific proteins was quantified from the density of bands on X-ray film using a Molecular Dynamics Computing Densitometer (Sunnyvale, California).

*Purification of NR, antiserum production, and characterization.* NR was purified by affinity chromatography using a modification of the protocol of Gao et al. (1993), based on the methods of Amy and Garrett (1974). A total of 160 L of log-phase culture (ca. 5 × $10^5$ cells·$mL^{-1}$) of *T. weissflogii*, grown in continuous irradiance, was harvested by continuous-flow centrifugation and frozen at −70 EC. Cells were thawed and broken into two 5-min cycles through a Yeda press (Linca Ltd., Tel Aviv, Israel) at 1500 psi. The crude extract was centrifuged at 10,000 × *g* for 10 min, bound in batch to Blue Sepharose (Pharmacia CL6B), packed into a 30 × 1 cm column, and washed according to Gao et al. (1993). NR was eluted in a 0- to 35-μM gradient of NADH and collected in 2-mL fractions at a flow rate of 1.5 mL·$min^{-1}$. The three fractions showing the greatest NR activity were pooled, and protein was precipitated with 45% ammonium sulfate, resuspended in binding buffer, and frozen at −70 EC.

Before immunization, a final gel purification was performed using SDS-PAGE under denaturing conditions. The dominant band, migrating at 98 kD, was visualized using a reversible copper stain (BioRad 161–0470) and cut from the gel for use in immunization. Antibodies were prepared in rabbits by Cocalico Biologicals Inc. (Reamstown, Pennsylvania) using an initial injection of 100 μg of antigen mixed with Freund's incomplete adjuvant, followed by boosts of 50 μg at 14, 21, and 49 days.

The antiserum produced was characterized in three ways. First, the activity of partially purified NR protein was measured in the presence of different concentrations of antibody (1:100000 to 1:10). Second, a dilution series of crude extracts from *T. weissflogii* was prepared, blotted, and challenged with the antiserum (1:2000). Third, a culture of *T. weissflogii* was grown as described above but with nitrate replaced with 100 μM of ammonium. Under these conditions, NR activity was undetectable (see Berges et al. 1995). Samples of nitrate- and ammonium-grown cultures were loaded on an equal protein basis, separated, and blotted to nitrocellulose as described above and then probed with NR antiserum (1:2000 dilution).

To determine the cross-reactivity of the NR antiserum from *T. weissflogii* with NR in other species, cultures of several microalgae were grown under continuous light with nitrate as the sole nitrogen source. The following species were harvested in logarithmic growth phase: the diatoms *Thalassiosira pseudonana* (Hustedt) Hasle et Heimdal (clone 3H ), *Skeletonema costatum* (Greville) Cleve (clone SKEL), *Phaeodactylum tricornutum* Bohlin (Culture Collection of Marine Phytoplankton (CCMP) clone 630), *Ditylum brightwellii* (T. West) Grunow ex van Huerck (North East Pacific Culture Collection (NEPCC) clone 8), and *Chaetoceros* spp. (CCMP 1424); the dinoflagellates *Amphidinium carterae* Hulburt (NEPCC 629) and *Scrippsiella trochoidea* (Stein) Loblich III (NEPCC 15); the chromophytes *Emiliania huxleyi* (Lohman) Hay et Mohler (CCMP 374) and *Isochrysis galbana* Parke (CCMP 1323); the chlorophyte *Dunaliella tertiolecta* Butcher (clone DUN); and the cyanobacterium *Synechococcus* sp. (WH 5701). Proteins, loaded equally in all lanes, were separated by PAGE and transferred to nitrocellulose and probed with NR antiserum as described.

*Ammonium effects on NR.* Different concentrations of ammonium were added to replicate, logarithmically growing cultures of *T. weissflogii* that had previously been growing with nitrate as the sole nitrogen source. NR activities and NR protein levels were measured 2 h after additions. For one ammonium concentration (100 μM), the time course of change in NR was followed for 4 h after ammonium was added. Because previous studies have shown that inhibition of NR does not occur if ammonium incorporation is blocked using the glutamine synthetase inhibitor methionine sulfoximine (MSX) (see Syrett 1981), the effect of adding 100 μM of MSX with and without 100 μM of ammonium was tested.

*Diel periodicity of NR.* One-liter replicate cultures of *T. weissflogii* were grown on a 12:12 h LD cycle and maintained in logarithmic growth phase for eight generations before sampling commenced. Cultures were sampled every 3 h until culture volume was exhausted. Samples were collected for cell counts, cell volume de-

termination, CHN analyses, and NR activity and NR protein measurements. The complete experiment was repeated twice.

In some cases, to improve the accuracy of quantitation when it was necessary to apply samples to more than one western blot, NR protein was normalized to Rubisco protein. In *T. weissflogii*, cellular levels of Rubisco protein were unaffected by experimental treatments; the turnover of Rubisco is lower than that of NR, and its abundance is not affected by irradiance (Sukenik et al. 1987). Polyclonal antiserum raised against the Rubisco holoenzyme of *Isochrysis galbana* (Falkowski et al. 1989) cross-reacts with the large subunit of Rubisco in diatoms. This antiserum was used at a dilution of 1:10000 simultaneously with the NR antiserum.

To clarify whether the diel periodicity of NR is evidence of an endogenous circadian rhythm, the experiments were repeated, but cultures were moved from a 12:12 h LD photoperiod, to which they had been acclimated for eight generations, to continuous irradiance. NR activity and NR protein were followed for an additional 48 h of sampling as above, except that CN measurements could not be made because of restricted volumes of culture.

In separate experiments performed on cultures grown on a diel cycle as described above, the effects of different metabolic inhibitors on the diel pattern of NR activity and protein were examined. Subsamples of cultures were taken and maintained in 50-mL flasks. Inhibitors were added 4 h before the onset of the light period, and flasks for monitoring NR activity and protein were taken every 3 h. Inhibitors included the RNA synthesis inhibitor actinomycin D (10 $\mu g \cdot mL^{-1}$), the protein synthesis inhibitor cycloheximide (100 $\mu g \cdot mL^{-1}$), and the photosynthetic electron transport inhibitors DCMU, 2,5 dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), and methylamine. Hydrophobic inhibitors were dissolved in 100 $\mu M$ of DMSO to give a final concentration of 10 $\mu M$. Control experiments indicated that DMSO at up to 1 mM did not affect the growth rate of *T. weissflogii*.

## RESULTS

*NR purification and antibody characterization.* The purification of NR led to an increase in specific activity by a factor of approximately 30,000 from the crude homogenate. The fractions with the highest specific activity isolated from the affinity column showed only four to six proteins on silver- or Coomassie-stained denaturing gels. The density of the NR band indicated that NR was almost 100-fold more abundant than any other protein and was well separated from other proteins (Fig. 1A). The molecular mass of the polypeptide recognized by the antibody was calculated as 98 kD (Fig. 1), which is comparable to that reported for individual NR subunits from other sources (Solomonson and Barber 1990). Applied at a 1:2000 dilution, the crude antiserum detected a single band with an apparent molecular mass of 98 kD in crude protein extracts of *T. weissflogii*. The relationship between protein concentration in the extract and the NR detected was linear over a 10-fold range.

NR activity in partially purified preparations was unaffected by the addition of crude antiserum at titers up to 1:10, and attempts to immunoprecipitate NR using the antiserum and protein-A Sepharose were unsuccessful, both in crude extracts and with the partially purified protein (data not shown). In contrast to nitrate-grown cells, ammonium-grown cultures showed neither NR activity nor detectable NR protein (Fig. 1B).

The polyclonal antiserum raised against NR from

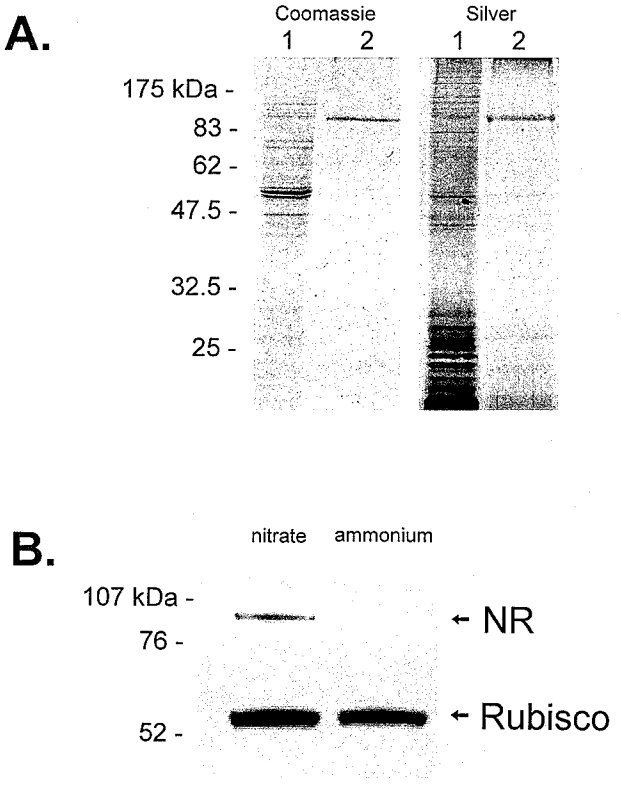


FIG. 1. (A) Comparison of whole-cell homogenates of *T. weissflogii* (lane 1) or NR partially purified by Blue Sepharose affinity chromatography followed by ammonium sulfate precipitation (lane 2), stained with either Coomassie R250 or silver stains. (B) Western blot of total protein from two cultures of *Thalassiosira weissflogii* grown either with nitrate or ammonium as sole nitrogen source and challenged with antisera to NR from *T. weissflogii* (1:2000) and to *Rubisco* from *Isochrysis galbana* (1:10,000) and detected using chemiluminescence techniques.

*T. weissflogii* did not cross-react with any of the non-diatom species tested (data not shown). When prolonged exposures or increased antibody titer was used, proteins stained nonspecifically in proportion to their abundance. Among the diatoms, several cross-reacting peptides were found (Fig. 2). The strongest response was observed in the congener *T. pseudonana*. A similar-sized peptide in *Skeletonema costatum* cross-reacted, but there was also a smaller band that reacted with equal intensity. *Ditylum brightwellii* exhibited slight cross-reactivity at a similar molecular mass, whereas *Phaeodactylum tricornutum* cross-reacted at a substantially lower molecular mass. Of the diatom species tested, only *Chaetoceros* sp. lacked cross-reactivity.

*Effects of ammonium addition on NR.* The addition of ammonium caused parallel decreases in both NR activity and NR protein (Fig. 3). The decrease in activity was unaffected by the ammonium concentrations >100 $\mu M$; at all concentrations, NR activity dropped to about 50% of initial levels 2 h following the addition (Fig. 3A). The decrease in NR occurred at approximately the same rate as the declines caused by the addition of actinomycin D or cyclo-

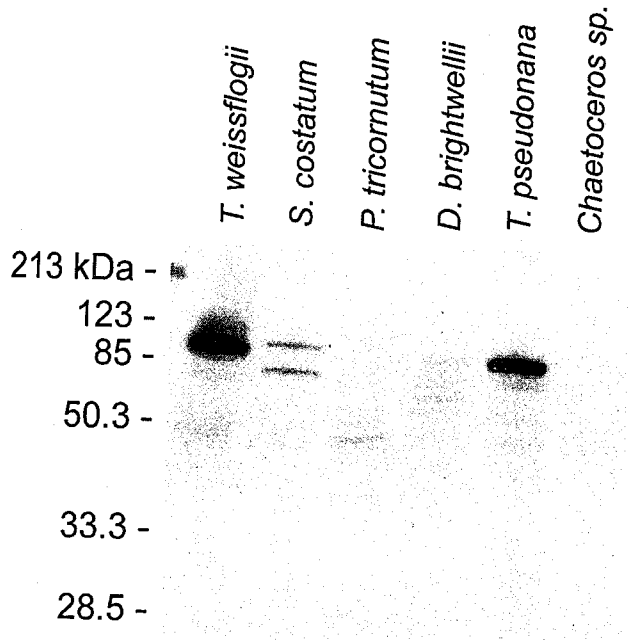


FIG. 2. Western blot of total protein extracts from 6 diatom species challenged with antiserum to *T. weissflogii* NR. Diatoms were all grown under continuous irradiance with nitrate as the sole nitrogen source and were harvested in early to mid-logarithmic growth phase. Samples from *Thalassiosira* species have been purposely overexposed to highlight weaker cross-reactions in other species. Other details are as in Figure 1.

heximide (data not shown), and changes in NR protein paralleled those in NR activity (Fig. 3B). The loss of NR caused by the addition of ammonium was not prevented by the addition of MSX (Fig. 3C).

*Diel periodicity of NR.* When grown on a 12:12 h LD cycle, both NR expression and catalytic activity showed a diel periodicity (Fig. 4A). The periodicity in protein levels and enzyme activity was highly autocorrelated throughout the light cycle. Although cell composition (C, N, or total protein) and cell volume varied over the light cycle (see below), the variability was far smaller than that for NR activity or protein. Thus, scaling NR measurements to other cellular parameters does not affect the basic patterns observed (cf. Berges and Harrison 1995a). As is previously established for diatoms in general and in *Thalassiosira* species in particular (Berges et al. 1995, Chisholm 1981), cell division is not synchronized by the LD cycle, and cell numbers increased monotonically throughout both light and dark periods.

Both NR protein levels and enzyme activity increased prior to the beginning of the light period, reached a maximum in the middle of the light period, and fell toward the end of the light period, remaining low for most of the dark period. There was a small but consistent lag between the peak of

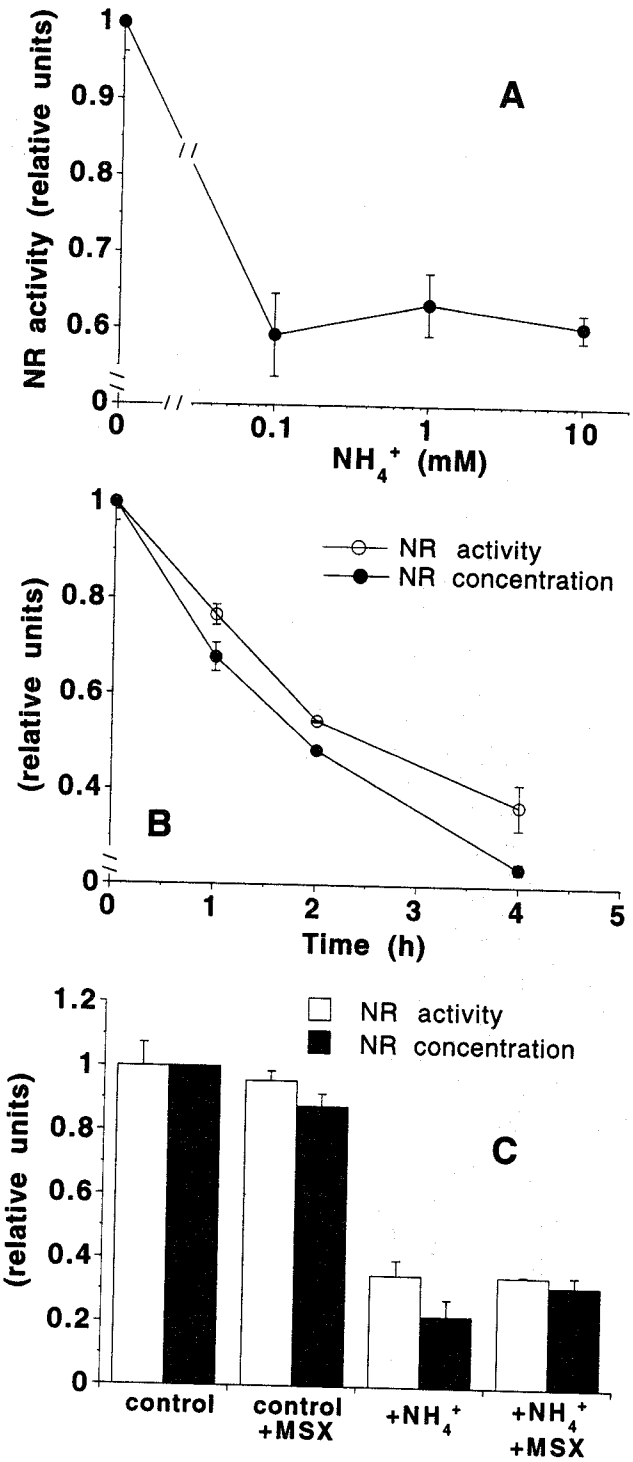


FIG. 3. Ammonium inhibition of NR in log-phase cultures of *Thalassiosira weissflogii* grown in nitrate. (A) Effect of concentration of ammonium addition on NR activity 2 h after addition; (B) time course of decline of NR activity and NR protein following a pulse of 100 μM of ammonium; (C) effects of ammonium (100 μM) and MSX (0.1 mM) on NR activity and NR protein 4 h after ammonium addition. Error bars represent means (±SE) of determinations from two replicate cultures.

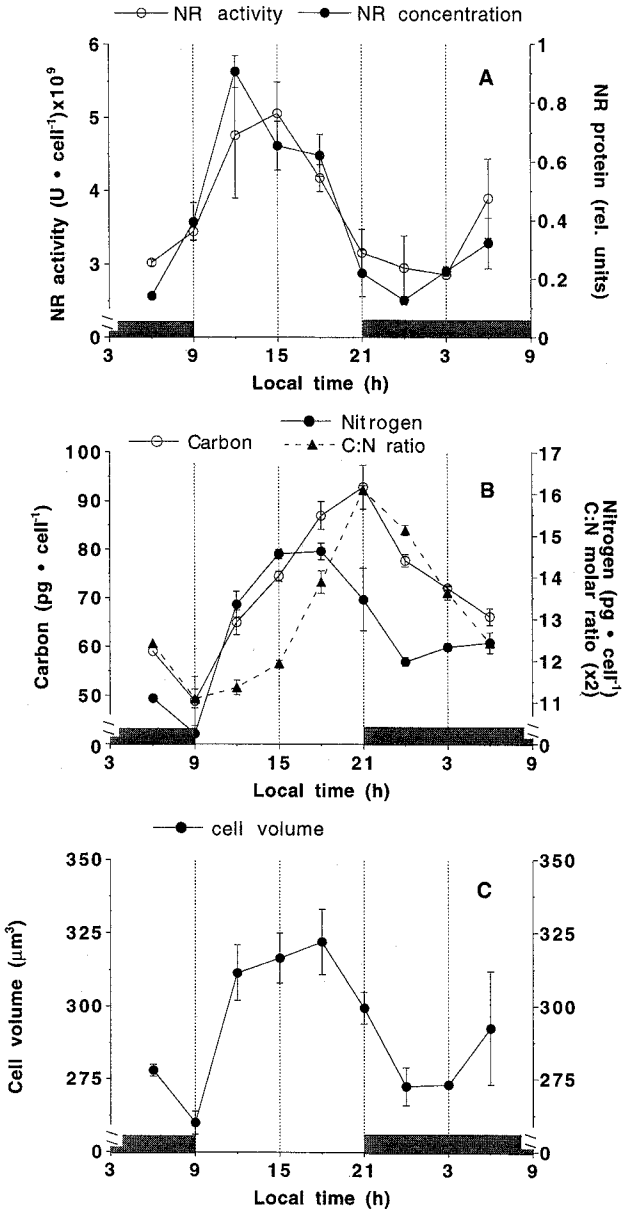


FIG. 4. Diel cycles of NR and cell constituents in log-phase cultures of *Thalassiosira weissflogii* grown on a 12:12 h LD cycle. (A) NR activity and NR protein; (B) cellular C and N content and C:N molar ratio; (C) cell volume. Symbols represent the mean (±SE) of determinations from two replicate cultures. Cells were acclimated to photoperiod for more than eight generations before sampling.

NR protein levels and the subsequent peak in NR activity. Cellular carbon content increased monotonically during the light period and declined monotonically in the dark (Fig. 4B). Both cell nitrogen content and cell volume showed a more complex pattern that was more similar to the pattern of NR (Fig. 4B, C). Carbon:nitrogen (C:N) ratios followed the pattern of C content closely, and variation in the ratio was determined largely by changes in carbon rather than nitrogen pools.

Following transfer from an LD cycle to continuous light, both NR expression and catalytic activity rapidly reached time-invariant, steady-state levels that fell between the maxima and minima observed during diel cycles (Fig. 5A). In contrast, cell volume maintained a cyclic pattern in continuous light, indicative of a circadian rhythm (Fig. 5B).

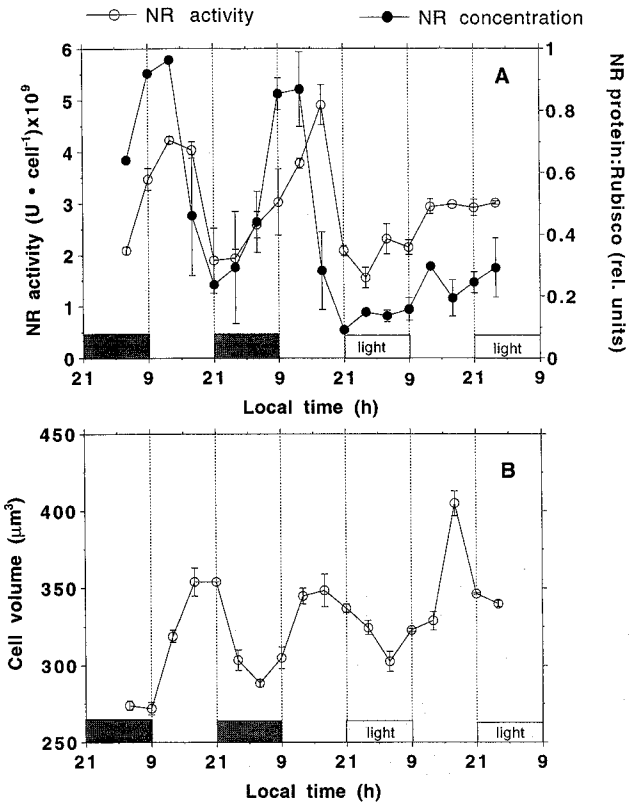


FIG. 5. Effect of transition from LD cycles to continuous irradiance on NR and cell volumes in log-phase cultures of *Thalassiosira weissflogii*. White rectangles labeled >light= indicate the normal timing of the dark period. Other details are as in Figure 4.

The addition of actinomycin D and cycloheximide 4 h before the onset of the light period had identical effects, abolishing the normal diel oscillation of both NR activity and protein levels (Fig. 6). The addition of DCMU resulted in significantly lower NR activity (ANOVA, $F_{5,25} = 9.36$, $P < 0.00005$) but not in a statistically significant lower NR protein level, whereas DBMIB did not affect either NR activity or protein concentration. Methylamine had no significant effect (Fig. 6A). Two hours following the onset of the light period, NR protein levels were significantly higher for DCMU and DBMIB treatments (Fig. 6B), although this difference did not persist later in the cycle.

## DISCUSSION

*NR purification and antibody characterization.* Only proteins from diatoms cross-reacted with the polyclonal antiserum raised against NR from *T. weissflogii*. The observed cross-reactivity is largely consistent with taxonomic relationships; the species found to

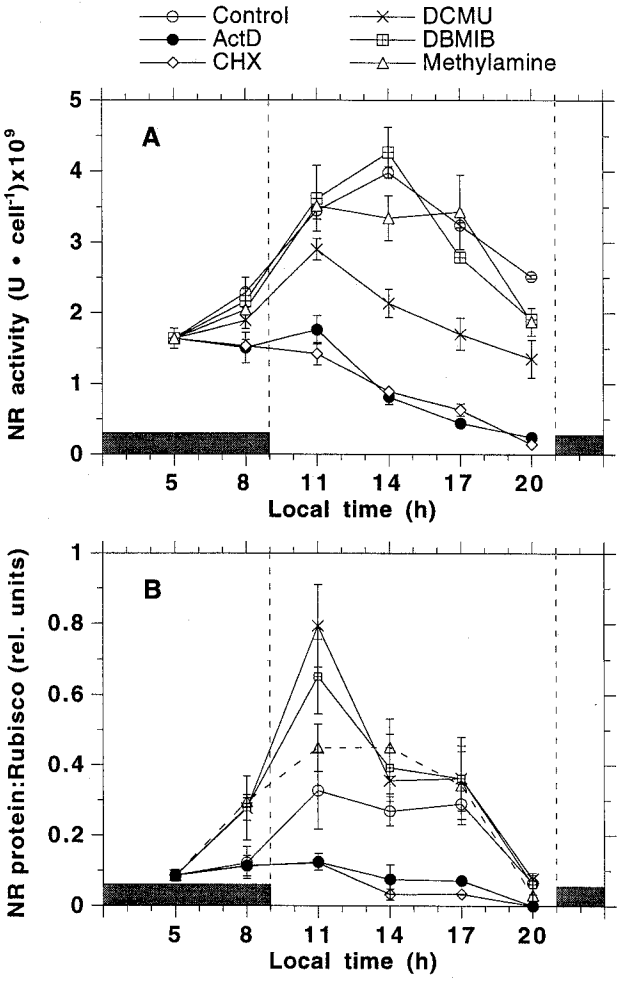


FIG. 6. Effect of inhibitors of RNA and protein synthesis, actinomycin D (ACT-D) (10 μg·mL$^{-1}$) and cycloheximide (CHX) (100 μg·mL$^{-1}$), and photosynthetic electron transport inhibitors DCMU, DBMIB, and methylamine (10 μM in 100 μM DMSO) on NR activity and NR protein in log-phase cultures of *Thalassiosira weissflogii* grown on 12:12 h LD cycles. Inhibitors were added at the beginning of the experiment. Details are as in Figure 5.

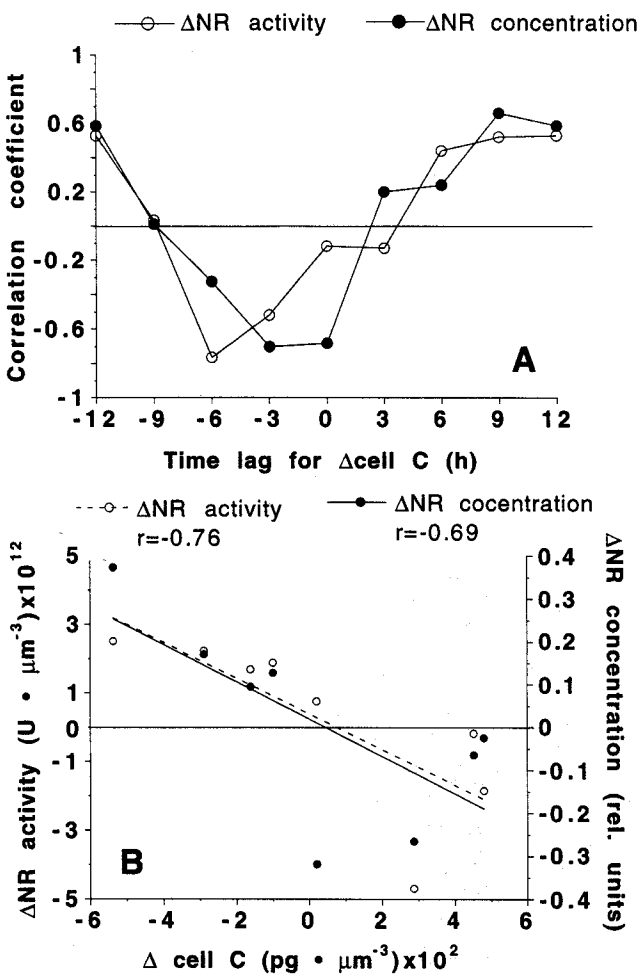


FIG. 7. Cross-correlation between changes with respect to time (Δ) in either NR activity or NR protein and changes in cell carbon content (ΔC). Time lag for ΔC corresponds with the sampling interval (3 h). Data are those shown in Figure 4B. Negative relationship between ΔNR activity and ΔC using a time lag of 6 h and between ΔNR protein and ΔC using a time lag of 3 h.

cross-react most strongly in the present study (*T. pseudonana* and *S. costatum*) are from the same family as *T. weissflogii* (Thalassiosiraceae), whereas *D. brightwellii* and *Chaetoceros* sp. are from different families within the same order (Centrales), and *P. tricornutum* is from a distinct order (Penales) (Bold and Wynne 1985). Only one other detailed comparison of cross-reactivity among diatom NR has been reported (Gao et al. 1993), and results obtained were similar; antiserum raised against NR from *Skeletonema costatum* cross-reacted weakly with *T. pseudonana* and showed two cross-reacting bands at lower molecular masses in *Phaeodactylum tricornutum*, whereas nondiatom species did not cross-react. However, in contrast to the results of Gao et al. (1993), we found that an antibody to NR from squash (a gift of Dr. W.H. Campbell) did not cross-react with diatom polypeptides in the 100-kD range, even at titers as high as 1:250 (data not shown). Although recognizing that differences in cross-reactivity can be characteristic also of the particular antibodies used, we believe that these results support previous biochemical evidence suggesting that there are substantial differences between epitopic sites in NR from diatoms and those in other taxa and between diatom taxa (see Berges and Harrison 1995a).

The fact that antibodies against NR from *T. weissflogii* did not inhibit NR activity in partially purified preparations suggests that the epitopes recognized by the antibodies either are not at the active sites of the enzyme, which are largely conserved (cf. Gao et al. 1993, Campbell, 1996), or do not recognize the native trimeric protein but, rather, are epitopes hidden in the native conformation. The failure of NR to immunoprecipitate with the antiserum and protein-A Sepharose supports the latter hypothesis.

*Effects of ammonium addition on NR.* Whereas the repression of NR by ammonium is well documented in unicellular algae (Syrett 1981, Zeilerand Solo-

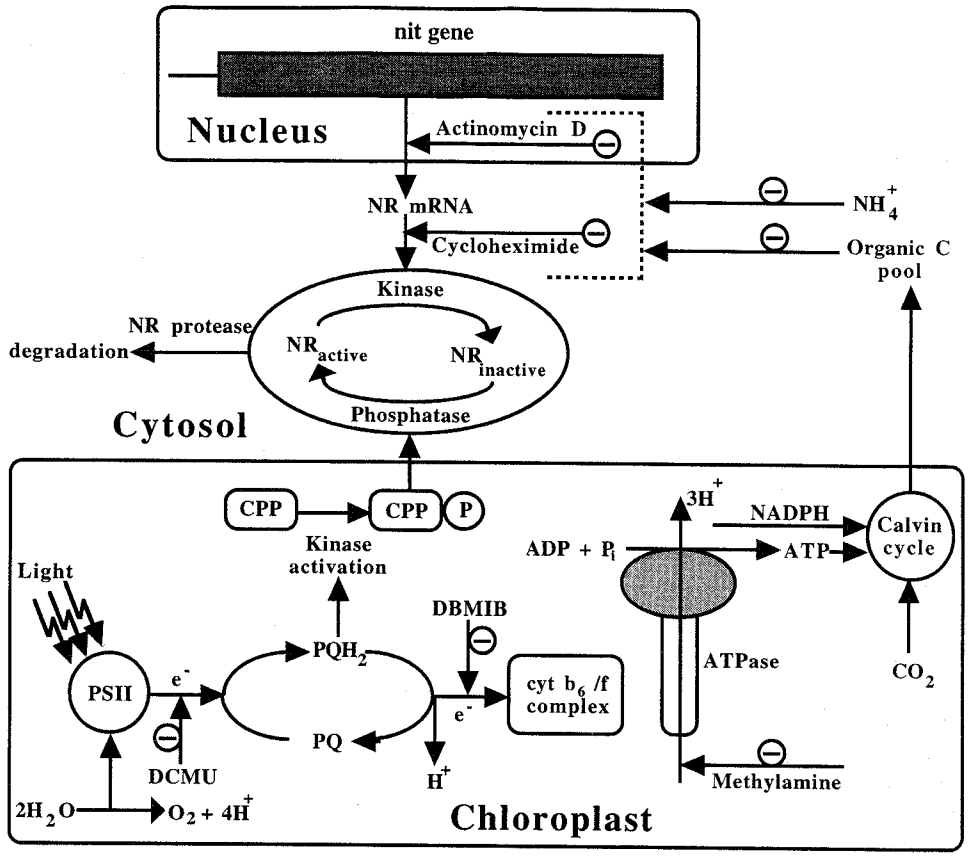


FIG. 8. Working model of the regulation of NR in *Thalassiosira weissflogii*. Negative signs indicate inhibitory effects on the associated process. Question marks denote pathways that remain unclear.

monson 1989), until now the distinction between the repression of NR activity and protein level has been unclear. Our results indicate that the repression is the consequence of a decreased level of the protein. The addition of ammonium to cells growing on nitrate led to declines in NR activity and protein levels at rates similar to those found with both cycloheximide and actinomycin D. These results suggest that (1) NR turns over rapidly *in vivo*, (2) the rate of turnover is regulated by the rate of synthesis of the protein rather than by an alteration in the rate of degradation of the enzyme, and (3) ammonium represses the synthesis of NR. We conclude that ammonium rapidly represses NR transcription by means of a secondary, indirect pathway rather than having a direct allosteric or post-translational effect on NR activity.

It is generally believed that ammonium per se is not responsible for the repression of NR activity but rather that the repression is mediated by a product of ammonium assimilation, such as an amino acid (see Collos 1989, Flynn 1991). This conclusion is supported by the observation that MSX sometimes prevents ammonium from repressing NR (see Syrett 1981). Despite clear evidence that the incorporation of ammonium in diatoms is through glutamine synthetase (Zehr and Falkowski 1988), MSX failed to prevent the decline of NR in *T. weissflogii* in the present study. Interestingly, other studies have failed to show an effect of MSX in green algae and higher plants (Díez and López-Ruiz 1989, Kaiser and Brendle-Behnisch 1991). This suggests either that MSX might not be as effective as is sometimes maintained or that the effect of ammonium is more direct; that is, a product of ammonium assimilation is not required to down-regulate NR expression. Kaiser and Brendle-Benisch (1991) proposed that the effects of ammonium might be mediated by cellular alkalinization following ammonia incorporation; however, were this so, we might expect that methylamine would induce a response similar to that of ammonium. It did not. It is possible that the effect of ammonium is principally on uptake of nitrate (see Collos 1989); if ammonium inhibited nitrate transport and reduced nitrate levels within the cell, and if nitrate were required to maintain levels of NR synthesis, then NR protein level and activity would be affected indirectly. Alternatively, a change in the redox status of the photosynthetic electron transport system due to the differences between nitrate and ammonium incorporation might be responsible (Turpin 1991, Escoubas et al. 1995).

*Diel periodicity of NR.* The diel cycle of NR expression and enzyme activity observed is qualitatively

similar to that found in unialgal cultures and natural assemblages of marine diatoms (Berges et al. 1995). Previous studies have considered multiple interacting factors, including shifts in irradiance and nitrogen source, as the underlying cause of NR variability in diatoms (Smith et al. 1992); however, this study is the first to document that the diel pattern is a consequence of variation in NR expression.

What causes the diel variation in NR activity? One possibility is that NR is directly regulated by light by means of a photosensitive element (Roldan and Butler 1980). In *T. weissflogii,* NR protein and activity increase prior to the onset of the photoperiod and vary during the photoperiod when irradiance is constant, making direct photoregulation unlikely. An alternative possibility is reversible post-translational modification, for example, phosphorylation involving a light-regulated protein kinase (see Huber et al. 1994). Like Gao et al. (1993), we were unable to demonstrate an effect of magnesium on NR activity that would support a phosphorylation mechanism similar to that in higher plants (see Huber et al. 1992). However, there is evidence for post-translational regulation in the down-regulation of NR activity but not in protein levels in DCMU-treated cells (Fig. 6). It is possible that we simply observed the synthesis of an immunoreactive but catalytically inactive NR protein in the presence of DCMU; however, the lag between peaks of NR protein levels and enzyme activity in the normal diel cycle also supports a post-translational mechanism. Because DCMU prevents the reduction of plastoquinone, these results suggest that the redox state of electron carriers on the acceptor side of Photosystem II, or downstream of Photosystem I, affects the activity of NR by means of a post-translational modification. Indeed, the plastoquinone redox system is coupled to a protein kinase (Race and Hind 1996) that has been implicated in a phosphorylation cascade in the cytosol (Escoubas et al. 1995). If so, it might be anticipated that DBMIB (which affects the oxidation of plastoquinone) would have an opposite effect; this inhibitor is more difficult to use, and our negative results are not conclusive. Nonetheless, the close correspondence of NR activity and protein suggests that post-translational modification of NR plays a relatively minor role in the overall determination of enzyme activity compared with the regulatory elements controlling protein abundance.

The diel periodicity in NR could be directly coupled to an endogenous circadian rhythm (Ronnenberg 1996). Such rhythms have been hypothesized to occur with respect to NR in higher plants (Lillo and Ruoff 1989, Pilgrim et al. 1993) and in a marine dinoflagellate (Ramalho et al. 1995). Although the transition from an LD cycle to continuous light resulted in an almost immediate loss of the cycle in NR levels, diel patterns in cell volume (Fig. 5B) were maintained. Maintenance of a diel cycle in chlorophyll content during transition from light to darkness has also been documented (Post et al. 1984). These results suggest that NR is not directly controlled by a circadian rhythm, although it should be noted that circadian phenomena in diatoms appear to differ from those of many other eucaryotic photoautotrophs (Chisholm 1981).

An alternative hypothesis to account for the diel variations in NR levels is a coordination with photosynthesis or some product of photosynthesis (Turpin et al. 1988, Turpin 1991, Sheen 1994, Crawford 1995). To explore this hypothesis, we analyzed changes in NR activity and NR protein levels in relation to changes in cell carbon content. This analysis was complicated by potential delays in responses of carbon and nitrogen metabolism and so was developed in two stages. First, we performed a cross-correlation with data from several diel experiments to assess the difference in timing between the diel cycle of NR activity/protein and cell carbon. This analysis revealed that changes in NR activity and changes in cell carbon content were strongly negatively correlated with a time lag of 6 h, whereas changes in levels of NR protein and changes in cell carbon content were best correlated with a time lag of 0–3 h (Fig. 7A). Put simply, cellular carbon content at the end of the photoperiod (when the pool of carbon is maximal) is highly negatively correlated with NR protein level in the first 3 h of darkness (when the NR protein pool is at a minimum). The maximum in the cell carbon pool is highly correlated with a minimum in NR enzyme activity 6 h later. Using these time lags, we plotted changes in carbon pools and changes in NR activity and protein (Fig. 7B). These results suggest the possibility of a feedback between cell carbon and NR levels. Examining the patterns of changes in intermediate compounds might be a logical next step (e.g. Turpin et al. 1988) in an effort to determine the specific molecules that mediate the regulation of NR levels.

We propose a working model for the regulation of NR in *Thalassiosira weissflogii* (Fig. 8) that suggests a variety of testable hypotheses. The level of NR protein in the cell at any moment in time is given as the difference between the rate of synthesis and degradation. The similar rates of NR loss in the presence of cycloheximide, actinomycin D, and ammonium at a given growth rate suggest that the rate of NR protein degradation is not highly regulated. As a first approximation, we assume that, for a given growth rate, the rate of NR degradation (by an NR-specific protease) is relatively constant and follows first-order kinetics. For example, we estimate a rate constant of 0.3 $h^{-1}$ for the cells growing at 0.75 $d^{-1}$ (Fig. 4). The constant loss of NR is offset by variable synthesis rates that are transcriptionally regulated (see also Smith et al. 1992). We hypothesize that the rate of synthesis is directed by the rate of accumulation of photosynthetic products (i.e. the organic carbon pool). As well, ammonium has a direct effect on NR synthesis rates through an unknown mecha-

nism. Although the level of NR activity is closely tied to the level of accumulated product, it can be post-translationally modified. Whether this regulation is by means of protein phosphorylation remains to be elucidated; however, we have suggested a plastiquinone-linked phosphorylation mechanism in Figure 8. Thus, the primary source of regulation of NR in *Thalassiosira weissflogii* is by means of transcriptional control of protein synthesis, with a secondary post-translational modification of enzyme activity. This relatively simple control pathway explains why simple assays of NR activity provide information about cell growth and nitrate incorporation in natural phytoplankton communities dominated by diatoms (Berges and Harrison 1995a, b, Berges et al. 1995, Berges 1997). Although the daily synthesis and degradation of a protein might seem wasteful, it is not necessarily inefficient. NR protein represents a very small investment in cell protein in diatoms, on the order of 0.1% (Gao et al. 1993). This strategy of regulation contrasts with that found in higher plants, in which alternative carbon sinks are available and protein turnover and cell division comparatively slow.

We thank Dr. S. Huber for discussions about phosphorylation mechanisms and Drs. R. M. McKay and J. Shanklin for guidance in immunochemical detection protocols. Conversations with Dr. T. Roenneberg helped clarify issues concerning circadian oscillations. Two anonymous referees helped substantially to improve the presentation of the data. The research was supported by the U.S. Department of Energy under contract DE-ACO2-98CH10886. J.J.V. was supported by a postdoctoral F.P.U. fellowship from the Spanish Ministry of Education and Science and J.A.B. by a postdoctoral fellowship from the Natural Science and Engineering Research Council, Canada.

Amy, N. K. & Garrett, R. H. 1974. Purification and characterization of the nitrate reductase from the diatom *Thalassiosira pseudonana*. *Plant Physiol.* 54:629–37.

Berges, J. A. 1997. Algal nitrate reductases. *Eur. J. Phycol.* 32:3–8.

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.* 124:259–69.

Berges, J. A. & Falkowski, P. G. 1996. Cell-associated proteolytic enzymes from marine phytoplankton. *J. Phycol.* 32:566–74.

Berges, J. A. & Harrison, P. J. 1995a. 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.

——— 1995b. Relationships between nitrate reductase activity and rates of growth and nitrate incorporation under steady-state light or nitrate limitation in the marine diatom *Thalassiosira pseudonana* (Bacillariophyceae). *J. Phycol.* 31:85–95.

Bold, H. C. & Wynne, M. J. 1985. *Introduction to the Algae.* Prentice-Hall, Englewood Cliffs, New Jersey, 720 pp.

Campbell, W. H. 1996. Nitrate reductase biochemistry comes of age. *Plant Physiol.* 111:355–61.

Chisholm, S. W. 1981. Temporal patterns of cell division in unicellular algae. *Can. Bull. Fish. Aquat. Sci.* 210:150–81.

Collos, Y. 1989. A linear model of external interactions during uptake of different forms of inorganic nitrogen by microalgae. *J. Plankton Res.* 11:521–33.

Crawford, N. M. 1995. Nitrate: nutrient and signal for plant growth. *Plant Cell* 7:859–68.

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.* 29:239–47.

Díez, J. & López-Ruiz, A. 1989. Immunological approach to the regulation of nitrate reductase in *Monoraphidium braunii*. *Arch. Biochem. Biophys.* 268:707–15.

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.* 7:150–4.

Escoubas, J. M., Lomas, M., LaRoche, J. & Falkowski, P. G. 1995. Light intensity regulation of cab gene transcription is signalled by the redox state of the plastoquinone pool. *Proc. Natl. Acad. Sci. USA* 92:10237–41.

Evans, H. J. & Nason, A. 1953. Pyridine nucleotide-nitrate reductase from extracts of higher plants. *Plant Physiol.* 28:233–54.

Falkowski, P. G. & Raven, J. A. 1997. *Aquatic Photosynthesis.* Blackwell, Cambridge, 375 pp.

Falkowski, P. G., Sukenik, A. & Herzig, R. 1989. Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). II. Relative abundance of chloroplast proteins. *J. Phycol.* 25:471–8.

Flynn, K. J. 1991. Algal carbon-nitrogen metabolism: a biochemical basis for modelling the interactions between nitrate and ammonium uptake. *J. Plankton Res.* 13:373–87.

Gao, Y., Smith, G. J. & Alberte, R. S. 1992. Light regulation of nitrate reductase in *Ulva fenestrata* (Chlorophyceae). I. Influence of light regimes on nitrate reductase activity. *Mar. Biol.* 112:691–6.

——— 1993. Nitrate reductase from the marine diatom *Skeletonema costatum*. Biochemical and immunological characterization. *Plant Physiol.* 103:1437–45.

Geider, R. J., LaRoche, J., Greene, R. M. & Olaizola, M. 1993. Response of the photosynthetic apparatus of *Phaeodactylum tricornutum* (Bacillariophyceae) to nitrate, phosphate, or iron starvation. *J. Phycol.* 29:755–66.

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.

Greene, R. M., Geider, R. J. & Falkowski, P. G. 1991. Effect of iron limitation on photosynthesis in a marine diatom. *Limnol. Oceanogr.* 36:1772–82.

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.

Huber, J. L., Redinbaugh, M. G., Huber, S. C. & Campbell, W. H. 1994. Regulation of maize leaf nitrate reductase activity involves both gene expression and protein phosphorylation. *Plant Physiol.* 106:1667–74.

Kaiser, W. M. & Brendle-Behnisch, E. 1991. Rapid modulation of spinach leaf nitrate reductase activity by photosynthesis I. Modulation *in vivo* by $CO_2$ availability. *Plant Physiol.* 96:363–7.

Kaiser, W. M. & Huber, S. C. 1994. Postranslational regulation of nitrate reductase in higher plants. *Plant Physiol.* 106:817–21.

Lillo, C. 1994. Light regulation of nitrate reductase in green leaves of higher plants. *Physiol. Plant.* 90:616–20.

Lillo, C. & Ruoff, P. 1989. An unusually rapid light-induced nitrate reductase mRNA pulse and circadian oscillations. *Naturwissenschaften* 76:526–8.

Montagnes, D. J. S., Berges, J. A., Taylor, F. J. R. & Harrison, P. J. 1994. Estimating carbon, nitrogen, protein and chlorophyll a from cell volume in marine phytoplankton: a comparison of optical and electronic particle counting techniques and the effect of fixation with Lugol's iodine. *Limnol. Oceanogr.* 39:1044–60.

Ninnemann, H. 1987. Photoregulation of eukaryotic nitrate reductase. *In* Senger, H. [Ed.] *Blue Light Responses: Phenomena*

*and Occurrence in Plants and Microorganisms.* CRC Press, New York, pp. 17–29.

Packard, T. T. & Blasco, D. 1974. Nitrate reductase activity in upwelling regions. 2. Ammonia and light dependence. *Tethys* 6:269–80.

Pilgrim, M. L., Caspar, T., Quail, P. H. & McClung, C. R. 1993. Circadian and light-regulated expression of nitrate reductase in *Arabidopsis. Plant Mol. Biol.* 23:349–64.

Post, A. F., Dubinsky, Z. & Falkowski, P. G. 1984. Kinetics of light-intensity adaptation in a marine planktonic diatom. *Mar. Biol.* 83:231–8.

Race, H. L. & Hind, G. 1996. A protein kinase in the core of Photosystem II. *Biochemistry* 35:13006–10.

Ramalho, C. B., Hastings, J. W. & Colepicolo, P. 1995. Circadian oscillation of nitrate reductase activity in *Gonyaulax polyedra. Plant Physiol.* 107:225–31.

Roenneberg, T. 1996. The complex circadian system of *Gonyaulax polyedra. Physiol. Plant.* 96:733–7.

Roldan, J. M. & Butler, W. L. 1980. Photoactivation of nitrate reductase from *Neurospora crassa. Photochem. Photobiol.* 32:375–81.

Shanklin, J., DeWitt, N. D. & Flanagan, J. M. 1995. The stroma of higher plant plastids contains ClpP and ClpC, functional homologs of *Escherichia coli* ClpP and ClpA: an archetypal two-component ATP-dependent protease. *Plant Cell* 7:1713–22.

Sheen, J. 1994. Feedback control of gene expression. *Photosynth. Res.* 39:427–38.

Sherman, T. D. & Funkhouser, E. A. 1989. Induction and synthesis of nitrate reductase in *Chlorella vulgaris. Arch. Biochem. Biophys.* 274:525–31.

Smith, P. K., Krohn, R. I., Hermanson, T. I., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenck, D. C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76–85.

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 Mol. Biol.* 41:225–53.

Sukenik, A., Bennet, J. & Falkowski, P. 1987. Light-saturated photosynthesis: limitation by electron transport or carbon fixation? *Biochim. Biophys. Acta* 891:205–15.

Syrett, P. J. 1981. Nitrogen metabolism of microalgae. *Can. Bull. Fish. Aquat. Sci.* 210:182–210.

Turpin, D. H. 1991. Effects of inorganic N availability on algal photosynthesis and carbon metabolism. *J. Phycol.* 27:14–20.

Turpin, D. H., Elrifi, I. R., Birch, D. G., Weger, H. G. & Holmes, J. J. 1988. Interactions between photosynthesis, respiration, and nitrogen assimilation in microalgae. *Can. J. Bot.* 66:2083–97.

Zehr, J. P. & Falkowski, P. G. 1988. Pathway of ammonium assimilation in a marine diatom determined with the radiotracer $^{13}N$. *J. Phycol.* 24:588–91.

Zeiler, K. G. & Solomonson, L. P. 1989. Regulation of *Chlorella* nitrate reductase: control of enzyme activity and immunoreactive protein levels by ammonia. *Arch. Biochem. Biophys.* 269:46–54.