J. Phycol. **41,** 84–94 (2005)
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DOI: 10.1111/j.1529-8817.2005.04115.x

RESPONSE OF GLUTAMINE SYNTHETASE GENE TRANSCRIPTION AND ENZYME ACTIVITY TO EXTERNAL NITROGEN SOURCES IN THE DIATOM *SKELETONEMA COSTATUM* (BACILLARIOPHYCEAE)[1]

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To understand the enhanced ability of marine diatoms to assimilate nitrogen (N), we measured changes in the transcript abundance and enzyme activity of glutamine synthetase (GS), one of the key enzymes that link carbon (C) and N metabolism, in the common diatom *Skeletonema costatum* (Greville) Cleve. Transcript abundance of *glnII* (the gene that encodes the GSII isoenzyme), measured by quantitative reverse transcriptase-PCR, and total GS activity increased 2 to 3.5 times above background in the cells taking up nitrate (NO_3^-) but not the cells taking up ammonium (NH_4^+). A background level of *glnII* mRNA was maintained at a steady level up to 15 days of N starvation before decreasing to below detection after 21 days. These results confirm that transcription of *glnII* is induced to assimilate NH_4^+ derived from reduction of NO_3^-. Because of this role of GSII in diatoms assimilating NH_4^+ derived from NO_3^- reduction rather than from the environmental NH_4^+, quantification of *glnII* mRNA promises to be a useful indication of new production by phytoplankton.

Key index words: **diatom; gene expression; glutamine synthetase real-time PCR; *Skeletonema costatum***

Abbreviations: **GOGAT, glutamate synthase; GS, glutamine synthetase; NR, nitrate reductase; RT-QPCR, reverse transcriptase-quantitative PCR**

Phytoplankton are the most numerous and ubiquitous primary producers in oceans around the world. It is difficult, however, to determine the individual species' contributions to carbon and nitrogen assimilation of the community. In a typical coastal upwelling environment, diatoms dominate over other eukaryotic phytoplankton such as dinoflagellates and coccolithophorids as a result of their exceptional responsiveness to the increase in NO_3^- (Estrada and Blasco 1979). Data from culture experiments showing that NO_3^- uptake rates and specific growth rates are generally higher for diatoms than for flagellates at comparable substrate concentrations (Eppley et al. 1969, Paasche et al. 1984) further support the diatoms' superior NO_3^- assimilation mechanisms.

Diatoms actively take up NO_3^- and NH_4^+ from the environment (Dortch 1990) and assimilate inorganic N into organic molecules through the coordinated activities of assimilatory nitrate reductase (NR, EC 1.6.6.1), nitrite reductase (EC 1.7.99.3), glutamine synthetase (GS, EC 6.3.1.2), and glutamate synthase (GOGAT, EC 1.4.1.13, 1.4.1.14, and 1.4.7.1; Fig. 1). Nitrate reductase is cytosolic, and its activity is quantitatively correlated to NO_3^- assimilation rates and qualitatively with ambient NO_3^- concentration (Berges and Harrison 1995). It is regulated at the transcriptional and translational levels in many photosynthetic eukaryotes, including diatoms (Vergara et al. 1998, Kay et al. 1991). In *Chlorella* and other algae, NR is not induced in the presence of NH_4^+, but rapid synthesis occurs upon NO_3^- addition (Dawson et al. 1996, Jochem et al. 2000, Song and Ward 2004). Furthermore, posttranslational mechanisms are involved in both up- and down-regulation of NR activity (Glaab and Kaiser 1996). Nitrite (NO_2^-), once reduced from NO_3^-, induces oxidative stress in the cytosol and is actively transported into the chloroplast where it is further reduced to NH_4^+ by nitrite reductase (Galvan et al. 2002).

Within most photosynthetic eukaryotes, a chloroplast GS isoenzyme catalyzes the formation of glutamine using NH_4^+ produced from nitrite reduction and glutamate produced via GOGAT. In contrast,

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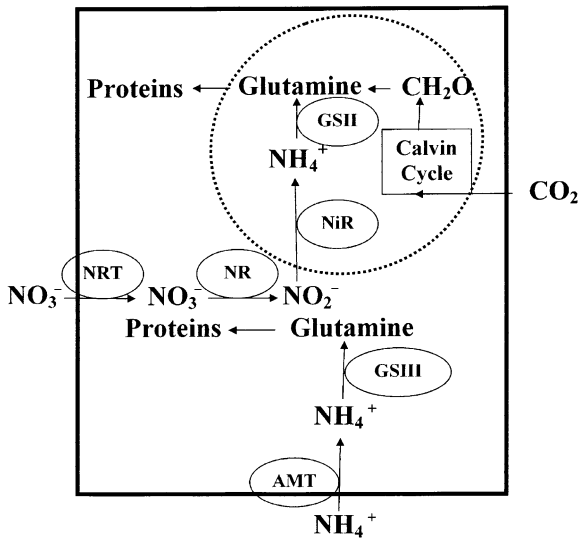

FIG. 1. A simplified schematic showing the major nitrogen assimilation pathways and enzymes (in ovals) in a diatom cell. The boundary of the chloroplast is shown as a dotted line. AMT, ammonium transporter; GSII and III, glutamine synthetase II and III; NiR, nitrite reductase; NR, nitrate reductase; NRT, nitrate transporter.

NH_4^+ that enters the cell from external sources or is produced within the cytosol by processes such as protein catabolism may be assimilated into glutamine by a cytosolic form of GS (Lam et al. 1996, Woodall et al. 1996). Total GS enzyme activity has been used as a measure of both regenerated (based on NH_4^+) and total productivity (based on NO_3^- and NH_4^+) and is commonly measured in *in situ* and *in vivo* studies of algal physiology (Slawyk and Rodier 1988, Rees et al. 1995). However, environmental and cellular cues important in the regulation of GS transcription and expression in diatoms are unknown.

The two forms of GS enzymes that confer activity in diatoms are members of distinct gene families. The GSII isoenzyme is encoded by a nuclear gene, and sequence analysis has revealed the presence of a chloroplast transit sequence at the N-terminus of the protein in *Skeletonema costatum* and *Thalassiosira pseudonana* (Robertson et al. 1999, Armbrust et al. 2004). The other isoenzyme is a member of the GSIII gene family and also is nuclear encoded. Sequence analysis of GSIII from *T. pseudonana* did not reveal a transit peptide, suggesting this enzyme functions in the cytosol (Armbrust et al. 2004, unpublished data). A third GS gene was identified in the *T. pseudonana* genome that shares homology with the GSI family of enzymes. Although GSI previously had not been detected in diatoms, it appears to be transcribed in *T. pseudonana* (unpublished data). Although GSI genes have also been described for vascular plants and fungi, there is no evidence that the GSI protein is enzymatically active (Lee and Adams 1994, Mathis et al. 2000, D'Souza et al. 2001). The genes encoding the GSI, II, and III isoenzymes in diatoms are designated as *glnA*, *glnII*, and *glnN*, respectively (http://genome.jgi-psf.org/thaps0/thaps0.home.html).

Studies of vascular plants, green algae, and cyanobacteria revealed that transcription, translation, and enzymatic activity of GS isoenzymes are regulated by external cues such as inorganic nitrogen sources and light as well as cellular fluxes of carbon and nitrogen (Patriarca et al. 1992, Chen and Silflow 1996,Oliveira and Coruzzi 1999, Spinosa et al. 2000). The mechanisms and direction of regulation by external nitrogen sources, however, differ widely among groups of organisms and also among members of the GS gene family. Generally, ammonium and nitrate up-regulate GS transcription and/or activity in vascular plants provided the genes are inducible. In contrast, in the unicellular green alga *Chlamydomonas reinhardtii*, external ammonium represses transcription of the cytosolic GS_1 (one of two GSII isoforms), whereas nitrate induces GS_1 transcription of this gene (Chen and Silflow 1996). In a nondiazotrophic cyanobacterium, *Synechococcus* sp., expression of GSIII, which contributes to a lesser degree to overall GS activity than GSI in this particular organism, is strongly induced in the initial stages of nitrogen starvation (Sauer et al. 2000). This evidence, along with the fact that *glnN* (gene encoding GSIII) has not been found in nitrogen-fixing cyanobacteria, implies that GSIII is functional under conditions of nitrogen deficiency in some photosynthetic cyanobacteria (Sauer et al. 2000).

Application of molecular techniques offers a suite of opportunities to measure physiological and enzymatic activities and gene expression along metabolic pathways within a particular taxon of interest. Abundance of mRNA is often used as a direct measure for gene expression, and the recently developed reverse transcriptase-quantitative PCR (RT-QPCR), also known as RT real-time PCR, is one of the more rapid and quantitative methods for determining the level of transcripts. In QPCR systems, the accumulating PCR products are quantitatively detected by capturing fluorescent signals generated by different chemistries (Bustin 2000). Nucleic acid templates are quantified by measuring the number of cycles needed for the PCR products to reach a threshold level in their exponential accumulation phase. The RT-PCR enzymatically amplifies defined sequences of RNA (Rappolee et al. 1988). It is effective in characterizing the expression patterns of mRNA of particular genes even from little sample material (Wang and Brown 1999). Application of RT-QPCR has become explosively popular in clinical studies (Bustin 2000) but has only been used in a few studies of phytoplankton (Wawrik et al. 2002, Gray et al. 2003). In this study, we used the RT-QPCR method to determine the effects of external N availability on the expression of *glnII* in batch cultures of the diatom *S. costatum*.

MATERIALS AND METHODS

Algal culture. An axenic strain of *S. costatum* (CCMP 1332) was obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA) and grown axenically in f/2 media (Guillard 1975) at 17° C

TABLE 1. Initial concentrations of NO_3^- and NH_4^+ used in treatment cultures.

Treatment	NO_3^- concentration (μM)	NH_4^+ concentration (μM)
Control	0	0
I	300	5
II	400	5
III	300	50
IV	400	50
V	0	50

with a 12:12-h light:dark cycle at either 60 μmol photons $\cdot m^{-2} \cdot s^{-1}$ for 100% light treatments or 30 μmol photons $\cdot m^{-2} \cdot s^{-1}$ for 50% light treatments. Illumination was provided from two sides by four Vita-Lite® Plus Power Twist fluorescent tubes (color rendering index-91, Duro-Test® Corp, Philadelphia, PA, USA). Cultures were maintained axenically, and lack of bacterial contamination was verified by observation with epifluorescence microscopy and negative PCR amplification with prokaryote-specific 23S rRNA primers (LSU190F and LSU19332-R, DeLong et al. 1999). Cells in late log phase were filtered at 5 psi onto 0.45-μm pore-size polyvinylidene fluoride Durapore® membrane filters (Millipore, Bedford, MA, USA) and carefully transferred to and maintained in presterilized f/2 medium without NO_3^- for 10 days to eliminate the internal pool of nitrogen in the cells. Cells in 250 mL of the above culture were then transferred to 4-L batch culture for each experimental treatment. All treatments contained f/2 levels of all nutrients except nitrogenous compounds. The initial concentrations of NO_3^- and NH_4^+ in treatments are summarized in Table 1. Control treatments did not receive nitrogenous additions.

Sampling and nutrient measurements. Each culture was sampled aseptically at 6, 24, 48, 72, 120, 168, and 288 h after the cell transfer. The 288-h sampling was not possible for the control treatment, because larger volumes of the culture were needed to obtain sufficient biomass for analyses of the early samples. All samples, except at 6 h, were collected 4 h after the start of the light period because transcription of GS in vascular plants peaks in the early part of the light period and NR activity in diatoms is high (Gao 1997, Vergara et al. 1998, Matt et al. 2001). At each sampling, cell concentration was measured in triplicate using 5 mL of culture and a Cytobuoy flow cytometer and its analytical software Cytoclus (CytoBuoy, Bodegraven, The Netherlands).

The numbers of cells in known volumes of cultures were counted by sorting *S. costatum* chains into size clusters empirically predetermined by previous experiments using manual comparison of data collected in Cytoclus (unpublished data). Cells from experimental cultures were harvested by filtration as described above, cells from 200 mL of the culture were used for RNA analysis, and cells from 500 to 1000 mL of the culture were collected for enzyme analysis at 24, 178, and 288 h. Volumes varied depending on the cell density at the time of the collection. Filtrates from each sample were used for nutrient measurements.

The NH_4^+ concentration in the media was measured (using 25-mL samples) with the modified protocol of Solorzano (1969): 1 mL phenol solution, 1 mL sodium nitroprusside, and 2.5 mL oxidizing reagent were added to 25 mL of filtrate (diluted 1/10 for the NH_4^+-containing treatments). Standards of known concentrations ranging between 0.2 and 10 μM were prepared daily and treated in the same manner as the samples. Absorption at 640 nm was measured spectrophotometrically using a 10-cm cell (8452A diode array spectrophotometer, Hewlett Packard, Palo Alto, CA, USA). Concentrations of NO_3^- (in conjunction with NO_2^-), silicate, and phosphate in the media were measured using a four-channel nutrient autoanalyzer (Auto-Analyzer 2, Bran and Luebbe) as described in Wilkerson et al. (2002).

Preparation of nucleic acids. Membrane filters with collected cells were placed into 2-mL polypropylene screw-cap tubes that contained 800 μL of the extraction buffer of Totally RNA Extraction Kit (Ambion Inc., Bartleville, TX, USA) and 50 mg of muffled sterile glass beads (Biospecs Products, Bartlesville, OK, USA). Cells were disrupted by shaking vigorously three times at maximum speed for 45 s in a bead beater (FastPrep™ FP120, Thermo Savant, NY, USA). The DNA and RNA were simultaneously extracted from the lysate following the manufacturer's instructions (Ambion Inc.).

Total RNA was incubated with RQ1 RNase-free DNase (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The cDNA was generated by RT of DNase-treated total RNA using sequence-specific reverse primers designed for real-time PCR analysis of the *glnII* or the 18S rRNA gene (see below). Each RT reaction contained 50 nmol sequence-specific reverse primer, 5 U AMV RT, 10 nmol each dNTP, 2.5 μL 5 × reaction, RNasin® ribonuclease inhibitor (all from Promega) and sterile water to make up to 12.5 μL total volume.

Control RNA. Each gene analyzed (*glnII* and 18 s RNA) was amplified from *S. costatum* cDNA using the primer sequences designed for TaqMan assays (see below). The amplified DNA was cloned into plasmids (TOPO pCR2.1, Invitrogen, Carlsbad, CA, USA). Plasmids, purified from *Escherichia coli* cultures using the Wizard® Plus SV Miniprep DNA Purification System (Promega), were linearized by digestion with *Hind*III (Promega), and the cloned sequences were transcribed into RNA using *in vitro* transcription with T7 RNA polymerase at 37° C for 1 h. The DNA was removed by treatment with RQ1 DNase (Promega), and RNA concentration was estimated spectrophotometrically. Serial dilutions of this RNA template were reverse transcribed in the same manner as the sample RNA above and used simultaneously in RT-QPCR. The cycle number at which the relative fluorescence of these serially diluted standards crossed the set threshold of amplification detection was highly reproducible (Fig. 2) and was used as a control for the RT-QPCR analysis of mRNA isolated from experimental cultures.

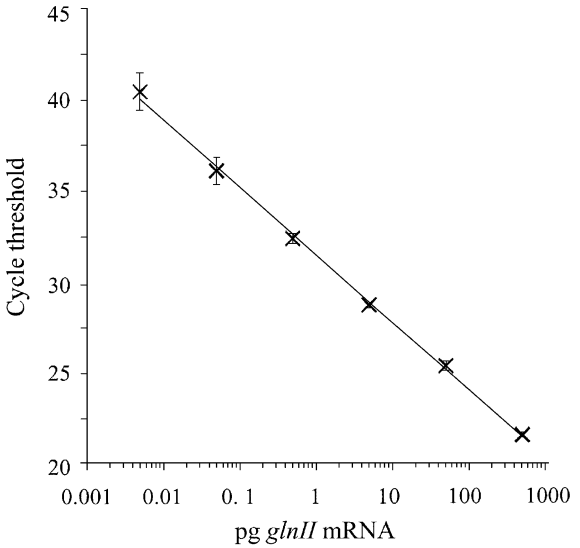

FIG. 2. A typical correlation ($r^2 > 0.99$) between cycle threshold of *in vitro* transcribed mRNA of *glnII* QPCR RNA template.

TaqMan® QPCR. The TaqMan® assays (primers and probe) were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) for *glnII* (forward primer: 5′-TCCTGCTGGAGAGGCTATCC-3′, reverse primer: 5′-TCGTCTTCCTTGCCTTCGAA-3′) and 18S rRNA (forward primer: 5′-GATTACGTCCCTGCCCTTTG-3′, reverse primer: 5′-TCACCGGACCATTCAATCG-3′) genes of *S. costatum* (GenBank AF064638 and X85395, respectively). Probes were synthesized with the reporter dye 6-carboxyfluoroscein covalently linked at the 5′ end and the quencher dye 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine linked to the 3′ end. Probe sequences were 5′-TACCAACACTCGTGCTATTGCCGCC-3′ and 5′-ACACACCGCCCGTCGCACC-3′ for *glnII* and 18S, respectively. The *glnII* Taqman® probe was designed to align with *S. costatum* cDNA sequence at an exon–exon boundary, determined by alignment of cDNA and genomic DNA sequences, so that the assay was cDNA specific and avoided amplification of contaminating genomic DNA. This was not possible for 18S rDNA.

Quantitative PCR reactions were performed in triplicate in an ABI PRISM® 5700 sequence detection system (Foster City, CA, USA) in a total volume of 25 µL. 18S rRNA was reverse transcribed and used as an internal reference in QPCR amplifications for the amount and quality of RNA extracted from each sample. Additional reactions, one without RT and another without the addition of template, were run to control for contamination. The reaction mix contained 12.5 µL 2 × TaqMan® universal master mix (containing AmpliTaq Gold® DNA polymerase, AmpErase UNG, dNTPs, and a passive reference dye), 10 pmol each of forward and reverse primers, 5 pmol of the oligonucleotide probe, and 5 µL cDNA template. The thermocycle conditions of all runs were 50° C for 2 min, 95° C for 10 min, and 45 cycles of 95° C for 15 s and 60° C for 1 min.

Relative quantification of mRNA. For each set of QPCR reactions, linearity of QPCR was determined with serial dilutions of plasmid-derived RNA template, the lack of contamination with genomic DNA was ascertained from the no RT control, and the lack of contamination of reagents was determined with no template control. For each sample, the mean *glnII* RT-QPCR cycle threshold values were normalized to the corresponding mean 18S RT-QPCR cycle threshold values to account for variability in RNA template concentrations due to extraction and Rt. The delta cycle threshold values of samples were then standardized to that of the control in 100% light at 6 h to obtain the relative mRNA values for *glnII*. All statistical analyses were performed using Statview 5.0.1 (SAS Institute, Cary, NC, USA).

Enzyme analysis. Cells were thawed on ice in GS extraction buffer (50 mM imidazole [pH 7.3], 4 mM 2-mercaptoenthanol, 1 mM glutathione, 6 mM dithiothreitol, 1 mM EDTA, 10 mM $MgSO_4$, 5 mM Glu, 10 mM $MnCl_2$) and disrupted by sonication for a total of 60, 1-s pulses. Cellular debris was pelleted by centrifugation at 10,000 *g* for 10 min at 4° C, and cell-free supernatants were used in the measurement of GS transferase activity. The GS transferase activity was assayed using the methods described by Bressler and Ahmed (1984) with the following modifications. For each sample, 100 µL of the cell-free extract was added to 400 µL of assay buffer (48 mM imidazole [pH 7.3], 33 mM Gln, 0.33 mM $MnCl_2$, 0.44 mM KH_2SO_4, 67 mM hydroxylamine). One reaction was stopped immediately by the addition of 0.5 mL of stop buffer (3% $FeCl_3$, 2% TCA, 0.25 N HCl); replicate reactions were incubated at 37° C for 30 min and the reactions were stopped by the addition of stop buffer. The production of γ-glutamylhydroxamate was measured spectrophotometrically at 500 nm (8452A diode array spectrophotometer, Hewlett Packard) and enzyme activity was

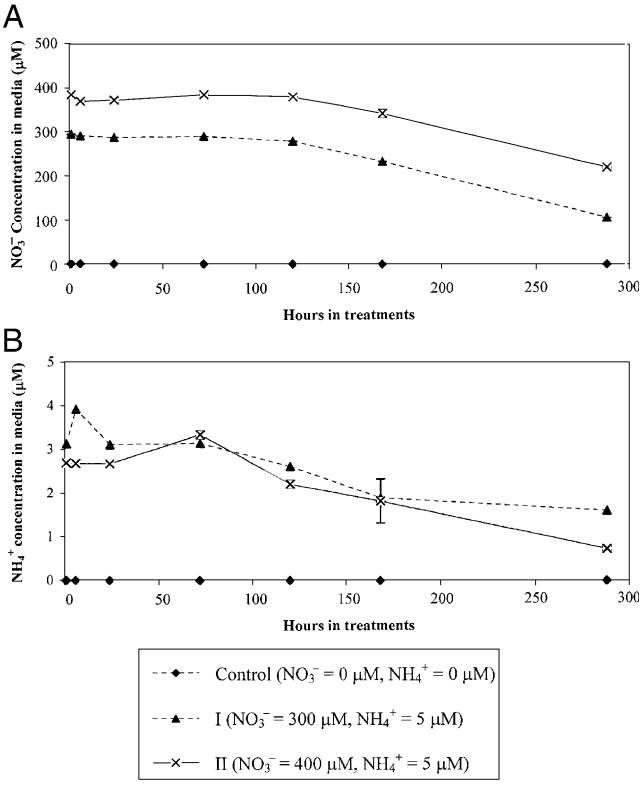

FIG. 3. Changes in mean (± SD). (A) NO_3^- and (B) NH_4^+ concentrations in the media over time in Control and low NH_4^+ treatments (treatments I and II). Initial concentrations of NO_3^- and NH_4^+ for each treatment are indicated in the legend.

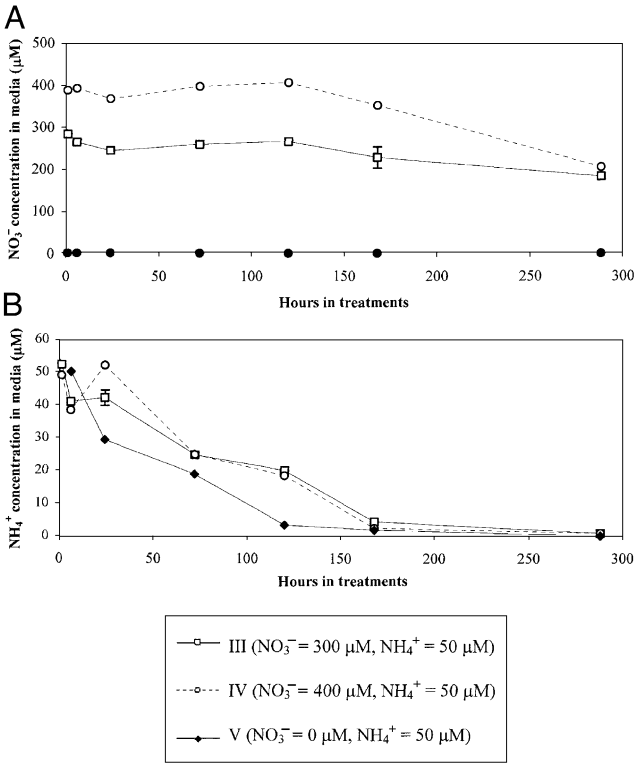

FIG. 4. Changes in mean (± SD). (A) NO_3^- and (B) NH_4^+ concentrations in the media over time in high NH_4^+ treatments (treatments III–V). Initial concentrations of NO_3^- and NH_4^+ for each treatment are indicated in the legend.

calculated as the amount of γ-glutamylhydroxamate formed in duplicate samples after subtraction of the absorbance of the blank. Protein concentration in the cell-free lysates was measured colorimetrically using duplicate samples with BSA as the standard.

RESULTS

No notable differences were observed in any measured parameters between cells grown at the two irradiance levels. Therefore, only the results from treatments exposed to 100% light (60 μmol photons $\cdot m^{-2} \cdot s^{-1}$) are presented here (Figs. 3–7 and 9).

Ammonium and nitrate uptake. In all nitrate-containing treatments, NO_3^- depletion began once the NH_4^+ concentration was reduced to approximately 2 μM. This was regardless of whether the initial NH_4^+ concentration was 5 μM (Fig. 3) or 50 μM (Fig. 4). Ammonium in the media was depleted at a higher rate in treatments containing initial concentration of 50 μM compared with 5-μM treatments, ranging 0.17–0.19 μM $\cdot h^{-1}$ and 0.005–0.019 μM $\cdot h^{-1}$, respectively, over the 288-h period (Figs. 3 and 4). The uptake of nitrate and ammonium did not vary among cultures grown at difference irradiance levels (data not shown).

Cell growth. Cell concentration in control cultures that did not receive nitrate or ammonium slowly decreased throughout the experiment (the control in Fig. 5). In all nutrient-replete treatments, the exponential phase of cell growth began between 72 and 120 h after cells were transferred to new culture media, and cell growth did not reach stationary phase before the end of the experiment at 288 h (Fig. 5). The exception to this pattern was treatment V, where 50 μM NH_4^+ and no NO_3^- were added and exponential growth began earlier than in other treatments (between 24 and 72 h; Fig. 5). The cell growth rate (μ) in this treatment (V) averaged 0.37 d^{-1} and 0.40 d^{-1} in 100% and 50% light, respectively, and reached a maximum around 178 h (Fig. 5), shortly after NH_4^+

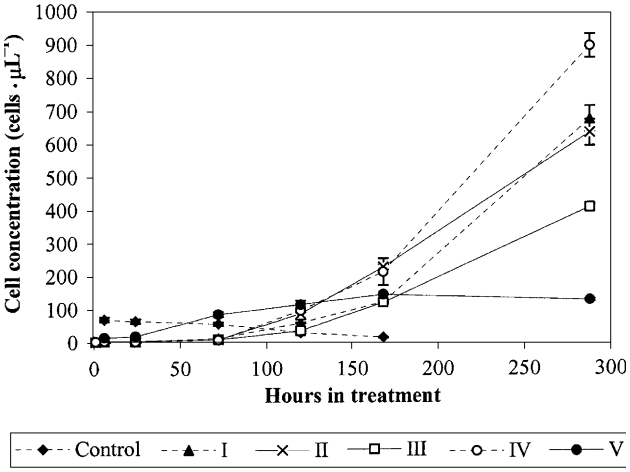

FIG. 5. Changes in mean (± SD) cell concentration in all treatments over time.

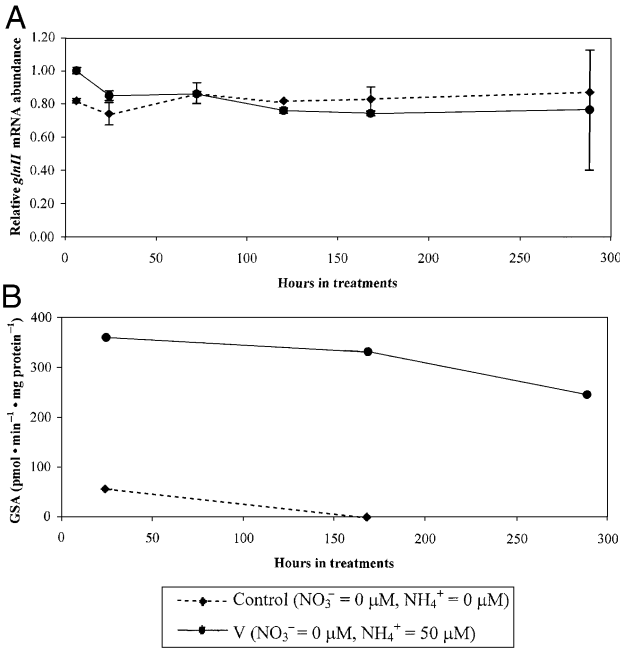

FIG. 6. (A) Mean (± SD of relative *glnII* mRNA abundance normalized to 18S transcription, and (B) GS total enzymatic activity normalized to total protein in the control and ammonium only treatment (V). Initial concentrations of NO_3^- and NH_4^+ for each treatment are indicated in the legend.

was depleted from the media (Fig. 4B). Cells grown with 50% light (30 μmol photons $\cdot m^{-2} \cdot s^{-1}$) tended to have lower μ than those grown with 100% light (60 μmol photons $\cdot m^{-2} \cdot s^{-1}$), but this was not consistently observed throughout the nutrient treatments. The average μ during the exponential growth stage in nitrate-containing treatments (between 72 and 288 h) varied between 0.41 and 0.52 d^{-1}, and the fastest rate was measured in treatment IV (high NO_3^- and high NH_4^+; Fig. 5).

glnII *transcription and total GS enzyme activity.* Measurable levels of *glnII* transcripts were observed in all cultures (Figs. 6 and 7). In cells maintained in the absence of external N, *glnII* transcript abundance remained relatively constant for the duration of the experiment and was within approximately 20% of the initial level (Fig. 6A). When cells were grown in the presence of 50 μM NH_4^+ and no NO_3^- (treatment V), *glnII* transcript abundance remained constant throughout the experiment (Fig. 6A). Total GS activity in this treatment was approximately 350 pmol $\cdot min^{-1} \cdot \mu g^{-1}$ protein at 6 and 168 h and declined in samples measured at 288 h (Fig. 6B). Despite apparent constant *glnII* transcript levels in the control, total GS activity declined from a low level at 24 h (29 and 56 pmol $\cdot min^{-1} \cdot \mu g^{-1}$ protein for 100% and 50% light treatments, respectively) to below detection at 178 h (Fig. 6B).

In all nitrate-containing treatments (treatments I–IV), there was little change in the abundance of *glnII* transcripts until external levels of NO_3^- began decreasing, typically between 120 and 168 h (Fig. 7A), and transcript abundance began to increase. In treatment I (300 μM NO_3^- and 5 μM NH_4^+), the increase in *glnII* transcript abundance was detected after the NH_4^+ concentration had been lowered to approximately 3 μM, and NO_3^- uptake occurred as indicated by the reduc-

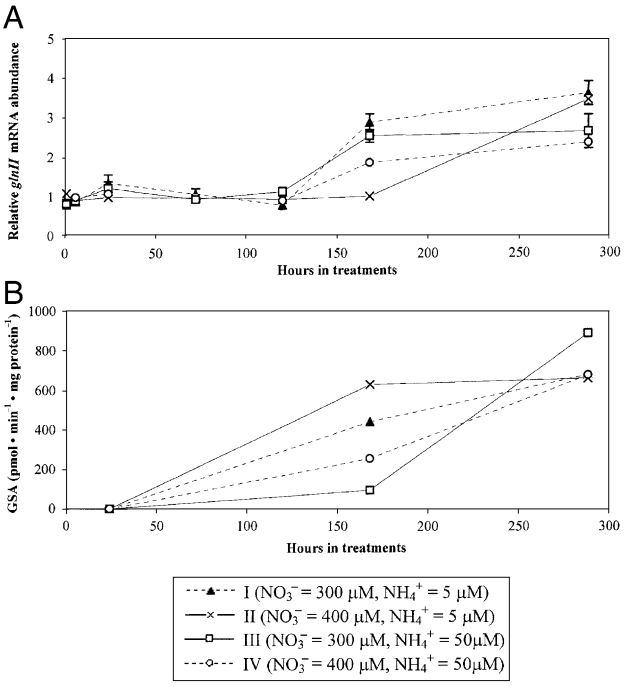

FIG. 7. (A) Mean (± SD) of relative *glnII* mRNA abundance normalized to 18S transcription, and (B) GS total enzymatic activity normalized to total protein in the control and nitrate-containing treatments (I–IV). Initial concentrations of NO_3^- and NH_4^+ for each treatment are indicated in the legend.

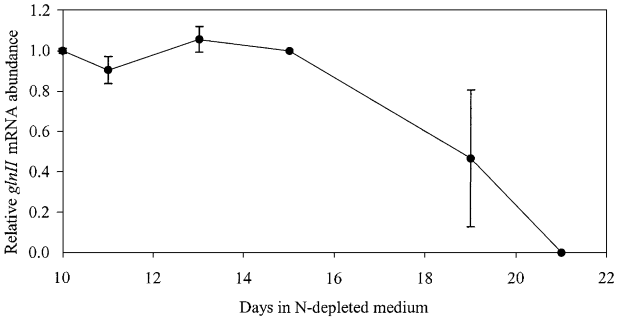

FIG. 8. Relative *glnII* transcription levels in cells incubated in N-deplete media with the ambient light at 60 μmol photons · $m^{-2} \cdot s^{-1}$.

tion in NO_3^- concentration of the medium to 250 μM (approximately 17% reduction). The GS activity was below the level of detection at 24 h and increased in samples collected at 172 and 288 h similarly to the pattern observed for *glnII* mRNA levels (Fig. 7B). GS activity was higher in cells grown at higher irradiance (data not shown). In treatment III, in which a higher concentration of NH_4^+ was added (50 μM) along with 300 μM NO_3^-, *glnII* transcript abundance increased (Fig. 7A) as levels of NH_4^+ decreased to approximately 2 μM and NO_3^- had been taken up from the medium, as indicated by a reduction from approximately 300 μM to approximately 225 μM (a 25% decrease; Fig. 4). As in treatment II, GS activity accompanied the increase in relative *glnII* mRNA abundance and was maximal at 288 h (Fig. 7B). Treatments II and IV paralleled those of I and III except a higher initial concentration of NO_3^- was used (400 μM vs. 300 μM), but again *glnII* transcription did not increase (Fig. 7A) until NH_4^+ levels were reduced to approximately 2 μM and NO_3^- had decreased from 400 to about 250 μM (37.5% decrease) and 350 μM (18% decrease), respectively (Figs. 3 and 4). In both treatments, total GS activity increased with the increased relative RNA abundance of *glnII* (Fig. 7B).

The absolute quantity of the initial level of *glnII* transcript abundance in the control is unknown, but

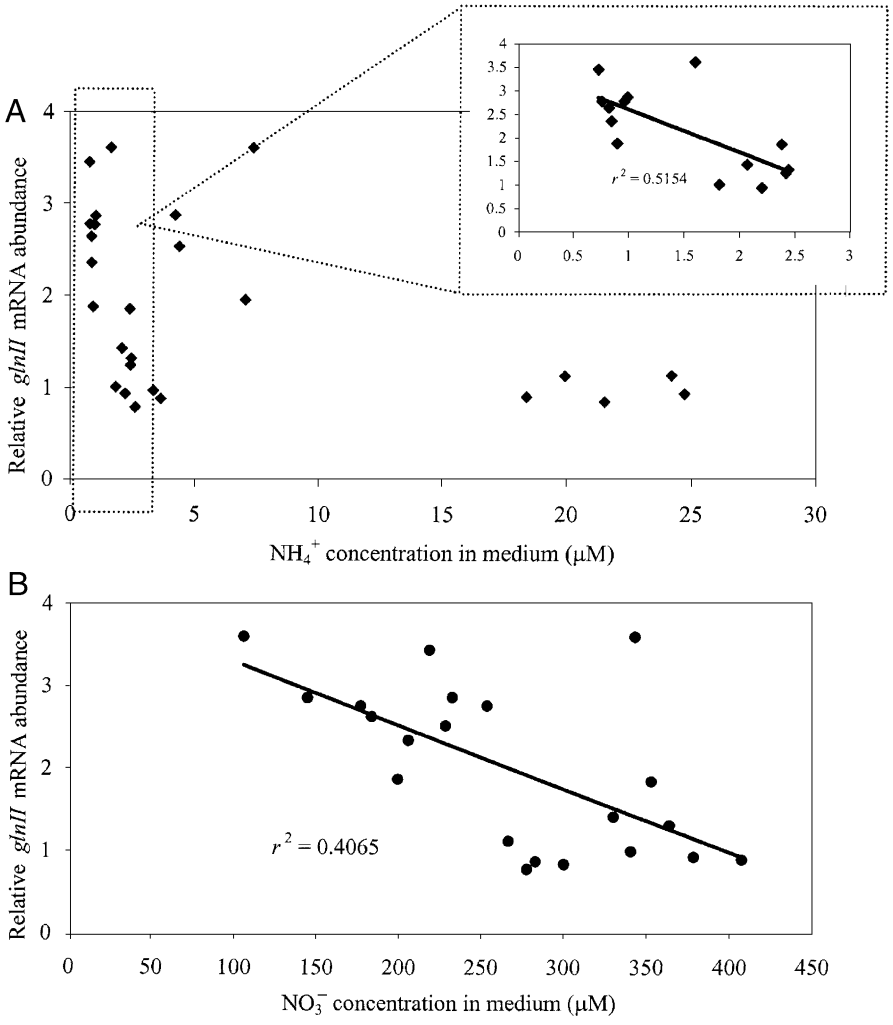

FIG. 9. Relationships between external nutrient concentrations and *glnII* transcript levels at 72, 120, 168, and 288 h. (A) NH_4^+ concentrations versus relative *glnII* transcript abundance. Data points <2.5 μM NH_4^+ are shown enlarged in the inserted graph with r^2 for the linear correlation. (B) NO_3^- concentrations versus relative *glnII* transcript abundance with r^2 for the linear correlation.

interestingly this transcript level was maintained until at least 15 days after f/2-grown cells were transferred to N-deprived medium. After this time, transcript levels decreased and were below detection by 21 days (Fig. 8).

The data from these experiments were combined to examine relationships between *glnII* transcription and external nutrient concentrations. There was no transcription above the background level at higher NH_4^+ concentrations (Fig. 9A). External NH_4^+ concentrations below 2.5 μM showed a linear correlation ($P < 0.01$) with *glnII* transcript abundance ($r^2 = 0.51$; Fig. 9A), suggesting that once external NH_4^+ concentrations were reduced to below a threshold value, there was a negative correlation between external NH_4^+ concentration and *glnII* transcript abundance. This is probably an indirect result of NO_3^- uptake causing an increase in *glnII* transcript abundance, shown as a negative correlation between external NO_3^- and *glnII* transcript abundance (Fig. 9B). Only data after 120 h were used for these correlation analyses because of the suspected lag period of nitrogen assimilation after a period of starvation. Total enzymatic activity of GS, including all GS isoforms, tended to increase throughout the experiment in treatments that contained NO_3^- (Fig. 7B). The timing and extent of increase in total GS activity varied among treatments, however, and was not necessarily synchronized or quantitatively correlated with increases in *glnII* gene transcription.

DISCUSSION

The ecological importance of diatoms in coastal upwelling system has long been appreciated, and the diatoms' exceptional abilities to assimilate NO_3^- into cellular mass have been clearly shown both *in vitro* and *in vivo* (Lomas and Glibert 2000, Clark et al. 2002). However, the biochemical and genetic regulation of N assimilatory pathways are only beginning to be unveiled in these organisms. This is despite the fact that N uptake and assimilation have been extensively studied in vascular plants (Stitt et al. 2002) and, to a lesser extent, in green algae (Huppe and Turpin 1994). Some aspects of N assimilation in diatoms have received more attention than others. For example, the regulation of NR activity has been well studied (Berges and Harrison 1995, Gao 1997), whereas less is known regarding the regulation of the glutamine–glutamate cycle that stands at the junction of C and N assimilatory metabolisms.

Glutamate-ammonia ligase, or GS, is one of the two enzymes that catalyze the glutamine–glutamate (GS–GOGAT) cycle. As observed in vascular plants and green algae, multiple GS isoenzymes are expressed in diatoms. Two GS isoenzymes have been characterized from the diatoms *S. costatum* and *T. pseudonana*, and sequence analysis of the isoenzymes predicts that GSII is localized to the chloroplast, whereas GSIII is cytosolic (Robertson and Alberte 1996, Robertson et al. 1999, 2001, Armbrust et al. 2004). Although there is some redundancy, the general model emerging from studies of vascular plants and green algae predicts that the chloroplastic GS isoenzyme assimilates ammonium produced from the reduction of nitrate, whereas the cytosolic isoenzyme assimilates ammonium from sources external to the cell and from catabolic processes. Our preliminary studies indicated that gene and enzyme expression of GSII was induced by external nitrogen, whereas GSIII was constitutively expressed regardless of presence or absence of nitrogen in the diatoms *T. pseudonana* (unpublished data) and *S. costatum* (Robertson and Alberte 1996, unpublished data). We therefore hypothesized that *glnII* (the gene encoding GSII) transcription would be up-regulated in cells assimilating nitrate but not in cells assimilating NH_4^+ taken up directly from the environment.

The results from this study support the hypothesis that GSII expression is up-regulated in cells assimilating NO_3^-. The *glnII* mRNA levels were unchanged in cells grown in medium supplemented only with NH_4^+. In contrast, *glnII* transcript abundance increased in cultures containing NO_3^- when NH_4^+ inhibition of NO_3^- uptake was alleviated (Dortch 1990, Gao 1997). In all treatments, this increase in *glnII* mRNA prompted by NO_3^- uptake continued to 288 h of nutrient exposure without plateauing. Transcription and enzyme activity may be expected to reach a plateau if the cells were in a steady state, which the batch cultures in this experiment were not. The negative correlation between *glnII* mRNA abundance and external NO_3^- concentration suggests that *glnII* transcription is induced in *S. costatum* as cells take up and assimilate NO_3^-. This is in agreement with the observation that *S. costatum* is able to take up NO_3^- in a near linear fashion with increasing levels of external NO_3^-, at least up to 300 μM (Lomas and Glibert 2000). Further evidence for *glnII* transcription being induced by uptake of NO_3^-, and not external NH_4^+, is seen in the relationship between external NH_4^+ oncentration and *glnII* transcripts. Transcripts above the background level were not observed until NO_3^- began to be taken up by cells at the point where there was sufficient reduction of inhibitory external NH_4^+. As external NH_4^+ decreased further beyond this point and more NO_3^- uptake by cells was facilitated, *glnII* transcripts increased linearly in *S. costatum*.

Total GS enzyme activity also increased after the initiation of NO_3^- uptake, although the level of activity varied among treatments. In previous experiments examining transcript levels and protein abundance in *S. costatum* and other diatoms, GSIII appeared to be expressed constitutively (Robertson and Alberte 1996, unpublished data). The GS activity measured in these experiments is assumed to reflect the activity of both GSII and GSIII. However, GSII has not been characterized biochemically, and it is not known if the GS transferase assay used here is optimal for measuring GSII activity. Thus, the increase in GS activity that paralleled the increase in *glnII* transcript levels may

have been the result of an increase in GSII activity; however, a concomitant increase in GSIII activity cannot be excluded. Chromatographic separation of the isoenzymes or Western analysis using isoenzyme specific antiserum will contribute to our understanding of the relative activity of each isoenzyme.

There was not a strong relationship between either GS activity or *glnII* transcript abundance and growth rate in these experiments. Growth rate was maximal around 120 h for all treatments except treatment V (only NH_4^+ added). In treatment V, maximum growth peaked at 72 h. The consequent decreases in growth rates were due to limitation of other nutrients such as phosphate and silicate (data not shown). The growth rate maxima did not coincide with maxima in *glnII* transcript abundance or GS expression. This, along with present and previous observations that NO_3^-- and NH_4^+-replete diatoms can achieve comparable growth rate maxima (Clark 2001), suggests that the two GS isoenzymes are used interchangeably or in conjunction with each other to assimilate differentially derived NH_4^+ in diatoms.

The absolute quantity of *glnII* transcript per cell cannot be determined in this study because *glnII* transcript abundance was normalized to 18S RNA abundance. This relative quantification method is preferred over absolute quantification mRNA because it circumvents errors imposed by variability among samples due to differences in efficiency of biochemical reactions (i.e. RT and PCR amplification) and extractions of nucleic acids (Bustin 2000, Pfaffl 2001). This relative quantification approach, in turn, can possibility underestimate expression levels when cells are rapidly dividing under more favorable growth conditions because more rRNA may be synthesized to satisfy the growing demands for protein synthesis (Smith et al. 1992). This is unlikely to have affected our relative comparisons between treatments because all treatments achieved comparable growth rates, albeit at different times. The 18S rRNA gene was chosen as an endogenous control gene because it was the only "housekeeping" gene whose sequence was publicly available for *S. costatum* at the time of our experiment (August 2003). Other nonregulated reference genes with introns (so TaqMan assays can be cDNA specific) may be preferable for future studies using similar mRNA quantification technique. Similar considerations can be applied to the measurement of enzyme activity. In cultures receiving nitrogen additions, GS activity increased as cell number increased. The increase in activity was not simply due to increased biomass, because enzyme activity was standardized to the amount of protein extracted from the cells.

It is of interest that the background level of *glnII* transcripts was maintained for as long as 15 days. Because this is well beyond the regular longevity of eukaryotic mRNA (up to 24 h), we can presume that *glnII* was continuously transcribed at a background level (exact amount unknown) in the absence of external nitrogen during this period. *Skeletonema costatum* has an exceptional capacity to store NO_3^- and NH_4^+ compared with other diatoms and flagellates (Lomas and Glibert 2000), and this stored N may have been enough to induce *glnII* transcription after N was depleted from the medium. Alternatively, cells may have begun mobilizing N stored in macromolecules; however, the role of GS isoenzymes in N remobilization in diatoms is unknown. Strong mRNA expression in N-deprived media was also observed for the nitrate transporter gene, although nitrate transporter mRNA abundance levels were suppressed in short incubations in NH_4^+-supplemented media but not as much in longer incubations (Hildebrand and Dahlin 2000). Similar suppression of NR in the presence of NH_4^+ and lack of suppression in the absence of N sources have also been observed at the enzyme and mRNA level (Jochem et al. 2000, Song and Ward 2004). The *glnII* mRNA expression in this study was relatively low in the presence of higher concentrations of NH_4^+ but was detected in all samples (Fig. 9A). This baseline expression is attributed to the other functions of GS in cellular metabolism (e.g. amino acid biosynthesis).

Nutrient uptake and *glnII* transcription were not affected by the difference in ambient light between 30 and 60 μmol photons $\cdot m^{-2} \cdot s^{-1}$; however, GS activity was consistently higher in cells grown with higher irradiance than with lower irradiance (data not shown). The light saturation index of *S. costatum* is 203 μmol photons $\cdot m^{-2} \cdot s^{-1}$ under continuous light (Gilstad et al. 1993). The fact that both light levels used in this experiment were well below saturation levels and that diatoms can assimilate NO_3^- and NH_4^+ in the dark (Clark et al. 2002) probably resulted in the lack of a detectable light effect in this study. Furthermore, inhibition of NO_3^- uptake by NH_4^+ also occurred in darker treatments compared with lighter treatments (Clark et al. 2002). This was consistent with a previous study that described lack of such inhibition with light levels above 17 μmol photons $\cdot m^{-2} \cdot s^{-1}$ (Yin et al. 1998).

In conclusion, this study demonstrates that in the diatom *S. costatum*, *glnII* is up-regulated by processes involved in NO_3^- uptake and reduction but not by NH_4^+ transported directly from the medium. The present results further support that GSII is the chloroplastic isoform in the diatoms as predicted from the analysis of protein sequences (Robertson et al. 2001). A further exciting implication of our findings is that because *glnII* transcription is induced by NH_4^+ made available from NO_3^- reduction, it can function as a qualitative indicator of new production instead of growth sustained by regenerated nitrogen in coastal upwelling and open ocean systems. The next step is to apply these molecular approaches in concert with ^{15}N-labeled nitrate and ammonium uptake measurements in both culture and field experiments. Another approach that could be taken is to use RT-QPCR to combine *glnII* and *rbcL* transcription (Wawrik et al. 2002) to evaluate molecular estimates of the f-ratio for specific phytoplankton, allowing for comparison with biogeochemically measured estimates using ^{14}C fixa-

tion and $^{15}NO_3$ uptake rates by size-fractionated phytoplankton populations or blooms dominated by diatoms.

We are grateful to Al Marchi, Laila Barada, Gabriel Griffin, and Allison Lorenzi for their assistance with nutrient analyses and phytoplankton culturing. This work was supported by Department of Energy Biotechnology of Ocean Margins Program (no. DE-F903-97ER62450/A000) awarded to F. P. W. and NSF Career Award (IBN 0238426) awarded to D. L. R.

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