

Nitrogen fixation, uptake and metabolism in natural and cultured populations of *Trichodesmium* spp.

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ABSTRACT: Uptake rates of several combined N sources, N₂ fixation, intracellular glutamate (glu) and glutamine (gln) pools, and glutamine synthetase (GS) activity were measured in natural populations and a culture of *Trichodesmium* IMS101 grown on seawater medium without added N. In cultured populations, the ratio of GS transferase/biosynthetic activity (an index of the proportion of the GS pool that is active) was lower, and intracellular pools of glu and gln and the ratios of gln/glu and gln/ α -ketoglutarate (gln/ α kg) ratios were higher when N₂ fixation was highest (mid-day). There was an excess capacity for NH₄⁺ assimilation via GS, indicating that this was not the rate-limiting step in N utilization. In natural populations of *Trichodesmium* spp., the gln/glu ratio closely approximated the gln/ α kg ratio over the diel cycle. High gln/glu and gln/ α kg ratios were noted in near-surface populations. These ratios decreased in samples collected from greater depths. Natural populations of *Trichodesmium* spp. showed a high capacity for the uptake of NH₄⁺, glu, and mixed amino acids (AA). Rates of NO₃⁻ and urea uptake were low. NH₄⁺ accumulated in the culture medium during growth and rates of NH₄⁺ uptake showed a positive relationship with the NH₄⁺ concentration in the medium. Although rates of N₂ fixation were highest and accounted for the majority of the total measured N utilization during mid-day, rates of NH₄⁺ uptake exceeded rates of N₂ fixation throughout much of the diel cycle. In exponentially growing cultures, only 23% of the total daily N utilization was due to N₂ fixation while NH₄⁺ uptake accounted for more than 70%. Based on N₂ fixation alone, the N turnover time for this culture during exponential growth was on the order of 9 d. This is consistent with the observed chlorophyll-based growth rates for these cultures suggesting that N₂ fixation was responsible for net growth. Our results contrast with the view that natural populations of *Trichodesmium* spp. acquire their cell N exclusively through N₂ fixation. C productivity may overestimate N demand for net production if regenerated production is significant in these populations.

KEY WORDS: *Trichodesmium* · Nitrogen metabolism · N₂ fixation · Amino acid pools · Nitrogen uptake

INTRODUCTION

Trichodesmium spp. are common throughout the tropical and subtropical oligotrophic ocean (Capone et al. 1997). They occur as free trichomes or as colonial

aggregates that are spherical (puffs) or fusiform (tufts). Colonies and free trichomes are capable of fixing N₂, which allows them to alleviate N limitation in the seas in which they occur.

In natural populations of *Trichodesmium thiebautii* nitrogenase synthesis and activity exhibit a daily cycle (Capone et al. 1990, Zehr et al. 1993). Nitrogenase, the enzyme complex responsible for catalyzing the reduction of N₂, activity is confined to the daylight hours and rates of N₂ fixation are highest around mid-day, suggesting that light or photosynthesis might regulate nitrogenase activity (Saino & Hattori 1978, Capone et

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al. 1990). Chen et al. (1996) established in cultures of *Trichodesmium* IMS101 that this cycle is driven by an endogenous rhythm.

N metabolism in natural populations of *Trichodesmium* spp. has been shown to proceed via the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway (Carpenter et al. 1992), as for most diazotrophic cyanobacteria. GS activity was measured in populations of *Trichodesmium* spp. from the North Atlantic using the transferase assay. Rates did not vary significantly over a diel cycle although concentrations of GS protein were positively correlated with concentrations of nitrogenase protein in immuno-labeling studies (Carpenter et al. 1992). Cellular nitrogenase and GS concentrations both declined at night when cells were not fixing N₂.

Little is known about the growth cycle, the physiological status or the regulation of N₂ fixation in natural populations of *Trichodesmium* spp. The glutamine/glutamate (gln/glu) ratio has been used as an index of N-status in phytoplankton and cyanobacteria (Flynn et al. 1989, Flynn 1990, Flynn & Gallon 1990). Capone et al. (1994) found that intracellular glu and gln concentrations and the ratio of gln/glu varied on a diel basis in *Trichodesmium* spp., in parallel with the pattern of N₂ fixation.

N₂ fixation is generally held to be the primary mode of N acquisition for these species in nature (Carpenter & McCarthy 1975, Mague et al. 1977, Carpenter 1983b, Carpenter et al. 1997). While surface waters in the tropical oligotrophic ocean gyres are generally depleted in combined N, NH₄⁺ and dissolved organic N (DON) are rapidly recycled to support much of the apparent N demand of primary production in these systems (Eppley & Peterson 1979, Bronk et al. 1994). *Trichodesmium* spp. contributes directly to NH₄⁺ turnover in these systems by its release of amino acids (AA), DON, and possibly NH₄⁺ (Capone et al. 1994, Glibert & Bronk 1994, O'Neil et al. 1996). During oceanic blooms of *Trichodesmium*, dissolved N pools can become elevated as a result of recent N input (Devassy 1987, Karl et al. 1992). Based on these observations and the energy requirements for N₂ fixation, the absence of a capacity for the uptake of reduced N by *Trichodesmium* spp. seems enigmatic.

There is evidence that *Trichodesmium* spp. are capable of taking up combined N (e.g. NH₄⁺, NO₃⁻, DON). Natural populations of *Trichodesmium* spp. were shown to have a capacity to take up NH₄⁺, NO₃⁻, and urea in the Northwest Pacific (Saino 1977, Saino & Hattori 1978) and in the tropical North Atlantic (Goering et al. 1966). In contrast, very low rates of NH₄⁺, NO₃⁻, and urea uptake were reported for populations from the North Atlantic Ocean and the Caribbean Sea (Carpenter & McCarthy 1975, Glibert & Banahan

1988). A capacity for glu and gln uptake by colonial aggregates was demonstrated in natural populations of *Trichodesmium* spp. (Saino 1977, Carpenter et al. 1992, Capone et al. 1994).

We therefore examined the ability of *Trichodesmium* to assimilate combined N in cultures grown on medium without added N and in natural populations in order to evaluate the generalization that they are wholly dependent upon N₂ fixation and also to understand how the observed patterns of nitrogenase synthesis, activity and modification are regulated. Both the gln/glu ratio and the gln/ α -ketoglutarate (gln/ α kg) ratios were measured in the same samples to determine whether these ratios were comparable and whether these metabolites could be used to predict patterns of N utilization and metabolism. GS activity was measured using the transferase assay, as well as the forward reaction assay, to determine the *in vivo* potential for NH₄⁺ assimilation relative to the diel pattern of N₂ fixation. By characterizing the cellular biochemical and physiological conditions under which N₂ fixation and N uptake occur, we hoped to identify factors contributing to the control and regulation of these processes in *Trichodesmium* spp.

METHODS

Sample collection. Concentrations of intracellular metabolite pools of glu, gln and α -ketoglutarate and rates of N₂ fixation, N uptake, and GS activity were measured in natural assemblages of *Trichodesmium* spp. from the tropical North Atlantic Ocean during cruises aboard the RV 'Gyre' in May 1994 and the RV 'Seward Johnson' in April 1996, and the Eastern Caribbean Sea during cruises aboard the RV 'Seward Johnson' in January 1995 and October 1996. The same parameters were measured in *Trichodesmium* IMS101 isolated from coastal North Carolina and grown on an N-depleted seawater medium (Prufert-Bebout et al. 1993). Colonies of *Trichodesmium* spp. were collected in the field using a 202 μ m mesh plankton net towed at <1 knot for 10 to 15 min at depths <30 m (unless otherwise specified). The ship was stopped during the deployment and recovery of the net. After dilution of the cod end sample with filtered seawater, colonies were transferred into GF/F filtered seawater using inoculating loops. GF/F filters have a nominal pore size of 0.7 to 0.8 μ m. Bacterial-size cells pass through these filters and were likely present in the seawater.

Culture conditions. Cultures of *Trichodesmium* IMS101 were grown in an enriched seawater medium depleted in combined N as described by Prufert-Bebout et al. (1993). Cultures were maintained in incubators at 28°C on a 14:10 h light:dark schedule at the

Chesapeake Biological Laboratory (CBL). Light levels in the incubators were about 55 to 65 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ PAR supplied by banks of cool white fluorescent lighting. Cells were transferred using sterile techniques under a laminar flow hood and maintained in exponential growth to prevent excess bacterial accumulation. Non-*Trichodesmium* bacterial abundance in cultures was estimated as <1% of the total cell numbers based on microscopic examinations. Bacteria were not observed in the culture medium. Cultures were swirled daily in order to prevent cells and filaments from adhering to the sides of the culture vessels. Prior to each sampling, cultures were swirled to ensure homogenous sampling.

Nitrogenase activity. Rates of N_2 fixation were measured by the acetylene reduction method as described by Capone (1993). Ethylene production from acetylene was measured using a Shimadzu mini-II flame ionization gas chromatograph having a 2 m Porapak R column and quantified against an ethylene gas standard. Ten colonies were counted into 14 ml serum vials containing 10 ml of GF/F filtered seawater leaving 4 ml of headspace. Care was taken to select colonies of similar size and to approximate a uniform biomass among vials. Vials without added *Trichodesmium* colonies were prepared in parallel with some of the incubations to correct for background N_2 fixation in the filtered seawater. Vials were sealed and 1 ml of acetylene (>99.9%) was added to the headspace. A time-zero gas sample was extracted and the vials were placed in incubators on deck and supplied with a steady flow of seawater under neutral density screening to simulate mixing, temperature and light conditions in the upper water column. The duration of incubations for N_2 fixation ranged from several to 12 h. Measurements of ethylene production were made by removing 100 μl of headspace gas from each vial immediately after, and at intervals of every 1 to 2 h after, the addition of acetylene. Ethylene production in the sample vials was estimated relative to a C_2H_4 standard gas. Estimates of N_2 fixation rates were calculated using the equations of Capone (1993) using a conversion factor of 4. N_2 fixation rates were normalized per colony for natural populations.

Because cultured populations did not form colonies, rates of acetylene reduction in *Trichodesmium* IMS101 were measured by withdrawing 10 ml of a well-mixed parent culture and placing it into the serum vial. Vials were sealed and 1 ml of acetylene was introduced into the headspace. N_2 fixation rates were normalized to particulate N concentration in cultures. Control assays were conducted on 10 ml of the seawater medium to determine whether there was any acetylene reduction associated with the seawater medium. No activity was found. Replicate injections of sample and C_2H_4 standard gas were reproducible to within about 1%.

^{15}N uptake. Rates of N uptake were measured using ^{15}N tracer techniques as outlined in Glibert & Capone (1993), using highly (>98%) enriched ^{15}N substrates. For field studies, colonies previously picked into GF/F filtered seawater were counted into polycarbonate incubation bottles containing 50 or 100 ml GF/F filtered, low-nutrient seawater (20 colonies per incubation). Uptake experiments were initiated by adding a ^{15}N substrate (NH_4^+ , NO_3^- , urea, glu or a commercial mix of AA).

Because nutrients in the surface waters of the study sites were typically at the limit of analytical detection (0.03 μM), additions of 0.03 $\mu\text{M } ^{15}\text{N l}^{-1}$ were used. These additions represented enrichments of ambient concentrations and may have stimulated uptake. The duration of incubations was 1 or 2 h except when specifically noted. ^{15}N incubations were terminated by gently filtering (<125 mm Hg) the contents of the incubation bottles onto pre-combusted (450°C for 2 to 4 h) GF/F filters. Filters were rinsed 3 times with low N filtered seawater to remove any tracer that was not taken up by cells. Time-zero controls were filtered immediately after the ^{15}N addition and the concentration of ^{15}N was measured to correct for ^{15}N label adsorption to the filters. Samples were frozen and returned to the CBL for analysis.

The effect of substrate concentration on uptake rates was estimated for the different N compounds. Concentrations of 0.03, 0.3, 3.0, and 30 $\mu\text{M } ^{15}\text{N NH}_4^+$, NO_3^- , urea, glu and AA were added to 100 ml incubation bottles containing 20 colonies of *Trichodesmium* spp. and incubated for 2 h in the morning, as described above. The uptake velocities calculated from measurements of ^{15}N uptake were plotted by using Lineweaver-Burk transformations (S/v vs S , where S is the substrate concentration in μM and v is velocity h^{-1}) to estimate the half saturation constant (K_s) for each N source. Because S included some unknown substrate concentration less than the analytical detection limit for that nutrient (S_n) we expressed the half saturation constant as $K_s + S_n$.

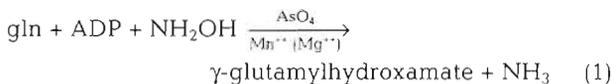
To measure ^{15}N uptake in cultures of *Trichodesmium* IMS101, 20 ml of a well-mixed parent culture was transferred into an acid-cleaned 24 ml scintillation vial and inoculated with 0.03 μM of either $^{15}\text{NH}_4^+$ or ^{15}N -enriched glu. Vials were returned to the culture incubator for 1 h and samples collected onto pre-combusted GF/F filters and rinsed 3 times with the N-depleted seawater medium as for field samples. Filters were frozen until analyzed.

Sample ^{15}N enrichment and total particulate nitrogen mass were measured by mass spectrometry on a Europa Scientific ANCA-SL 20-20 IRMS (Isotope Ratio Mass Spectrometer) against a peptone standard. The instrument was calibrated and tuned before each sample run. A full reference sample set (6 samples) was

analyzed at the beginning and end of each set of 25 samples. Two reference samples were inserted after every 5th sample throughout the sample run to verify instrument performance over the course of sample runs. Reference samples were reproducible to within 0.0001 atom %.

GS activity. In field studies, 20 to 100 colonies of *Trichodesmium* spp. were rinsed and then counted into incubation bottles containing fresh GF/F filtered seawater. The contents of these bottles were then filtered onto polycarbonate filters (3.0 to 8.0 μm pore size). Filters were flash frozen in liquid N_2 and stored at -80°C until analyzed. In culture studies with *Trichodesmium* IMS101, the parent culture was gently mixed and a known culture volume was gently filtered onto polycarbonate filters, frozen and stored in liquid N_2 until analyzed.

GS activity was measured on crude cell extracts either immediately or after storage in liquid N_2 for <2 wk. GS biosynthetic and transferase activities were measured using the γ -glutamyltransferase and γ -glutamylsynthetase assays of Stadtman et al. (1979) with the modifications described in Lee et al. (1988) after extraction in HEPES buffer solution. The GS transferase assay measures the amount of γ -glutamylhydroxamate produced in the following reaction catalyzed by GS:

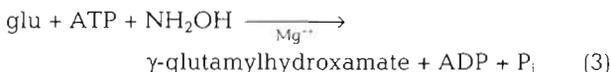


This reaction does not occur *in vivo* but the γ -glutamylhydroxamate produced can be measured spectrophotometrically and the reaction is analogous to the reverse of the *in vivo* biosynthetic reaction:



When Mn^{++} is present as the divalent cation in the assay, the activity of both modified and unmodified enzyme is stimulated. The cation Mg^{++} has been shown to inhibit the activity of modified enzyme in other species (Lee et al. 1988).

The GS biosynthetic assay measures the production of γ -glutamylhydroxamate from the forward GS reaction as follows:



This assay provides an estimate of the *in vivo* potential for gln synthesis via GS. It has the analytical advantage over 'true' biosynthetic assays of having a stable end product that is easily measured (Slawyk & Rodier 1988). In these studies we measured both GS transferase activity, in the presence of Mn^{++} , and GS biosynthetic activity, in the presence of Mg^{++} , and calculated the ratio between the 2 activities. Changes in

the ratio might be indicative of enzyme modification and the proportion of available enzyme that is biosynthetically active (Lee et al. 1988).

The HEPES extraction buffer and the reaction 'stop' mix were prepared fresh daily. Reaction mixtures were prepared fresh just prior to initiating assays and warmed to the assay temperature just prior to use. Transferase and biosynthetic assays were each set up in triplicate for each sample. Assays were initiated by adding cell homogenate to tubes containing the warmed reaction mixture and the reaction proceeded in a water bath or a heating block maintained at 30°C . Background absorption was corrected for by stopping the reaction in 1 of the 3 replicate assay tubes immediately after addition of the crude cell extract. Reaction times were optimized for both the GS transferase and GS biosynthetic assays by measuring activity over several time courses using cell extract from freshly harvested *Trichodesmium* spp. Reaction times were selected from the portion of the curve in which enzyme activity increased linearly over time, 10 min for transferase assay and 15 min for the biosynthetic assay. The reaction mixtures were adjusted to pH 7.7 (transferase) and 7.5 (biosynthetic) using 10 N NaOH prior to initiating the assays (Meeks pers. comm.).

The γ -glutamylhydroxamate produced from the biosynthetic and transferase reactions was measured using a Shimadzu UV-160 spectrophotometer and the concentration determined relative to the standard curve associated with each sample set. Replicate measurements varied by less than 0.010 absorption units for transferase assays and 0.001 absorption units for biosynthetic assays (usually <1%). Very little error was associated with the replicate assays. All standard curves had regression coefficients of $R^2 > 0.99$.

Protein analysis. Total cell protein was measured spectrophotometrically using the Lowry procedure on a TCA precipitate of the cell extracts used for the GS assays (Sigma Protein Assay Kit No. P5656). Absorbance was measured on a Shimadzu UV-160 spectrophotometer. Protein content was estimated in replicate assays against a standard curve of bovine serum albumin (BSA) ($R^2 > 0.99$). Estimates of protein content in replicate analyses of the same sample extract varied by <2%. Specific GS activity was calculated and rates of enzyme activity expressed as the nmoles of γ -glutamylhydroxamate produced per μg protein or per ml culture per hour.

Amino acid analysis. Samples for the analysis of intracellular AA pools were collected in the same manner as those designated for the GS assays. Samples collected in the field studies were immediately frozen and stored in liquid N_2 for the duration of the cruises and transported to the CBL on dry ice. They were stored at -80°C in the laboratory until analysis. Samples col-

lected during culture studies in the laboratory were frozen and stored in liquid N₂ until analysis. Intracellular pools were extracted in 0.3 N perchloric acid. The extract was neutralized and chloride precipitated with 2 M potassium carbonate as described by Senior (1975). AA were analyzed by OPA fluorescence using reverse-phase high performance liquid chromatography (HPLC) as described by Flynn (1988) and Cowie & Hedges (1992). Analyses were performed on a Waters system with a refrigerated autosampler and fitted with a Novapac column (150 × 3.9 mm, 4 μm, C-18 packing). The mobile phase, supplied at a rate of 1.5 ml min⁻¹, was a gradient (solvent A: 0.025 M sodium acetate, 2% tetrahydrofuran, 0.05% Brij; solvent B: 100% methanol). AA were resolved by modifying the gradient and pH of solvent A to achieve optimum separation. Individual AA peaks were identified by comparison with retention times from mixtures of pure AA standards. AA were quantified relative to a standard AA mix (Sigma A-2161) to which gln had been added. Standards were run after every 5 samples. Distilled water blanks were run with each sample set and subtracted from the sample measurement. Neutralized perchloric acid extraction blanks were run with each group of samples extracted. Reproducibility of measurements to within 1% was established by repeat injections. Standard curves were run at the beginning and end of each sample set (R² > 0.99). Only the glu and gln concentrations are reported here. αkg was measured enzymatically as described by Lowry et al. (1971) and Lowry & Passonneau (1972).

Inorganic nutrient analysis. Intracellular NH₄⁺ concentrations were measured on a number of occasions by rupturing cells with a combination of heat and osmotic shock (Thoresen et al. 1982). Interfilamental nutrient concentrations were approximated by placing 30 colonies into 30 ml nutrient-free filtered seawater, gently agitating them to disperse trichomes without disrupting cells, and then collecting the resultant filtrate for nutrient analysis, as described in Capone et al. (1994). Samples for measuring the ambient nutrient and dissolved free amino acid (DFAA) concentrations in the GF/F filtrate used in each study were collected and measured immediately or stored frozen (-20°C) until analysis. Nutrients (NH₄⁺, NO₃⁻, urea) were measured colorimetrically using a Technicon autoanalyzer II (Friederich & Whitley 1972) equipped with an Alchem autosampler and FASPac software. DFAA were measured by HPLC as described above. Concentrations were determined by comparison with standard curves (R² > 0.99). Repeat measurements were made from the same sample to verify reproducibility of measurements to within about 2%.

Field measurements were normalized per colony and culture measurements were normalized per unit

N. An average colony was estimated to be 0.1 to 0.2 μg N (Carpenter 1983a, Mulholland unpubl. results) although this level varied with colony size. Cultures ranged from 0.7 to 1.0 μg N ml⁻¹. N uptake measurements were expressed in units of reciprocal time to avoid the problems of variable colony size and variations in total biomass among incubations and to allow better comparisons with uptake rates measured previously and uptake rates measured in cultures in which colonies did not form.

Because the analytical error associated with the measurement of AA, GS activity, N₂ fixation by acetylene reduction and ¹⁵N uptake were negligible based on instrument performance and reproducibility between replicate assays, only that error associated with differences between replicate samples is presented in the results. Replicate measurements of acetylene reduction and ¹⁵N uptake were made for most studies and errors are presented as standard deviations among sample replicates. Due to biomass limitation in both natural and cultured populations and the handling time necessary for sample collection and preparation, replication was not possible in some of these experiments.

RESULTS

Natural populations

N₂ fixation and N uptake

Rates of N₂ fixation, as estimated by acetylene reduction, by *Trichodesmium* spp. varied among sampling stations and sampling dates. The highest N turnover rates attributed to N₂ fixation, up to 0.4% h⁻¹, (equal to 0.6 nmol N colony⁻¹ h⁻¹) were recorded at mid-day (Table 1). The daily pattern of nitrogenase activity for individual incubations and *Trichodesmium* spp. populations was a bell-shaped curve centered around mid-day as shown in previous studies (Table 1, Capone et al. 1990, 1994). While analytical precision was high (see 'Methods'), replicate incubations could vary by up to 50%. This is consistent with variability found in earlier studies (Carpenter & McCarthy 1975, Carpenter & Price 1977, Capone 1990, 1994). Activity decreased over the course of the afternoon and was absent by evening. N₂ fixation was not detected at night.

N uptake (NH₄⁺, NO₃⁻, urea, glu and a mixed AA substrate) was detected in *Trichodesmium* spp. collected from low nutrient areas of the North Atlantic Ocean and the Eastern Caribbean Sea and N uptake rates varied among populations, between sample replicates, and relative to calculated rates of N₂ fixa-

Table 1. Summary of measured rates of N uptake (% N h⁻¹) and N₂ fixation (% N h⁻¹) in *Trichodesmium* spp., by date. All samples were collected from the tropical and subtropical Northwest Atlantic Ocean. Tracer additions of ¹⁵N substrates were 0.03 μM. Ambient concentrations of NH₄⁺, NO₃⁻, and urea were at or below the limits of detection (0.03 μM) and ambient glutamate (Glu) and amino acid (AA) concentrations were <10 and <100 nM, respectively. The average colony had 0.1 μg at N colony⁻¹. Standard deviations are reported in parentheses. ND: no N uptake or N₂ fixation was detected. NR: no replicate samples

Date (d/mo/yr)	Time (h)	N ₂ fixation (% h ⁻¹)	NH ₄ ⁺ uptake (% h ⁻¹)	NO ₃ ⁻ uptake (% h ⁻¹)	Urea uptake (% h ⁻¹)	Glu uptake (% h ⁻¹)	AA uptake (% h ⁻¹)
12/06/94	04:00	ND			0.09 (0.08)		
20/10/96	07:00	0.01 (0.002)	0.067 (NR)	0.0043 (NR)	0.03 (NR)	0.09 (NR)	
12/06/94	09:48	0.23 (0.04)	0.27 (0.07)		0.03 (0.01)		
17/10/96	10:45		0.07 (NR)		ND		
27/10/96	11:10	0.34 (0.10)	0.06 (0.001)	0.0003 (0.0003)	0.002 (0.003)	0.015 (0.001)	0.011 (0.002)
19/10/96	11:15		0.44 (0.27)	ND	0.04 (0.006)	0.012 (0.006)	
16/10/96	13:45	0.40 (0.03)	0.70 (0.28)			0.37 (0.40)	0.32 (NR)
13/04/96	14:06		0.58 (0.43)				
06/11/96	15:00	0.023 (0.002)	1.84 (0.96)			0.04 (0.02)	
19/04/96	15:10	0.046 (0.03)	0.19 (0.06)				
12/06/94	15:23				0.11 (0.04)		
13/10/96	16:00	ND	0.65 (NR)				
28/10/96	18:50	ND	0.04 (0.001)	0.0004 (0.0001)	0.0007 (0.0001)	0.015 (0.001)	0.02 (0.006)
19/04/96	19:52	ND	0.08 (0.03)				
12/06/94	21:00	ND			0.11 (0.013)		

tion. Rates of ¹⁵NH₄⁺ uptake were generally higher than those measured for other N substrates. NH₄⁺ uptake exceeded rates of N₂ fixation throughout most of the day and proceeded during the night when there was no N₂ fixation (Table 1). The highest NH₄⁺ uptake rates, up to 1.8% h⁻¹, were measured during the day. Because ambient concentrations of NH₄⁺ were at or below the limits of detection, rates of ¹⁵N uptake may have been stimulated by 0.03 μM additions.

Rates of urea uptake were variable among studies and ranged from zero to about 0.11% h⁻¹. Glu and mixed AA uptake rates were lower but always detected. Ambient concentrations of AA were <30 nM. Rates of ¹⁵NO₃⁻ uptake were low or undetectable.

On average a total of about 12% of the cell N was turned over during a 24 h period, and about 2.3% was attributed to N₂ fixation. This constitutes a N based turnover time of about 8.5 d. Based on a 10 h period of N₂ fixation, a maximum of 4% of the total colony N biomass could be provided daily by this N utilization pathway. This would yield doubling times of about 25 to 40 d. N turnover due to N₂ fixation may be lower (and the doubling time greater) when we allow that cells fix N₂ at maximal rates only around mid-day.

Kinetic constants varied among stations and substrates. *Trichodesmium* spp. colonies had the highest capacity (V_{max}) for NH₄⁺ uptake and the lowest capacity for NO₃⁻ uptake (Fig. 1). Uptake of NO₃⁻ was not detected at concentrations less than 3.0 μM and so the low K_s values calculated may not reflect the actual

affinity for this substrate. Relatively low $K_s + S_n$ and high V_{max} values were calculated for NH₄⁺, glu and AA uptake, indicating a high affinity for these N substrates (Fig. 1). Urea uptake was not saturated at 30 μM and so $K_s + S_n$ values may be underestimated in our calculations.

Urea and NH₄⁺ concentrations were measured in interfilamental spaces in some populations of *Trichodesmium* spp. by gentle disaggregation of colonies. Extracellular concentrations of NH₄⁺ and urea associated with colonies were generally elevated relative to ambient water column concentrations (Table 2). Interfilamental concentrations of NH₄⁺ were as high as 211 μM while urea concentrations of up to 315 μM were measured. Because cells have a high uptake capacity and affinity for these 2 N substrates and since there are also high nutrient concentrations in the area around the cells, uptake of these compounds may be important in natural systems.

Intracellular metabolite pools

Intracellular αkg concentrations were highest early in the morning and declined in the afternoon (Fig. 2A,B). Although replicate assays varied by <1%, variation among samples collected at the same time varied more. Increases in gln concentrations generally increased in parallel with rates of N₂ fixation (Fig. 2C,D). Concentrations of gln (usually <1 nmol

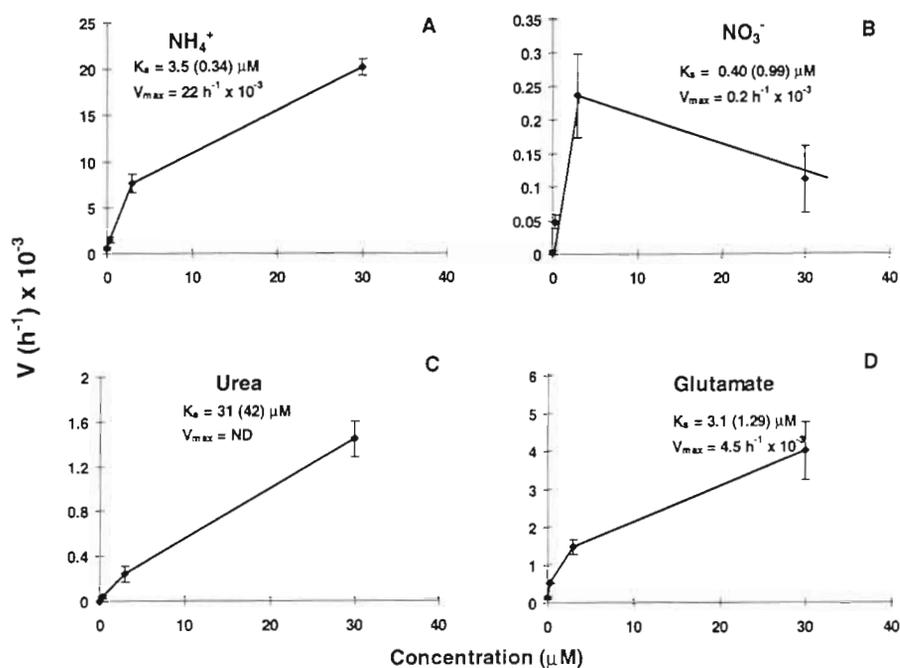


Fig. 1. Saturation curves for combined N sources: (A) NH_4^+ , (B) NO_3^- , (C) urea, and (D) glutamate in natural populations of *Trichodesmium* spp. from the tropical North Atlantic Ocean. Value for K_s + S_0 (standard deviation) and V_{\max} indicated in each panel. All incubations were done during the morning after sunrise. ND: value could not be determined

colony⁻¹) were always lower than concentrations of glu (<5 nmol colony⁻¹). As previously reported (Capone et al. 1994), the peak in intracellular glu concentrations was slightly later in the day than that for gln (Fig. 2C,D). There was an apparent diel pattern in metabolite ratios in *Trichodesmium* spp. The gln/ α kg was highest before or at mid-day and lowest during the night (Fig. 2E,F). The ratio ranged from about 0.2 to 2. The highest gln/glu ratios were measured before or about mid-day (range 0.1 to 0.6) (Fig. 2G,H).

Intracellular NH_4^+ pools ranged from 209 to 1822 μM (Table 3), similar to the ranges of values reported for glu and gln.

Table 2. Concentrations of nutrients in interfilamental areas of *Trichodesmium* spp. colonies from the North Atlantic Ocean. Calculations were based on an average colony size of 5 by 0.5 mm. Ten percent of the total cylindrical volume was interfilamental spaces (Capone et al. 1994). Standard deviations from 3 replicate analyses are reported in parentheses. ND: nutrient was not detected. The detection limit was 0.03 μM

Date (d/mo/yr)	NH_4^+ conc. (μM)		Urea conc. (μM)	
	Ambient	Interfilamental	Ambient	Interfilamental
31/05/94	ND	ND	ND	182 (4.9)
06/06/94	ND	211 (2.5)	ND	315 (9.9)

Intracellular pools of glu and gln and the ratio between these pools varied depending on the depth from which *Trichodesmium* colonies were collected. The glu and gln concentrations were highest in colonies collected from just below the surface (5 m) and then decreased with depth (Fig. 3A). The gln/glu ratio was highest at the surface and decreased with depth (Fig. 3B). The highest gln/glu and gln/ α kg ratios measured were about 0.7 and 7, respectively, in samples collected from near surface waters. The ratios rapidly decreased to between 0.2 and 0.4 for gln/glu and <2 for the gln/ α kg for samples collected deeper in the euphotic zone (Fig. 3C).

In experiments in which *Trichodesmium* spp. colonies were collected from 15 m and then incubated on deck at various simulated light levels, there were no consistent patterns in intracellular glu and gln concentrations with respect to light level (data not shown). Intracellular pools of α kg were lowest at the higher light levels and, therefore, the gln/ α kg ratio was slightly higher in the high light treatment but did not change much compared to the lower light treatments.

GS activity

GS transferase activity was, on average, between 8 and 16 nmol γ -glutamylhydroxamate μg^{-1} protein h^{-1} , more than 20 times higher than GS biosynthetic activ-

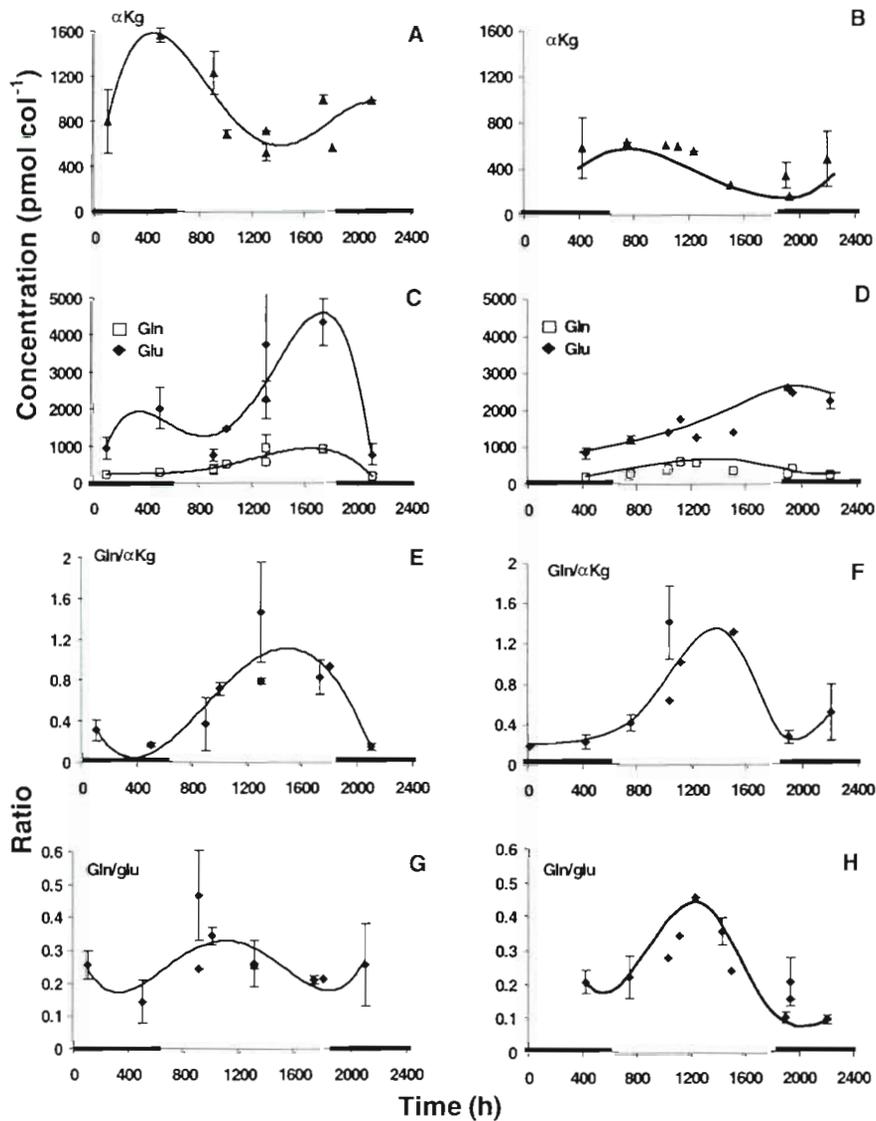


Fig. 2. Intracellular concentrations of (A,B) α -ketoglutarate, (C,D) glutamate and glutamine; intracellular ratios of (E,F) $\text{gln}/\alpha\text{kg}$ and (G,H) gln/glu in natural populations of *Trichodesmium* spp. (tufts) from (A,C,E,G) the tropical North Atlantic Ocean and (B,D,F,H) the Caribbean Sea. Error bars are standard deviations calculated from 3 *Trichodesmium* spp. samples. Bars on the x-axis indicate the dark period. Curves were drawn in Excel[®] using a 4th order polynomial regression.

Date (d/mo/yr)	Colony form	Time	NH_4^+ conc. (nmol colony^{-1})	NH_4^+ conc. (μM)
31/05/94	Tufts and puffs	1000	0.58	209
05/06/94 ^a	Tufts	1000	1.47	868
05/06/94 ^a	Puffs	1000	2.67	494
28/05/94	Tufts and puffs	1330	0.83	301
06/06/94 ^a	Tufts	1330	0.77	454
06/06/94 ^a	Puffs	1339	0.57	1050
31/05/94	Tufts	1500	3.42	809
31/05/94	Puffs	1500	2.46	1820

^aOn these days the average colony size was estimated to be about 100 trichomes colony^{-1} . On the other days, the average colony was comprised of about 250 trichomes.

Table 3. Intracellular NH_4^+ pools in *Trichodesmium* spp. collected from the tropical North Atlantic Ocean. All measurements are reported. Replicate samples were not collected on these days. Tufts represent fusiform bundles of trichomes and puffs are a radial arrangement of trichomes. Intracellular concentrations were calculated based on an average of 100 cells trichome^{-1} (Marumo 1975, McCarthy & Carpenter 1979) and cell volumes of $540 \mu\text{m}^3$ for spherical colonies (puffs) and $1690 \mu\text{m}^3$ for fusiform colonies (tufts) (Carpenter 1983a).

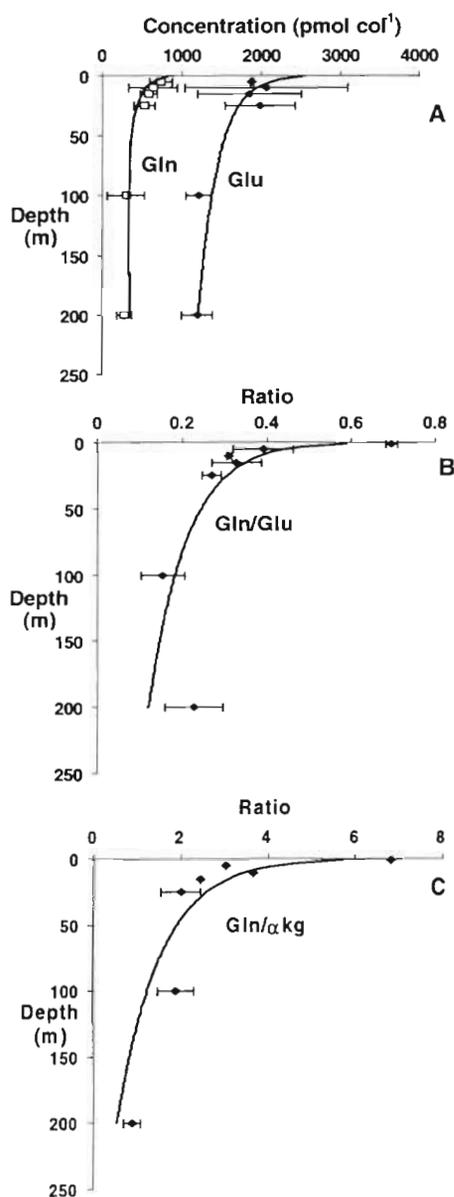


Fig. 3. Intracellular (A) glu and gln concentrations, (B) gln/glu ratios, and (C) gln/ α kg ratios in *Trichodesmium* spp. collected at a single station from different depths on the same day in the Caribbean Sea

ity (4 to 6 nmol γ -glutamylhydroxamate μg^{-1} protein h^{-1}). Levels of activity were highest after mid-day and lower during the early morning. The ratio between GS transferase and GS biosynthetic activity varied only slightly during the course of the day, averaging between about 18 and 25. There was a slight increase in the transferase/biosynthetic ratio in the afternoon but there was no significant trend. Few measurements were made during the dark period. As a result, discerning diel changes in activity is difficult.

Cultured populations

Biomass

The average N biomass of cultures of *Trichodesmium* IMS101 used in these experiments was $121 \mu\text{Mol N l}^{-1}$. At this density, cells were in exponential phase growth.

N₂ fixation

Assays for N₂ fixation were initiated at 4 points during the light period and twice during the dark and monitored over time. In cultures of *Trichodesmium* IMS101, the highest average rates of N₂ fixation ($1.05\% \text{ h}^{-1}$) were measured during the middle of the light period (Table 4). These rates exceeded the rates of N₂ fixation measured in natural populations of *Trichodesmium* spp. colonies. Activity increased after the onset of the light period and decreased during the latter half of the light period (Table 4). N₂ fixation was not detected during the dark period. Integrating over the day and assuming maximal rates of N₂ fixation throughout the 10 h light period, we estimate that there was a turnover of 11% of the cell N per day due to N₂ fixation. This translates to an N₂ fixation-based N doubling time of about 9 d.

Nitrogen uptake

Prior to the addition of *Trichodesmium*, NH_4^+ , glu and gln were not measurable in the seawater medium. NH_4^+ accumulated in the culture medium during growth of *Trichodesmium* IMS101, with concentrations ranging between 0.3 to $1.8 \mu\text{M}$ over the diel cycle (Table 4). Additions of ^{15}N -labeled glu represented 100% or more of ambient concentrations. Average rates of NH_4^+ uptake were highest (average of $0.68\% \text{ h}^{-1}$) just after the onset of the light period and then were lower at mid-day (0.2 to $0.3\% \text{ h}^{-1}$) when N₂ fixation rates were high (Table 4). Uptake of NH_4^+ represented about 20 to 40% of the N₂ fixation rate at mid-day. Rates of NH_4^+ uptake were greater than the rates of N₂ fixation except around mid-day when N₂ fixation rates were highest. In the afternoon, NH_4^+ uptake rates were lower than in the morning. Integrated over a day, NH_4^+ uptake accounted for 10 to 16.3% of the total cell N per day. There was a strong positive relationship ($R^2 = 0.90$) between the rate of NH_4^+ uptake and the NH_4^+ concentration measured in the culture medium (Fig. 4).

Table 4. Summary of N uptake and N₂ fixation rates by *Trichodesmium* IMS101 from experiments conducted on 12/06/96 using 0.03 μM additions of ¹⁵NH₄⁺ or ¹⁵N-glutamate. Rates are expressed as turnover rates of % cell N h⁻¹. The standard deviations from 2 replicate incubations are reported in parentheses. ND: rates were not detected

Time (h)	N ₂ fixation (% h ⁻¹)	NH ₄ ⁺ uptake (% h ⁻¹)	NH ₄ ⁺ in medium (μM)	Glu uptake (% h ⁻¹)	Glu in medium (μM)
02:39	ND	0.559 (0.19)	0.11 (0.04)	0.03 (0.002)	0.004 (0.001)
07:48	0.0086 (0.004)	0.337 (0.13)	0.08 (0.03)	0.03 (0.001)	0.005 (0.002)
10:20	0.022 (0.006)	0.681 (0.21)	0.13 (0.07)	0.03 (0.002)	0.006 (0.002)
13:38	1.046 (0.14)	0.269 (0.05)	0.04 (0.01)	0.03 (0.003)	0.002 (0.001)
17:40	0.019 (0.003)	0.45 (0.07)	0.09 (0.03)	0.03 (0.001)	0.004 (0.001)
21:57	ND	0.415 (0.08)	0.08 (0.03)	0.02 (0.003)	0.004 (0.001)

Glu uptake was low, but detectable, throughout the diel cycle (Table 4). Rates were about 0.03% h⁻¹, yielding an average daily N turnover due to glu of 0.72% d⁻¹. Glu concentrations in the culture medium ranged from about 0.02 to 0.06 μM. Therefore, the 0.03 μM ¹⁵N tracer additions represented 50 to 100% enrichments of the ambient glu concentration (Table 4). These high additions may have stimulated uptake and therefore the results should be considered as upper estimates. The uptake of glu did not correlate with the concentration of glu in the medium.

Intracellular metabolite pools

Intracellular pools of glu and gln increased during the light period in *Trichodesmium* IMS101 growing on medium without added N substrates (Fig. 5A). The highest concentrations were measured during the period of high N₂ fixation. Intracellular concentrations of glu were more than 10-fold higher than gln concentrations during most of the day. There was a strong diel pattern in the gln/glu ratio in *Trichodesmium* IMS101 (Fig. 5B). The gln/glu ratio rose to a peak of about 0.3 during and just after the period of highest N₂ fixation. However, during the dark period, when there was no N₂ fixation, ratios decreased to about 0.1

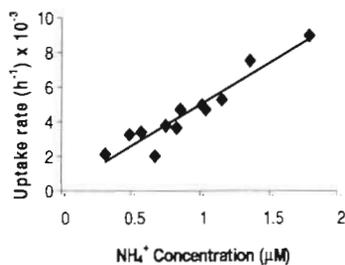


Fig. 4. NH₄⁺ uptake relative to concentrations of NH₄⁺ measured in the culture medium

GS activity

Both GS transferase and GS biosynthetic activity decreased after the period of high N₂ fixation in the afternoon (Fig. 6A). Similarly, the ratio of GS transferase/biosynthetic activity decreased from about 26 to 20 during the period when rates of N₂ fixation were high (Fig. 6B). Late in the light period the ratio increased to values over 30 and remained about 30 during the dark period.

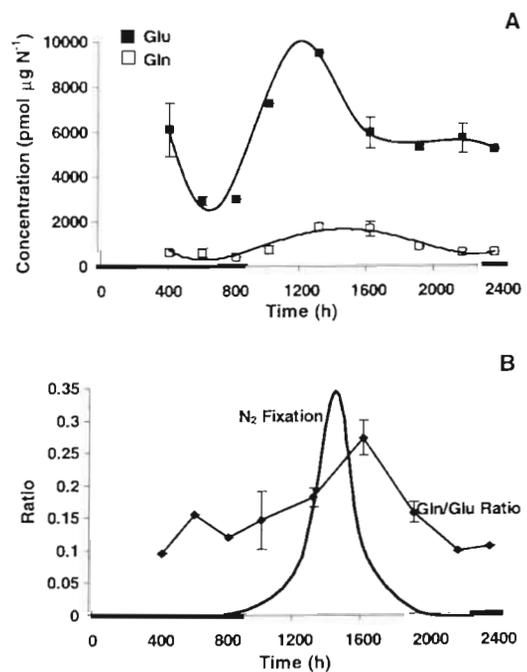


Fig. 5. Intracellular pools of (A) glu and gln and (B) the gln/glu ratio relative to the pattern of N₂ fixation measured (actual values shown in Table 4) in *Trichodesmium* IMS101 grown on seawater medium depleted in combined N substrates. Bars on the x-axis indicate the dark period (darkness from 22:30 to 08:30 h daily).

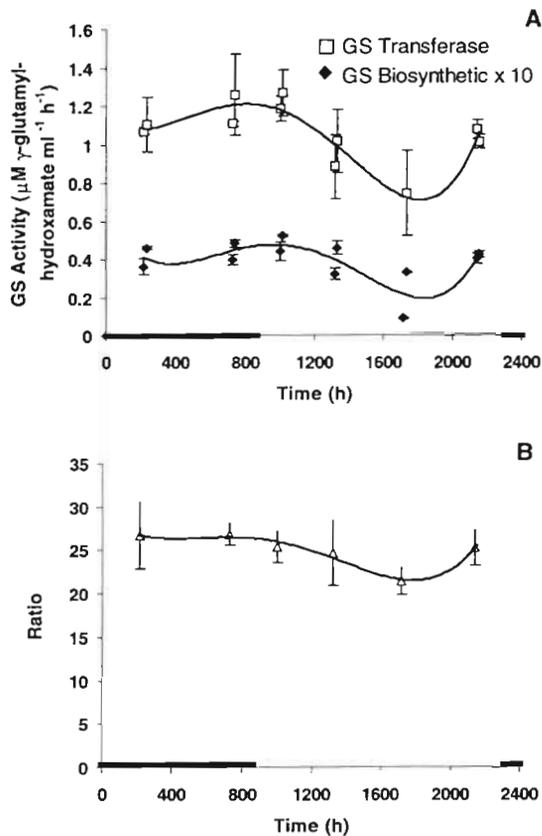


Fig. 6. (A) Glutamine synthetase (GS) transferase and biosynthetic activity and (B) the GS transferase/biosynthetic activity ratio relative to the pattern of N_2 fixation measured (see Table 5 for data) in *Trichodesmium* IMS101 grown on seawater medium depleted in combined N substrates. Bars on the x-axis indicate the dark period (darkness from 22:30 to 08:30 h daily)

DISCUSSION

N utilization

Rates of N_2 fixation vary widely among populations of *Trichodesmium* from different oceanic regions (Goering et al. 1966, Saino 1977, Saino & Hattori 1978). This may be because environmental factors, such as the availability of light and trace elements (e.g. Fe), and physiological factors, such as the growth stage, affect the rates at which N_2 fixation proceeds (Carpenter & Price 1976, Carpenter 1983a). Rates of combined N uptake by natural populations of *Trichodesmium* also vary among studies and sites (Table 5). As for N_2 fixation, this variability may be due to differences in the nutritional status of populations or differences in their growth stage at the time of sampling.

Similar to previous reports, rates of NO_3^- uptake in our studies were low or undetectable in both natural populations of *Trichodesmium* and in cultured popula-

tions growing on medium without added N (Table 5). An induction period for NO_3^- transport systems and NO_3^- assimilatory pathways or some minimum concentration may be necessary for NO_3^- utilization by natural and cultured populations (Flores & Herrero 1994, Mulholland et al. 1999).

In general, the rates of urea uptake measured in this study (Table 5) were much higher than those measured by Carpenter & McCarthy (1975). However, these rates are consistent with rates reported by Saino (1977). We also measured high concentrations of urea within interfilamental spaces of colonies of *Trichodesmium*, suggesting that urea may be produced and taken up within the colonial assemblage. The source of this urea is not known. It is possible that urea is produced by grazers living in association with colonies. Bacterial production of urea has been measured in sediments (Pedersen et al. 1993) and seawater (Slawyk et al. 1990) but its production in association with *Trichodesmium* spp. colonies has not been measured.

Trichodesmium spp. have the highest capacities for NH_4^+ and AA uptake. In natural populations of *Trichodesmium* we measured NH_4^+ uptake rates of 0.05 to $1.84\% \text{ h}^{-1}$. These rates are higher than those measured by Carpenter & McCarthy (1975) but similar to the rates reported by Saino (1977) and Goering et al. (1966). Bacterial utilization and regeneration of N substrates, particularly NH_4^+ and AA within incubation bottles can cause variability in measured rates of N uptake (Kirchman et al. 1989). While bacteria can consume NH_4^+ , Kirchman et al. (1989) demonstrated that high DON (specifically as AA) concentrations stimulate net regeneration of NH_4^+ by bacteria. Enrichment of interfilamental spaces with AA (Carpenter et al. 1992, Capone et al. 1994) may result in a net production of NH_4^+ from AA in *Trichodesmium* communities. Bacteria occur in association with *Trichodesmium* colonies and are both directly attached to filaments and in loose association with colonies (Paerl et al. 1989). Carpenter & Price (1977) estimated that <1 to 8.3% of *Trichodesmium* spp. trichomes were populated with bacteria.

Direct utilization of mixed AA by *Trichodesmium* spp. from the Eastern Caribbean Sea was observed by Paerl et al. (1989) using microautoradiography. AA can also be utilized indirectly. Extracellular AA oxidases have been identified in a variety of phytoplankton cultures (Palenik et al. 1988, Palenik & Morel 1990a,b), bacterial populations in Long Island Sound (Pantoja et al. 1993), and in incubations containing *Trichodesmium* spp. (Mulholland et al. 1998). Extracellular oxidation of AA results in the liberation of NH_4^+ which is subsequently available for utilization by cells. Glu uptake was much lower in cultured populations of *Tri-*

Table 5. Rates of N uptake by *Trichodesmium* spp. reported in the literature and those measured in these studies. Glu: glutamate, Gln: glutamine, AA: amino acids, ND: not detected

Substrate	Location	Addition (μM)	N uptake ($\% \text{ h}^{-1}$)	Uptake rate ($\text{pg N cell}^{-1} \text{ h}^{-1}$)	Uptake rate ($\text{pg N colony}^{-1} \text{ h}^{-1}$)	Source
NH_4^+	W Sargasso Sea	0.13–20.4	0.0006–0.018 ^c	0.02–0.59	0.6–17.7 ^b	Carpenter & McCarthy (1975)
	W Sargasso Sea	0.067	0.005–0.014			Carpenter & McCarthy (1975)
	W Sargasso Sea	14	0.0042–0.0048 ^c	0.14–0.16	4.2–4.8 ^b	Carpenter & McCarthy (1975)
	N Atlantic	2	0.005–0.08			Goering et al. (1966)
	N Atlantic	2	0.51 (Bloom)			Goering et al. (1966)
	NW Pacific	0.03	0.01			Saino (1977)
	NW Pacific	0.05	0.02			Saino (1977)
	NW Pacific	0.1–1.0	0.035–0.23			Saino (1977)
	NW Pacific	2.0–10	0.03–0.21			Saino (1977)
	N Atlantic	20	2.0			Saino & Hattori (1978)
NO_3^-	W Sargasso Sea	0.13–20.4	~0	~0	~0 ^b	Carpenter & McCarthy (1975)
	W Sargasso Sea	14	0.00012–0.0003 ^c	0.004–0.01	0.12–0.3 ^b	Carpenter & McCarthy (1975)
	N Atlantic	2	0–0.02			Goering et al. (1966)
	N Atlantic	2	0.175 (Bloom)			Goering et al. (1966)
	NW Pacific	10	0.0026–0.0028			Saino (1977)
	N Atlantic	0.03	ND–0.0005		4.4 ^a	Present study
Urea	W Sargasso Sea	0.13–20.4	~0	~0	~0 ^b	Carpenter & McCarthy (1975)
	W Sargasso Sea	14	0.00054–0.0006 ^c	0.018–0.02	0.54–0.60 ^b	Carpenter & McCarthy (1975)
	NW Pacific	10	0.0494–0.0534			Saino (1977)
	N Atlantic	0.03	ND–0.2		ND–420	Present study
Glu	NW Pacific	10	0.0085–0.0093			Saino (1977)
	Caribbean	0.07	< 0.2			Capone et al. (1994) ^d
	Caribbean	0.001	0.0018–0.0053 ^c		1.8–5.3 (am)	Carpenter et al. (1992) ^e
	Caribbean	0.001	0.025 ^c		25 (mid-day)	Carpenter et al. (1992) ^e
	Present study	N Atlantic	0.03		0.0081–0.69	2.8–952
Gln	Caribbean	0.001	0.0001–0.00007 ^c		0.1–0.07 (am)	Carpenter et al. (1992) ^e
	Caribbean	0.001	0.0025–0.005 ^c		2.5–5 (mid-day)	Carpenter et al. (1992) ^e
Glycine	N Atlantic	2	0.02			Goering et al. (1966)
AA	N Atlantic	0.03	0.01–0.023 ^c		2.3–410	Present study

^aMostly non-detectable
^bCalculated based on Carpenter & McCarthy's estimate of 30000 cells colony⁻¹
^cCalculated based on estimate of 100 ng-at N colony⁻¹
^dUsing ¹⁴C-labeled glu
^eUsing ³H-labeled glu and gln

chodesmium. This may reflect the lower abundance of the microbes necessary for regenerating NH_4^+ from glu in these populations.

We found that in natural populations of *Trichodesmium* spp., $K_s + S_n$ values for NH_4^+ and glu were consistent with those reported previously (Table 6). Our V_{max} estimates for NH_4^+ uptake were substantially higher than those previously reported. In cultures of *Trichodesmium* NIBB1067, apparent V_{max} decreased as incubation length increased, suggesting that recycling of nutrients, and consequent isotope dilution, may occur within the incubation bottles during longer incubations (Mulholland et al. 1999). Because our incubations were short (1 or 2 h), isotope dilution was minimal during these experiments.

N metabolism

Intracellular N and C dynamics and the availability of substrates for NH_4^+ assimilation have been implicated as important factors affecting the regulation of N_2 fixation and N metabolism in a variety of bacteria and cyanobacteria (Guerrero & Lara 1987, Flores & Herrero 1994). Gln/glu ratios have been utilized as an index of cellular C:N status and N limitation in a variety of microalgae (Flynn et al. 1989, Flynn & Gallon 1990). Flynn et al. (1989) suggested that phytoplankton cells with intracellular gln/glu ratios >0.5 are N-replete while those with ratios of <0.2 are N-depleted. Applying these criteria, *Trichodesmium* spp. cells are rarely N-depleted. We observed gln/glu ratios of 0.6 during

Table 6. Values of K_s (μM) and V_{max} ($\% \text{ N h}^{-1}$) reported for various N sources and those measured in these studies

Substrate	K_s (μM)	V_{max} ($\% \text{ h}^{-1}$)	Location	Source
NH_4^+	6.74	0.177 ^a	W Sargasso Sea	Carpenter & McCarthy (1975)
	0.3 to 12	0.045 to 0.25	NW Pacific	Saino (1977)
	3.1 to 3.9	2.2	N Atlantic	Present study
NO_3^-	0 to 2	0.02	N Atlantic	Present study
Glutamate	1.59 to 3.24		N Atlantic	Capone et al. (1994)
	1.8 to 4.4	0.45	N Atlantic	Present study
Amino acids	0.12 to 1.43	0.45 to 0.55	N Atlantic	Present study

periods of N_2 fixation, consistent with the earlier observations by Capone et al. (1994). Gln/glu ratios of about 0.2 were observed during the dark period. In these studies, the magnitude and timing of the daily peak in gln/glu ratios varied among sites and populations, consistent with the variability reported by Capone et al. (1994). The gln/ αkg ratio exhibited a similar pattern to the gln/glu ratio, suggesting that the gln/glu ratio may be a good indicator of cellular N status.

The higher gln/glu ratios observed in *Trichodesmium* colonies collected near the surface waters relative to colonies collected from depth may indicate light limitation of N_2 fixation. Higher rates of photosynthesis by surface populations may provide energy to support higher rates of N_2 fixation, resulting in higher gln/glu and gln/ αkg ratios in these populations. The high gln/glu ratios observed in surface samples in this study were comparable to ratios reported by Capone et al. (1994) in samples collected from about 5 m depth. We observed lower gln/glu ratios in colonies collected from between 20 and 30 m depth and in cultures of *Trichodesmium* IMS101 (a maximum of about 0.3). Cultures were grown under low light levels relative to those measured in tropical surface waters and N_2 fixation may be light limited in these culture systems.

Assimilation of fixed N_2 via GS does not appear to limit the rate of N_2 fixation. Rates of GS transferase and GS biosynthetic activity by *Trichodesmium* spp. increased in the afternoon when high rates of N_2 fixation and high gln/glu ratios were observed. The absence of a strong diel cycle of GS transferase activity suggests that the GS pool is conserved in *Trichodesmium*.

Our results indicate that *Trichodesmium* colonies have an excess biosynthetic capacity via GS. Based on an average $5 \mu\text{g}$ protein colony⁻¹, we calculated a maximum biosynthetic capacity of $0.72 \mu\text{Mol N}$ assimilation colony⁻¹ d⁻¹. Our average N biomass for a colony was 7.15 to 14.3 nmol N (0.1 to 0.2 $\mu\text{g N}$). Colonies

therefore had the potential to assimilate enough N to turn over their cell N at least 50 times d⁻¹, suggesting that N assimilation does not limit the rate of N utilization by cells, even when N_2 fixation rates are maximal.

Some investigators have reported that only a subset (<10 to 15%) of cells within a colony or along a filament of *Trichodesmium* contain nitrogenase and are capable of fixing N_2 (Janson et al. 1994, Fredriksson & Bergman 1995, 1997, Bergman 1999). Immuno-gold labeling indicates that while the GS enzyme is present in all cells within colonies, its concentrations are higher in cells containing

nitrogenase (Carpenter et al. 1992). This pattern is consistent with that observed in the genus *Anabaena*. For these species, high GS activities are associated with N_2 -fixing heterocysts. Rapid assimilation of NH_4^+ and transport of gln out of the N_2 -fixing regions is thought to be important for preventing feedback inhibition of GS and nitrogenase by accumulated N metabolites (Bergman et al. 1985, Guerrero & Lara 1987).

GS biosynthetic/transferase activity ratio decreased during the period of maximum N_2 fixation. Changes in the ratio between biosynthetic and transferase activity in enteric bacteria is attributed to changes in the adenylation state and consequent biosynthetic activity of the GS enzyme (Lee et al. 1988). We found that the ratio of GS transferase/biosynthetic activity varied by about 20 to 30% over a diel cycle. These changes may reflect an increase in the relative biosynthetic capacity of the GS pool associated with nitrogenase.

Tumer et al. (1983) found that there were different promoters for transcribing the gene encoding GS during growth on fixed N and molecular N_2 in *Anabaena* 7120, a heterocystous N_2 fixer. They suggested that there are 2, separately regulated pools of GS: a general pool that is conserved to assimilate N as it is encountered, and a nitrogenase-linked pool that is synthesized and activated in conjunction with nitrogenase. In *Trichodesmium*, GS mRNA transcript abundance exhibits 2 peaks, one before dawn corresponding with peak nitrogenase mRNA transcription (Wyman et al. 1996), and one during the late afternoon (Kramer et al. 1996). This observation is consistent with the idea that there may be 2, separately regulated pools of GS in *Trichodesmium*. If 2 pools of GS are present, the small changes in total filament or colony GS activity that we observed over a diel cycle may be significant in terms of the capacity for assimilating N from N_2 fixation.

N budgets

Based on total measured N utilization (N_2 fixation plus NH_4^+ and glu uptake), an N-based turnover time of about 3 to 5 d was calculated for *Trichodesmium* IMS101 during exponential phase growth. When only N_2 fixation was considered, 9 d were required for a doubling of the culture biomass. At similar biomass levels, *Trichodesmium* NIBB1067 doubles in 8 d and is in late exponential growth phase (Mulholland et al. 1999).

Independent of absolute growth demands, we calculated a daily N utilization budget for *Trichodesmium* IMS101 by integrating the relative contribution of 3 N sources (N_2 , NH_4^+ , and glu) to the total measured N turnover over a diel cycle (Fig. 7). Of the total daily N utilization, 72% was attributed to NH_4^+ uptake, 23% was due to N_2 fixation and 4% was from glu uptake. Because our cultures of *Trichodesmium* were closed systems to which no combined N had been added, all of the N used to support net growth must ultimately be derived from N_2 fixation.

Based on C:N ratios observed in colonies of *Trichodesmium* (Carpenter 1983a), the stoichiometry of C and N_2 fixation appear to be out of balance. For instance, Capone et al. (1998) calculated that N_2 fixation provided <20% of the daily N demand necessary to balance the observed C utilization by *Trichodesmium* populations within an Indian Ocean bloom. However, the low $\delta^{15}N$ of these populations suggested that N_2 fixation was still the primary N source for these populations. Other imbalances have been observed in

productivity estimates based on N_2 fixation versus C fixation in natural populations of *Trichodesmium*. In the Caribbean Sea, Carpenter & Price (1977) estimated that *Trichodesmium* spp. represented 20% of the carbon productivity but only 8% of the system's N demand was supplied by N_2 fixation. In *Trichodesmium* IMS101 cultures, we calculated that C and N turnover were nearly balanced relative to the reported C:N ratios (Carpenter 1983a, Carpenter & Romans 1991) only when we consider both N_2 fixation and NH_4^+ uptake as components of total N turnover. The N demand calculated from short-term C productivity measurements may overestimate N requirements for net growth. In cultures of *Trichodesmium* grown on medium without added N sources, N_2 fixation was a good indicator of net growth

Observations of high N turnover rates relative to rates of N_2 fixation combined with low $\delta^{15}N$ signatures may be reconciled if there is a tight coupling between N_2 fixation, NH_4^+ release or N regeneration and uptake of the low $\delta^{15}N$ derived from recently fixed N_2 . At present, there are no determinations of the $\delta^{15}N$ signature of the NH_4^+ pools in these systems to confirm this speculation. Zooplankton grazing on *Trichodesmium* and other N_2 -fixing organisms may contribute to an isotopically light NH_4^+ pool in the upper water column. Checkley & Miller (1989) have observed that oceanic zooplankton excrete NH_4^+ that is isotopically lighter than their body values.

Carpenter et al. (1999) reported that the relative contribution of NH_4^+ to the total N nutrition increased over time in a bloom of an N_2 -fixing symbiosis, *Hemialus/Richelia*. However, the population retained a light $\delta^{15}N$ signature. We speculate that *Trichodesmium* may exhibit a similar pattern whereby populations alleviate N limitation initially by fixing N_2 . Subsequently, newly fixed N may be recycled as NH_4^+ and DON. These N sources may be available for uptake by cells to support growth. Similarly, the relative proportions of N uptake and N_2 fixation to total N turnover may change over a growth cycle in cultures of *Trichodesmium*. Experiments are underway to test this hypothesis.

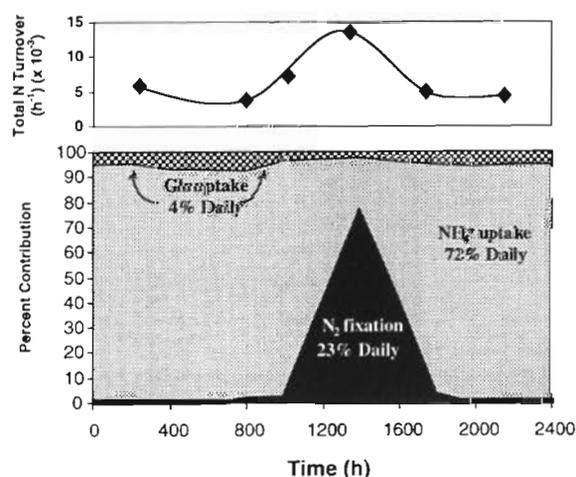


Fig. 7. Total daily N turnover by *Trichodesmium* IMS101 growing on medium depleted in combined N substrates (upper panel). Percent contribution of glu, NH_4^+ and N_2 fixation to the daily N turnover. N_2 fixation N and glu and NH_4^+ uptake rates were measured over a diel cycle and these rates were integrated over a 24 h day

Community N dynamics

A high affinity for NH_4^+ , coupled with the positive correlation between NH_4^+ concentration and NH_4^+ uptake in cultures of *Trichodesmium* IMS101, suggest that NH_4^+ release and uptake may be tightly coupled within *Trichodesmium* spp. communities. Tight coupling between N_2 fixation and NH_4^+ uptake and a capacity for concomitant N_2 fixation and NH_4^+ uptake may be an important adaptation whereby *Trichodesmium*

spp. can support growth and maintain their biomass in extremely oligotrophic environments. In closed culture systems, NH_4^+ is retained in the system and may accumulate in the culture medium for utilization by the cultured organism. By contrast, in natural systems that are open, NH_4^+ and DON produced by and released from cells or regenerated within colonial associations may diffuse away from cells and become part of the ambient nutrient pool available for utilization by all organisms in the community. Sometimes, high concentrations of NH_4^+ and glu were measured in the interfilamental areas of *Trichodesmium* colonies collected from natural populations (Capone et al. 1994, Table 2). This suggests that colonies may retain nutrients in microzones thereby preventing their diffusion away from cells.

There are a variety of potential NH_4^+ sources within the *Trichodesmium* consortia. AA and DON are released by *Trichodesmium* during growth (Capone et al. 1994, Glibert & Bronk 1994). AA oxidase activity associated with *Trichodesmium* spp. can liberate NH_4^+ from these compounds (up to 20 pmol N colony⁻¹ h⁻¹) (Mulholland et al. 1998). Bacteria can also regenerate NH_4^+ in environments rich in N-rich dissolved organic material (Kirchman et al. 1989). Nausch (1996) estimated that 0.32 to 15 nmol N colony⁻¹ h⁻¹ was released by peptidase and β -glucosamidase activity associated with bacteria residing among *Trichodesmium* spp. colonies. Zooplankton grazing can also result in rapid NH_4^+ recycling. O'Neil et al. (1996) reported NH_4^+ release rates of 2 nmol N copepod⁻¹ h⁻¹ from *Macrosetella gracilis* grazing on *Trichodesmium* spp. They estimated that 33 to 45% of a *T. thiebautii* colony and more than 100% of the N_2 fixed d⁻¹ could be consumed by grazing each day. The N content of a single colony is on average about 100 ng N colony⁻¹. *Trichodesmium* can also contain cyanophages (Ohki 1999). Viral cell lysis causes cells to burst, releasing the contents of the cell (including N metabolites and organic compounds) into the environment. Our analysis of intracellular NH_4^+ (0.57 to 3.42 nmol colony⁻¹), glu (1 to 5 nmol colony⁻¹), and gln (0.2 to 1 nmol colony⁻¹) concentrations would suggest that 3 to 9.5 nmol colony⁻¹ of these combined N sources could be released when cells lyse. Based on the high rates of N regeneration estimated from bacterial processes and grazing associated with *Trichodesmium* spp. colonies, sufficient NH_4^+ substrates are available to support the rates of NH_4^+ uptake reported here (up to 55 pmol N colony⁻¹ h⁻¹).

In contrast to results reported by Glibert & Banahan (1988), our results also support a substantive direct release of NH_4^+ by *Trichodesmium* despite excess biosynthetic capacity via GS. In this study, NH_4^+ con-

centrations increased to almost 2.0 μM in cultures grown on medium without added N substrates. Saino (1977) also observed release of NH_4^+ from *Trichodesmium* colonies over time.

Conclusions

The previously reported low rates of combined N uptake by *Trichodesmium* (Carpenter & McCarthy 1975, Glibert & Banahan 1988) and their low $\delta^{15}\text{N}$ signature (Carpenter et al. 1997) have led to the general conclusion that N_2 fixation is the primary mode of N acquisition by these species (Carpenter et al. 1997). Our results indicate that, while N_2 fixation may meet the bulk of the cellular N demand for net growth, *Trichodesmium* spp. assemblages can concurrently utilize combined N (mainly NH_4^+ and DON) and fix N_2 at high rates. If only 10 or 15% of *Trichodesmium* cells are able to fix N_2 , the release of fixed N_2 and its subsequent uptake may be an important mechanism for transporting N among cells that are fixing N_2 and those that are not.

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