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Margaret R. Mulholland  
*Old Dominion University*, [mmulholl@odu.edu](mailto:mmulholl@odu.edu)

Peter W. Bernhardt  
*Old Dominion University*

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## The effect of growth rate, phosphorus concentration, and temperature on N<sub>2</sub> fixation, carbon fixation, and nitrogen release in continuous cultures of *Trichodesmium* IMS101

Margaret R. Mulholland<sup>1</sup> and Peter W. Bernhardt

Department of Ocean, Earth and Atmospheric Science, Old Dominion University, 4600 Elkhorn Avenue, Norfolk, Virginia 23529-0276

### Abstract

With the use of continuous culture systems, rates of dinitrogen (N<sub>2</sub>) and carbon (C) fixation and nitrogen (N)- and C-based doubling times were assessed in *Trichodesmium* IMS101 growing exponentially at steady state dilution rates of 0.10, 0.20, and 0.33 d<sup>-1</sup> (doubling times of 10, 5, and 3 d—within the range reported for natural populations). Rates of C fixation, N<sub>2</sub> fixation, and N release were examined in replicate culture systems with several techniques. Biomass-specific C uptake varied little with population doubling time, but N<sub>2</sub> fixation and N release varied markedly among treatments. Total daily gross N<sub>2</sub> fixation rates and estimated N release rates were higher in cultures with higher dilution rates. Cultures grown at lower dilution rates had higher daily C:N<sub>2</sub> fixation ratios and lower N release rates. Consistent with other studies, it was estimated that *Trichodesmium* released about 80–90% of their recently fixed N<sub>2</sub> during growth. Turnover of cellular C estimated from carbon fixation was a good estimator of population growth rates in steady state cultures, whereas turnover of cellular N estimated from gross or net N<sub>2</sub> fixation was not. Small changes in temperature (24°C vs. 28°C) did not appear to affect gross N<sub>2</sub> fixation, whereas inorganic phosphorus (1 vs. 5 μmol L<sup>-1</sup>) supply had a large effect on N<sub>2</sub> fixation. These results suggest that continuous culture systems are excellent for elucidating physiological responses of *Trichodesmium* under ecologically relevant growth conditions and provide a framework for assessing highly variable field estimates of N<sub>2</sub> and C fixation.

*Trichodesmium* spp. are nonheterocystous cyanobacteria commonly found in tropical and subtropical oligotrophic oceans where they can contribute substantially to new production through dinitrogen (N<sub>2</sub>) fixation. Because these waters are generally nitrogen (N) deplete, *Trichodesmium* could be an important source of fixed N to ecosystems in which they occur. A number of studies have tried to quantify N<sub>2</sub> fixation by *Trichodesmium* in subtropical and tropical seas (e.g., see Capone et al. 1997; Karl et al. 1997; Orcutt et al. 2001); however, reported rates of N<sub>2</sub> fixation, carbon (C) fixation, and growth vary by orders of magnitude within and among studies (Mulholland and Capone 2000; Orcutt et al. 2001). For example, doubling times ranging from <1 to 243 d have been reported (e.g., Mulholland and Capone 2000). In general, C-based doubling times are faster than doubling times estimated from N<sub>2</sub> fixation. Similarly, the ratio of C:N<sub>2</sub> fixation ranges from 1.2 to 703 in natural populations, with the bulk of the estimates far in excess of canonical Redfield numbers (~6.6; Mague et al. 1977; McCarthy and Carpenter 1979; Orcutt et al. 2001), suggesting an imbalance in C and N metabolism or the use of unquantified N sources in nature.

The availability of combined N and other essential non-nitrogenous nutrients (e.g., phosphorus [P] and iron [Fe]), temperature, and the quality and quantity of light can all affect *Trichodesmium* growth rates and rates of N<sub>2</sub> fixation in nature. For example, sea surface temperature has been

used to define the geographic extent of this genus (Capone and Carpenter 1982) and predict ecosystem N<sub>2</sub> fixation rates (Bissett et al. 1999). Because Fe and P are also in short supply or near the limits of analytical detection in many oligotrophic gyres in which *Trichodesmium* spp. are found, these elements have been invoked as growth-limiting factors for *Trichodesmium* and phytoplankton production in general (Karl et al. 1995; Sañudo-Wilhelmy et al. 2001; Mills et al. 2004).

Light can also limit N<sub>2</sub> fixation rates in natural systems. *Trichodesmium* occur throughout the upper water column and frequently amass as large surface aggregations. High rates of N<sub>2</sub> fixation have been observed in surface populations (Capone et al. 1997), whereas lower N<sub>2</sub> fixation rates are characteristic of cells collected from depth and of cells incubated at low light levels (Letelier and Karl 1998). Because photosynthesis supplies energy and C skeletons for N uptake and assimilation, the availability of light and low photosynthetic rates might limit N<sub>2</sub> fixation at depth.

Because environments in which *Trichodesmium* occur vary, characterizing growth, N<sub>2</sub> fixation and release, and C fixation over a range of ecologically relevant conditions is necessary to assess the physiological limits of new N inputs from N<sub>2</sub> fixation by this diazotroph. The physiological status of natural populations is generally unknown at the time of sampling; consequently, it is difficult to assess their potential for N<sub>2</sub> and C fixation with respect to their nutritional physiology.

Culture systems have been employed successfully to study algal physiology under a wide range of environmental conditions. *Trichodesmium* has now been isolated from three sites, one from the Kuroshio area of the North Pacific Ocean (*Trichodesmium* NIBB1067), one from the coast of North Carolina in the Atlantic Ocean (*Trichodesmium* IMS101),

<sup>1</sup> Corresponding author (mmulholl@odu.edu).

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and one from a Great Barrier Reef Lagoon (*Trichodesmium* GBRTRL101). Culture studies using these isolates have provided important information with regard to the regulation of N<sub>2</sub> fixation with respect to light (Chen et al. 1996, 1998; Fu and Bell 2003a) and N (Ohki et al. 1991; Mulholland et al. 2001; Fu and Bell 2003a) and some information with regard to the regulation of N<sub>2</sub> fixation by P (Mulholland et al. 2002; Fu and Bell 2003b) and Fe (Berman-Frank et al. 2001a; Kustka et al. 2003; Fu and Bell 2003b). However, studies performed to date have described physiological properties only in batch cultures grown under low light, moderate temperature (24–28°C), saturating P (roughly  $\geq 50 \mu\text{mol L}^{-1}$ , with the exception of Mulholland et al. [2002] and Fu and Bell [2003a]), and trace metal conditions (with the exception of Berman-Frank et al. [2001a] and Fu and Bell [2003c]). In closed batch culture systems, the nutrient environment and physiological status of cells are constantly changing, with high nutrient concentrations at the outset and no subsequent input or output of nutrients or cells from the culture vessel. This contrasts with open natural systems in which the ambient levels of limiting nutrients are continuously low and nutrient and trace element concentrations rarely approach levels used in most culture media.

Continuous culture systems are excellent for addressing physiological questions because low nutrient conditions can be maintained over time, and equilibrium between nutrient input and growth can be established in a manner similar to natural conditions. At steady state, growth rate approximates the dilution rate, and the population comprises cells in the same physiological state. To better understand the physiological limits on N<sub>2</sub> fixation in the sea, we established stable continuous culture systems for *Trichodesmium* IMS101 and examined N<sub>2</sub> fixation rates with respect to growth rate (and carbon doubling time), temperature, and dissolved inorganic P (DIP) concentrations.

## Methods

Unialgal cultures of *Trichodesmium* IMS101 were established in continuous cultures in a walk-in temperature controlled incubator with light supplied on a 12:12 light:dark (LD) cycle. Although these cultures were not without bacterial contaminants, culture biomass was dominated by *Trichodesmium* during exponential growth (Mulholland and Capone 1999). Because *Trichodesmium* require a diel light cycle to continue to fix N<sub>2</sub> (with N<sub>2</sub> fixation confined to the light period), truly continuous cultures cannot be achieved. However, the terms “continuous culture” and “chemostat” will be used throughout this paper for simplicity. Recent culture work investigating circadian periodicity in cell division and death in another N<sub>2</sub>-fixing cyanobacterium, *Anabaena flos-aquae*, suggests that turbidostats and chemostats with 12:12 LD cycles can be successfully employed to study cyanobacterial systems (Lee and Rhee 1999a,b).

**Cultures.** *Trichodesmium* IMS101 was grown on artificial seawater (modified YBCII; Chen et al. 1996) in 2-liter Pyrex culture bottles with  $1 \mu\text{mol L}^{-1}$  or  $5 \mu\text{mol L}^{-1}$  phosphate and no added N compounds. Once cultures reached exponential growth phase, they were connected to a peristaltic

pump, which supplied continuous input of fresh medium and output of culture. All tubing used in each chemostat was acid-cleaned and dried, and glassware was acid-cleaned and then combusted at 450°C for at least 6 h. In initial tests of the continuous culture system, viable *Trichodesmium* cultures were maintained at a steady biomass for as long as 60 d and could be theoretically maintained indefinitely with fresh media supplies. In continuous as well as batch cultures, *Trichodesmium* IMS101 occurred almost exclusively as free filaments.

Duplicate chemostats were run at dilution rates of 0.10, 0.20, and 0.33 d<sup>-1</sup> with medium containing  $1 \mu\text{mol L}^{-1}$  phosphate and maintained at 24°C in temperature-controlled walk-in incubators under a 12:12 LD cycle. For all experiments, fluorescent lighting (GE or Phillips F20W T12CW Cool White bulbs) was supplied at 20 to 25  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . Cultures were continuously stirred by magnetic stir bars to prevent wall growth and surface accumulation. Chlorophyll concentrations were measured every 2 or 3 d throughout the experiments. Chemostats were considered to have reached steady state when biomass measurements were consistent for at least one generation. Cultures were maintained at steady state for at least four generations prior to the initiation of experiments. During each experiment, we measured N<sub>2</sub> fixation, C fixation, NH<sub>4</sub><sup>+</sup> release and uptake, and nutrient concentrations (described in “Analyses”). Future work should include microscopy to enumerate bacterial biomass in continuous cultures and on filters collected for rate estimates.

To determine whether temperature affected rates of N<sub>2</sub> fixation, one set of continuous cultures was established at 28°C at dilution rates of 0.33 d<sup>-1</sup> and  $1 \mu\text{mol L}^{-1}$  phosphate. Similarly, to determine whether P limited N<sub>2</sub> fixation rates, two sets of continuous cultures were maintained at dilution rates of 0.33 d<sup>-1</sup> and temperatures of 28°C, one set on medium containing  $5 \mu\text{mol L}^{-1}$  phosphate and one set on medium with  $1 \mu\text{mol L}^{-1}$  phosphate. These cultures were otherwise maintained as described previously. For these experiments, rates of N<sub>2</sub> fixation measured with the acetylene reduction technique are reported (see “Analyses” for details).

**Analyses.** For each experiment, rate measurements were initiated in steady state cultures at three points during the light period; 2 h after the onset of the light period, at midday, and 3 h before the onset of the dark period. At each time point, about a third of the culture volume was removed to measure nutrients, biomass, and rate processes. Nutrient samples were filtered through 0.2- $\mu\text{m}$  filters, and the filtrate was frozen until analysis. Chlorophyll *a* (Chl *a*) samples were collected on GF/F filters and frozen for less than a week before analysis (Parsons et al. 1984).

Rates of N<sub>2</sub> fixation were measured by two methods: (1) the acetylene (C<sub>2</sub>H<sub>2</sub>) reduction method (Capone 1993), which estimates gross N<sub>2</sub> fixation (Mulholland et al. 2004), and (2) the <sup>15</sup>N-labeled N<sub>2</sub> method, which estimates net N accumulation into particulate organic nitrogen (PON) (Montoya et al. 1996; Mulholland et al. 2004). Likewise, NH<sub>4</sub><sup>+</sup> regeneration was measured in two ways: (1) by adding <sup>15</sup>N<sub>2</sub> gas and quantifying the appearance of <sup>15</sup>N in the NH<sub>4</sub><sup>+</sup> pool

(Mulholland et al. 2004) and (2) by adding <sup>15</sup>NH<sub>4</sub><sup>+</sup> and measuring isotope dilution over time (Glibert et al. 1982).

For acetylene reduction assays, the production of ethylene (C<sub>2</sub>H<sub>4</sub>) from C<sub>2</sub>H<sub>2</sub> was measured by gas chromatography with a Shimadzu gas chromatograph equipped with a flame ionization detector. Assays were initiated by adding 2 ml of C<sub>2</sub>H<sub>2</sub> to the headspace of 25-ml serum vials containing 10 ml of culture. Immediately after the C<sub>2</sub>H<sub>2</sub> addition and at 1-h increments over the course of the day, 100 μl of headspace was removed, and ethylene production was measured. The theoretical ratio of 3:1 (mol C<sub>2</sub>H<sub>2</sub>:mol N<sub>2</sub> reduced) was used to convert rates of ethylene production (C<sub>2</sub>H<sub>2</sub> reduction) to N<sub>2</sub> fixation (Montoya et al. 1996). Deviations between C<sub>2</sub>H<sub>2</sub> reduction and <sup>15</sup>N<sub>2</sub> uptake-based estimates were assessed.

Rates of N<sub>2</sub> and NH<sub>4</sub><sup>+</sup> uptake were measured by tracer additions (<10%) of highly enriched (99%) <sup>15</sup>N<sub>2</sub> and <sup>15</sup>NH<sub>4</sub><sup>+</sup> (Montoya et al. 1996; Mulholland et al. 2004). For <sup>15</sup>N<sub>2</sub> uptake experiments, combusted (450°C overnight) Pyrex bottles (159 ml total volume) were filled to overflowing before being sealed with a septum cap (Teflon-lined butyl rubber). Remaining gas was removed with a syringe. A gas-tight syringe was then used to inject 160 μl of <sup>15</sup>N<sub>2</sub> (Cambridge Isotopes Laboratories) into each incubation bottle as described by Montoya et al. (1996). Sample bottles were then replaced in the incubator. Ambient N<sub>2</sub> concentrations in the culture bottles were calculated with the equations of Weiss (1970), assuming that cultures were at equilibrium with the atmosphere at the start of incubations. The resulting <sup>15</sup>N<sub>2</sub> additions were ~10%. <sup>15</sup>N<sub>2</sub> uptake rates were calculated by the equations outlined in Montoya et al. (1996) and Orcutt et al. (2001).

Production of <sup>15</sup>NH<sub>4</sub><sup>+</sup> from <sup>15</sup>N<sub>2</sub> fixation was measured in the filtrate in the <sup>15</sup>N<sub>2</sub> uptake experiments after isolating the NH<sub>4</sub><sup>+</sup> pool by solid phase extraction (Dudek et al. 1986; Mulholland et al. 2004). NH<sub>4</sub><sup>+</sup> regeneration was also measured by isotope dilution (Glibert et al. 1982) after isolating the NH<sub>4</sub><sup>+</sup> pool from the filtrate of NH<sub>4</sub><sup>+</sup> uptake experiments. Rates of NH<sub>4</sub><sup>+</sup> production from N<sub>2</sub> fixation were calculated with Eq. 1.

#### NH<sub>4</sub><sup>+</sup> production

$$= \left\{ \left[ (\text{atom \% NH}_4^+)_{\text{final}} - (\text{atom \% NH}_4^+)_{\text{initial}} \right] \div \left[ (\text{atom \% enrichment N}_2 - \text{atom \% NH}_4^+)_{\text{initial}} \times \text{time} \right] \right\} \times (\text{NH}_4^+) \quad (1)$$

As in previous studies, rates of <sup>15</sup>NH<sub>4</sub><sup>+</sup> production from <sup>15</sup>N<sub>2</sub> were calculated with N<sub>2</sub> as the source pool (Glibert and Bronk 1994). This assumes that intracellular pools of NH<sub>4</sub><sup>+</sup> are minimal and that release of these compounds occurs before their assimilation into PON. Therefore, mixing is between the enriched N<sub>2</sub> and NH<sub>4</sub><sup>+</sup> pools.

Rates of NH<sub>4</sub><sup>+</sup> regeneration by isotope dilution were estimated by Eq. 2, assuming changing isotopic signatures and NH<sub>4</sub><sup>+</sup> concentrations over the course of incubations.

#### NH<sub>4</sub><sup>+</sup> regeneration

$$= \left\{ \left[ \ln(\text{atom \% NH}_4^+)_{\text{final}} - \ln(\text{atom \% NH}_4^+)_{\text{initial}} \right] \div \left\{ \left[ (\text{atom \% NH}_4^+)_{\text{produced}} - (\text{atom \% NH}_4^+)_{\text{initial,avg}} \right] \times \text{time} \right\} \right\} \times (\text{NH}_4^+)_{\text{avg}} \quad (2)$$

The (atom % NH<sub>4</sub><sup>+</sup>)<sub>produced</sub> is the initial atom percentage of the particulate pool (assuming PON is the source of NH<sub>4</sub><sup>+</sup>), and the averages are exponential averages (Glibert et al. 1982). This is a modification of the equations outlined by Glibert et al. (1982) and Glibert and Capone (1993) but is consistent with the model of Caperon et al. (1979) and accounts for the atom percentage of all relevant pools.

Carbon fixation was measured simultaneously by adding H<sup>13</sup>CO<sub>3</sub><sup>-</sup> to the <sup>15</sup>N<sub>2</sub> or <sup>15</sup>NH<sub>4</sub><sup>+</sup> incubations (Mulholland and Capone 2001). C fixation rates were calculated essentially as described above by Eq. 3,

#### H<sup>13</sup>CO<sub>3</sub><sup>-</sup> uptake

$$= \left\{ \left[ (\text{atom \% POC})_{\text{final}} - (\text{atom \% POC})_{\text{initial}} \right] \div \left[ (\text{atom \% enrichment HCO}_3^- - \text{atom \% POC})_{\text{initial}} \times \text{time} \right] \right\} \times (\text{POC}) \quad (3)$$

where POC is particulate organic C. This is essentially the mixing model described by Montoya et al. (1996), and the initial concentration of HCO<sub>3</sub><sup>-</sup> was calculated (Parsons et al. 1984) on the basis of the salinity of the growth medium at the start of the incubations, assuming that the medium was in equilibrium with the atmosphere. The pH of the cultures at steady state was not significantly different from the pH of the medium used to dilute cultures (~8.1).

Uptake of <sup>15</sup>N<sub>2</sub> and H<sup>13</sup>CO<sub>3</sub><sup>-</sup>, production of <sup>15</sup>NH<sub>4</sub><sup>+</sup>, and uptake and isotope dilution of NH<sub>4</sub><sup>+</sup> were measured in 1–2-h incubations terminated by filtration through precombusted (450°C for 2 h) GF/F filters. Filters were dried and encapsulated in tin discs and analyzed on a Europa 20/20 mass spectrometer equipped with an ANCA-GSL preparation module. Total N release was also estimated as the difference between N<sub>2</sub> fixation estimates made using gross C<sub>2</sub>H<sub>2</sub> reduction and net <sup>15</sup>N<sub>2</sub> uptake (Mulholland et al. 2004).

Nutrient concentrations were measured with an Astoria-Pacific automated nutrient analyzer. Dissolved free amino acid (DFAA) concentrations were measured by high-performance liquid chromatography (Cowie and Hedges 1992). POC and PON concentrations and isotopes were analyzed on a Europa GSL 20/20 isotope ratio mass spectrometer.

Daily rates of C and N<sub>2</sub> fixation were calculated by multiplying the morning and afternoon hourly rates by 3 h and the midday rate by 6 h (total of 12 h light).

## Results

In all of the cultures grown on medium with 1 μmol L<sup>-1</sup> DIP, concentrations of inorganic phosphorus (P<sub>i</sub>) were near the analytical detection limit at steady state (Table 1). There was no measurable NH<sub>4</sub><sup>+</sup> in any of the culture media before being introduced into the culture vessels. However, in the steady state cultures, inorganic NH<sub>4</sub><sup>+</sup> concentrations were of-

Table 1. Average extracellular  $\text{PO}_4^{3-}$ ,  $\text{NH}_4^+$ , Chl *a*, PON, and POC concentrations and POC:PON ratios in *Trichodesmium* IMS101 growing in continuous cultures at dilution rates of 0.10, 0.20, or 0.33  $\text{d}^{-1}$ . Standard deviations from replicate cultures are in parentheses.

Dilution ( $\text{d}^{-1}$ )	$\text{PO}_4^{3-}$ ( $\mu\text{mol L}^{-1}$ )	$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	Chl <i>a</i> ( $\mu\text{g L}^{-1}$ )	PON ( $\mu\text{mol N L}^{-1}$ )	POC ( $\mu\text{mol C L}^{-1}$ )	POC:PON
0.10	0.03(0.04)	0.73(0.08)	23.1(1.2)	55.0(3.6)	273.1(6.4)	5.0
0.20	0.02(0.01)	0.12(0.13)	31.7(1.2)	63.3(6.0)	325.3(56.4)	5.1
0.33	0.04(0.01)	0.78(0.35)	28.3(3.4)	47.1(5.8)	203.1(16.9)	4.3

ten measurable but always  $<1 \mu\text{mol L}^{-1}$ . In general, Chl *a* concentrations were higher in cultures grown at higher dilution rates; however, PON and POC and the POC:PON ratios were lower (Table 1).

Extracellular N concentrations in the culture medium changed over the course of the day (Table 2). Extracellular  $\text{NH}_4^+$  concentrations were highest at the beginning of the day, and DFAA concentrations were highest later in the day. No measurements were made at night because of volume constraints. Previous results suggest that extracellular  $\text{NH}_4^+$  and DFAA concentrations vary somewhat over diel cycles and that uptake of  $\text{NH}_4^+$  and its concentration are correlated in culture systems (Mulholland and Capone 1999). DFAA pools were on the order of 100 to 500  $\text{nmol L}^{-1}$ , most of which was serine, glycine, or aspartate (data not shown).

There were sizeable intracellular  $\text{NH}_4^+$  and free amino acid pools in the cultured cells and  $>50\%$  of the intracellular amino acid pool was as glutamine (gln) and glutamate (glu), primary metabolites from  $\text{NH}_4^+$  assimilation (Table 2). Intracellular gln:glu ratios ranged from 0.19 at midday to 0.72 early in the day, values consistent with earlier culture and field studies (Mulholland and Capone 1999; Mulholland et al. 1999). Intracellular  $\text{NH}_4^+$  pools ranged from 0.28 to 0.58  $\mu\text{mol L}^{-1}$ , with higher concentrations earlier in the day.

Natural abundance estimates (SD) for PON and POC indicate an average for all cultures of  $-1.49$  (0.38) and  $-34.25$  (2.04) for  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ , respectively. Higher chlorophyll-specific gross  $\text{N}_2$  fixation rates (measured by the  $\text{C}_2\text{H}_2$  reduction technique) were observed at higher growth rates, particularly during the second half of the light period (Fig. 1). Net chlorophyll-specific  $^{15}\text{N}_2$  uptake showed a similar pattern to gross  $\text{N}_2$  fixation and was low at the beginning of the light period and reached a maximum in the middle of the day (Fig. 2). However, biomass-specific rates of  $^{15}\text{N}_2$  uptake were an order of magnitude lower than gross  $\text{N}_2$  fixation rates. Midday  $\text{N}_2$  uptake varied among cultures growing at different dilution rates.

Chlorophyll-specific C fixation rates did not vary much

among cultures growing at different dilution rates (Fig. 3), but on a volumetric basis, they were higher in cultures with higher biomass (Table 3).

Turnover times for cellular C from carbon fixation approximated the culture dilution or growth rate, suggesting that C turnover and photosynthesis were tightly coupled with growth in these P-limited cultures (Table 3). In contrast, estimated cellular N turnover from gross  $\text{N}_2$  fixation underestimated culture growth by a factor of three, and cellular N turnover from  $^{15}\text{N}_2$  uptake underestimated culture growth by a factor of 20 or more. This suggests that there were either unquantified N sources or that there was substantial and rapid recycling of N within the culture vessels. Because there were no measurable quantities of either dissolved inorganic N or dissolved organic N (DON) in the media reservoirs, we rule out the presence of unquantified N sources in these cultures.  $\text{NH}_4^+$  and DFAA were detectable in the media during culture growth, but concentrations remained low because of dilution and, possibly, simultaneous consumption. The differences in rates of gross  $\text{N}_2$  fixation measured by the acetylene reduction technique and net  $\text{N}_2$  fixation measured by  $^{15}\text{N}_2$  uptake (Table 4) support the idea that there was substantial recycling within cultures.

When we examined  $\text{NH}_4^+$  release rates and uptake of  $^{15}\text{N}_2$  into intracellular  $\text{NH}_4^+$  pools, release rates and assimilation into intracellular pools were insufficient to account for the difference between gross  $\text{N}_2$  fixation and net  $\text{N}_2$  uptake (Table 4). Measured production of extracellular  $\text{NH}_4^+$  from  $^{15}\text{N}_2$  uptake was minimal, as in previous studies, suggesting that the fairly large intracellular pools might preclude the release of  $^{15}\text{NH}_4^+$  in our short-term incubations (1–2 h). However, we also observed little accumulation of  $^{15}\text{NH}_4^+$  in intracellular pools (Table 4). In contrast, estimates of  $\text{NH}_4^+$  regeneration from isotope dilution could equal or exceed estimates of N release on the basis of the difference between gross and net  $\text{N}_2$  fixation estimates. On the basis of the difference between gross and net  $\text{N}_2$  fixation, chlorophyll-specific N release rates represented 80–90% of recently fixed  $\text{N}_2$  and

Table 2. Daily changes in extracellular  $\text{NH}_4^+$  and dissolved free amino acid (DFAA) concentrations and intracellular (IN)  $\text{NH}_4^+$  and free amino acids (INFAA), glutamine (gln) and glutamate (glu) concentrations, gln:glu ratios, and the percentage of the intracellular DFAA pool that is gln and glu from replicate cultures of *Trichodesmium* IMS101 growing in continuous cultures at dilution rates of 0.33  $\text{d}^{-1}$ . Standard deviations from replicate cultures are in parentheses.

Time (h)	$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	DFAA ( $\text{nmol L}^{-1}$ )	IN $\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	INFAA ( $\text{nmol L}^{-1}$ )	Gln ( $\text{nmol L}^{-1}$ )	Glu ( $\text{nmol L}^{-1}$ )	Gln:glu	% gln and glu
0900	0.79(0.35)	269(169)	0.58(0.04)	587(227)	146(21)	210(75)	0.72(0.16)	57–68
1200	0.04(0.01)	103(36)	0.40(0.05)	481(48)	55(2.5)	286(13)	0.19(0.004)	64–79
1500	0.06(0.05)	547(68)	0.28(0.0)	648(8)	114(5.7)	391(18)	0.29(0.03)	76–80

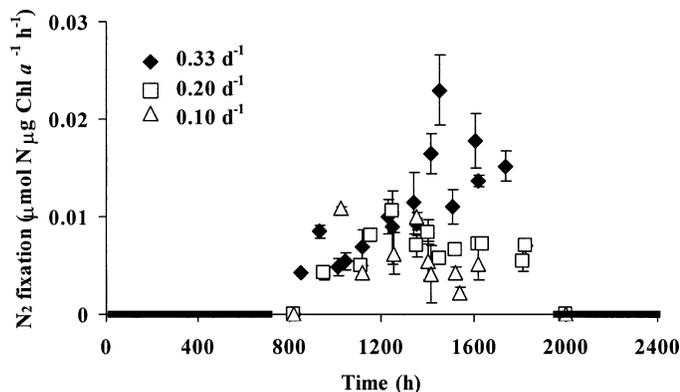


Fig. 1. Hourly gross N<sub>2</sub> fixation rates estimated by the acetylene reduction technique and a conversion factor of 3:1 (mol C<sub>2</sub>H<sub>2</sub> reduced: mol N<sub>2</sub> fixed) in steady state continuous cultures of *Trichodesmium* IMS101 growing on medium with 1 µmol L<sup>-1</sup> phosphate at dilution rates of 0.10, 0.20, and 0.33 d<sup>-1</sup> and at temperatures of 24°C. Error bars are standard deviations from replicate cultures.

increased with growth rate (Table 4). Interestingly, at our intermediate growth rate, biomass and rates of gross and net N<sub>2</sub> fixation and NH<sub>4</sub><sup>+</sup> uptake were highest, whereas rates of NH<sub>4</sub><sup>+</sup> regeneration from isotope dilution and NH<sub>4</sub><sup>+</sup> concentrations were lowest.

Although chlorophyll-specific daily N<sub>2</sub> fixation rates increased with increasing growth rates, carbon fixation rates did not change much with growth rate. Consequently, the molar ratio of C:N<sub>2</sub> fixation estimated by acetylene reduction assays decreased with increasing growth rate from about 20 to 13.4 (Table 5). Molar C:N<sub>2</sub> fixation ratios were much greater if net <sup>15</sup>N<sub>2</sub> uptake rates were used in this calculation. Calculated ratios are greater than the observed C:N ratio of cellular material (4.3–5.1; Table 1).

For comparison with cultures grown at 24°C, we measured gross N<sub>2</sub> fixation in continuous cultures growing at 28°C on medium with 1 µmol L<sup>-1</sup> DIP and dilution rates of 0.33 d<sup>-1</sup>. N<sub>2</sub> fixation rates were not significantly different in cultures growing under similar conditions but at different temperatures (Fig. 4).

We also compared N<sub>2</sub> fixation rates in replicate cultures growing on media containing either 1 or 5 µmol L<sup>-1</sup> DIP at temperatures of 28°C and dilution rates of 0.33 d<sup>-1</sup>. Rates of N<sub>2</sub> fixation were significantly higher in cultures growing at higher P concentrations (Fig. 5).

## Discussion

We have developed continuous culture systems suitable for physiological studies of *Trichodesmium* and have determined with these that biomass-specific N<sub>2</sub> fixation rates vary with growth rate and P supply, whereas biomass-specific C fixation estimates do not.

**Nutrient dynamics**—Concentrations of intracellular amino acid pools and NH<sub>4</sub><sup>+</sup> pools were comparable to those observed in a recent batch culture study (Mulholland et al. 2004). The composition of the intracellular amino acid pool was dominated by glutamate and glutamine, consistent with

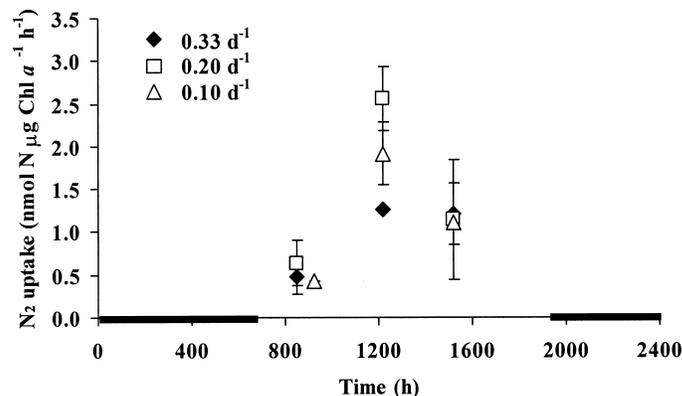


Fig. 2. Hourly net N<sub>2</sub> uptake rates measured with <sup>15</sup>N<sub>2</sub> in steady state continuous cultures of *Trichodesmium* IMS101 growing on medium with 1 µmol L<sup>-1</sup> phosphate at dilution rates of 0.10, 0.20, and 0.33 d<sup>-1</sup> and at temperatures of 24°C. Error bars are standard deviations from replicate cultures.

previous observations (Capone et al. 1994; Mulholland and Capone 1999; Mulholland et al. 1999). The dissolved free amino acid pool, on the other hand, was dominated by serine, glycine, and aspartic acid (also among the dominant constituents of the DFAA pool in nature), suggesting that direct release of intracellular amino acids was low, there was rapid uptake of any released glutamate or glutamine, or amino acids were produced in the culture medium by some other process (e.g., degradation of cellular material or microbial activity in nonaxenic cultures). Release of glutamine and glutamate has been suggested for *Trichodesmium* (Capone et al. 1994); however, uptake of these compounds by *Trichodesmium* appears to be minimal (Mulholland and Capone 1999; Mulholland et al. 1999).

**N<sub>2</sub> fixation**—Rates of N<sub>2</sub> fixation by *Trichodesmium* can vary by over two orders of magnitude within and among studies of natural populations (e.g., see Mulholland and Capone 2000; Orcutt et al. 2001) and cultures (Table 6). All three culture isolates (*Trichodesmium* IMS101, NIBB1067,

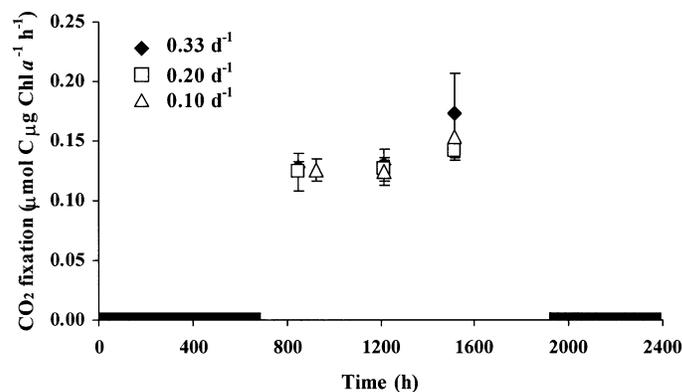


Fig. 3. Hourly C fixation rates measured with H<sup>13</sup>CO<sub>3</sub><sup>-</sup> in steady state continuous cultures of *Trichodesmium* IMS101 growing on medium with 1 µmol L<sup>-1</sup> phosphate at dilution rates of 0.10, 0.20, and 0.33 d<sup>-1</sup> and at temperatures of 24°C. Error bars are standard deviations from replicate cultures.

Table 3. Estimated daily volumetric rates of carbon fixation, N<sub>2</sub> fixation (measured by C<sub>2</sub>H<sub>2</sub> reduction with a ratio of 3:1) and <sup>15</sup>N<sub>2</sub> uptake relative to the turnover times of the particulate C and N pools. Daily rates were estimated by multiplying the measured hourly rates from the ~0900 h and ~1500 h timepoints by three and the ~1200 h hourly rate by six and then summing over the 12 h. Standard deviations from replicate cultures are in parentheses.

Dilution (d <sup>-1</sup> )	Carbon fixation (μmol C L <sup>-1</sup> d <sup>-1</sup> )	POC initial (μmol C L <sup>-1</sup> )	POC turnover (d <sup>-1</sup> )	N <sub>2</sub> fixation from C <sub>2</sub> H <sub>2</sub> (μmol N L <sup>-1</sup> d <sup>-1</sup> )	PON initial (μmol N L <sup>-1</sup> )	PON turnover from C <sub>2</sub> H <sub>2</sub> (d <sup>-1</sup> )	<sup>15</sup> N <sub>2</sub> uptake (μmol N L <sup>-1</sup> d <sup>-1</sup> )	N turnover from <sup>15</sup> N <sub>2</sub> uptake (d <sup>-1</sup> )
0.10	36.7	275(17)	0.13	1.82	55(5)	0.033	0.36	0.0066
0.20	50.5	294(59)	0.17	4.65	61(3)	0.077	0.66	0.011
0.33	48.8	165(37)	0.30	3.92	38(10)	0.103	0.35	0.0094

and GBRTRLI101) used to date have been studied in batch culture systems in low light, under which they fix N<sub>2</sub> at comparable rates (Table 6). Rates of N<sub>2</sub> fixation from these continuous culture studies are within the ranges reported for batch culture studies with *Trichodesmium* IMS101 and NIBB1067. Both of these isolates have been tentatively identified as *Trichodesmium erythraeum*. Similarly, N<sub>2</sub> fixation rates reported here are within the ranges reported from natural systems. Because *Trichodesmium* are thought to acquire the bulk of their cellular N through N<sub>2</sub> fixation in nature, the wide range of N<sub>2</sub> fixation rate estimates has led to widely varying estimates of N-specific growth rates. These continuous culture studies demonstrate that N<sub>2</sub> fixation rates vary with growth rate but that they could be poor predictors of growth (Table 3).

Variation in and limitation of N<sub>2</sub> fixation rates in natural systems have been attributed to the availability of non-nitrogenous macronutrients (e.g., P) and trace metals (e.g., Fe), light, and temperature. Steady state continuous cultures with residual P concentrations fixed N<sub>2</sub> at much higher rates than cultures that were P depleted at steady state (Fig. 5). This is consistent with the observation that batch cultures of *Trichodesmium* GBRTRLI101 fixed N<sub>2</sub> at higher rates when there was more DIP in the medium (Fu and Bell 2003a). However, P additions (1 μmol L<sup>-1</sup>) rarely stimulated short-term N<sub>2</sub> fixation rates in colonies collected from the Caribbean Sea (Mulholland unpubl. data). This is consistent with the lack of short-term responses in N<sub>2</sub> fixation rates to N additions observed in cultures and field populations (Mulholland et al. 2001; Fu and Bell 2003a). In contrast, longer term incubation experiments conducted in the Sargasso Sea suggested that N<sub>2</sub> fixation rates are limited by the availability of P and that alleviating P limitation of N<sub>2</sub> fixation alleviates

systemwide N limitation of phytoplankton growth (Mills et al. 2004).

Doubling times for *Trichodesmium* appear to be faster in tropical than subtropical seas (see Mulholland and Capone 2000). Although only one dilution rate was tested in this study, we found very little difference in biomass-specific N<sub>2</sub> fixation rates in cultures growing at 24°C and 28°C, consistent with the results of Chen et al. (1998). Further examination of variations in N<sub>2</sub> fixation under a wider range of ecologically relevant physical and chemical conditions, including temperature, is necessary for a more complete evaluation of physiological plasticity associated with N<sub>2</sub> fixation. However, these initial results suggest that continuous culture systems are useful for determining physiological constraints on N<sub>2</sub> fixation.

*Acetylene reduction and <sup>15</sup>N<sub>2</sub> uptake*—The relationship between estimates of N<sub>2</sub> fixation made on the basis of <sup>15</sup>N<sub>2</sub> uptake and C<sub>2</sub>H<sub>2</sub> reduction is dependent on a number of factors, as discussed in Mulholland et al. (2004). Ratios derived from field studies that were based on comparisons between moles of acetylene reduced (or moles of ethylene produced) and net uptake of <sup>15</sup>N<sub>2</sub> range from 0.67 to 10.4 (Mague et al. 1977; Orcutt et al. 2001 and references therein). Extensive field studies (191 paired comparisons) suggest a mean ratio of C<sub>2</sub>H<sub>2</sub> reduction to net <sup>15</sup>N<sub>2</sub> uptake of about 3.6:1 (Capone et al. pers. comm.). Similarly, Orcutt et al. (2001) reported an average ratio of about 3:1, with considerable variance around that mean, for a multiyear study at the Bermuda Atlantic Time Series (BATS) station. In earlier culture studies, N<sub>2</sub> fixation estimated by C<sub>2</sub>H<sub>2</sub> reduction and ratios of 3:1 (Mulholland and Capone 2001) or 4:1 (Mulholland

Table 4. Chl *a*-specific rates of NH<sub>4</sub><sup>+</sup> production, regeneration, and uptake in steady state continuous cultures of *Trichodesmium* IMS101 growing at dilution rates of 0.10, 0.20, and 0.33 d<sup>-1</sup>. Included are estimates of N release based on the estimated difference between gross N<sub>2</sub> fixation measured by acetylene reduction (ratio of 3:1) and net <sup>15</sup>N<sub>2</sub> uptake and the percentage of newly fixed N<sub>2</sub> released (estimated as the difference between gross and net N<sub>2</sub> fixation). Standard deviations from replicate cultures are in parentheses.

Dilution (d <sup>-1</sup> )	NH <sub>4</sub> <sup>+</sup> production from <sup>15</sup> N <sub>2</sub> (μmol N [μg Chl <i>a</i> ] <sup>-1</sup> d <sup>-1</sup> )	NH <sub>4</sub> <sup>+</sup> uptake into IN NH <sub>4</sub> <sup>+</sup> pools (μmol N [μg Chl <i>a</i> ] <sup>-1</sup> d <sup>-1</sup> )	NH <sub>4</sub> <sup>+</sup> regeneration from isotope dilution (μmol N [μg Chl <i>a</i> ] <sup>-1</sup> d <sup>-1</sup> )	NH <sub>4</sub> <sup>+</sup> uptake (μmol N [μg Chl <i>a</i> ] <sup>-1</sup> d <sup>-1</sup> )	Released N from C <sub>2</sub> H <sub>4</sub> - <sup>15</sup> N <sub>2</sub> (μmol N [μg Chl <i>a</i> ] <sup>-1</sup> d <sup>-1</sup> )	Release of fixed N <sub>2</sub> (%)
0.10	0.0020(0.0004)	0.0023(0.0009)	0.26 (0.05)	0.0063(0.004)	0.063	80
0.20	0.0016(0.0006)	0.0016(0.0003)	0.036(0.02)	0.12 (0.01)	0.077	79
0.33	0.0015(0.0007)	0.0012(0.0003)	0.121(0.03)	0.0013(0.001)	0.113	90

Table 5. Stoichiometric balance between C and N<sub>2</sub> fixation for continuous cultures growing at dilution rates of 0.10, 0.20, or 0.33 d<sup>-1</sup> with estimates of gross N<sub>2</sub> fixation (C<sub>2</sub>H<sub>2</sub> reduction assay, 3:1 ratio) and net <sup>15</sup>N<sub>2</sub> uptake on a daily basis in steady state cultures. Standard deviations from replicate cultures are in parentheses.

Dilution (d <sup>-1</sup> )	Carbon fixation (μmol C [μg Chl a] <sup>-1</sup> d <sup>-1</sup> )	N <sub>2</sub> fixation from C <sub>2</sub> H <sub>2</sub> (μmol N [μg Chl a] <sup>-1</sup> d <sup>-1</sup> )	<sup>15</sup> N <sub>2</sub> uptake (μmol N [μg Chl a] <sup>-1</sup> d <sup>-1</sup> )	C:N fixation from C <sub>2</sub> H <sub>2</sub>	C:N fixation from <sup>15</sup> N <sub>2</sub> uptake
0.10	1.58(0.098)	0.079(0.028)	0.016(0.001)	20.0	98
0.20	1.56(0.065)	0.098(0.003)	0.021(0.001)	15.9	75
0.33	1.68(0.130)	0.126(0.007)	0.013(0.003)	13.4	133

et al. 2004) more closely predicted increases in PON or PON plus released DON and NH<sub>4</sub><sup>+</sup>, respectively.

Variability in the measured ratio (mol C<sub>2</sub>H<sub>2</sub>:mol N<sub>2</sub> reduced) from the theoretical ratio could also be a result of differences in the physiological status and prehistory of *Trichodesmium* populations at the time of sampling and simultaneous N uptake or release of N during growth. It has been estimated that >50% of recently fixed N<sub>2</sub> is released as DON (Capone et al. 1994; Glibert and Bronk 1994), NH<sub>4</sub><sup>+</sup> (Mulholland et al. 2004), or both, even in short incubations (*see following discussion*). This release would result in lower observed rates of net <sup>15</sup>N<sub>2</sub> uptake relative to gross N<sub>2</sub> fixation and bias paired comparisons of <sup>15</sup>N<sub>2</sub> uptake and acetylene reduction.

We applied a ratio of 3:1 to estimate gross N<sub>2</sub> fixation from C<sub>2</sub>H<sub>2</sub> reduction in these studies because it resulted in more conservative N-based doubling times. However, this assumption results in higher estimates of release rates on the basis of the difference between gross N<sub>2</sub> fixation and <sup>15</sup>N<sub>2</sub> uptake (when the 4:1 ratio is applied, estimated release rates are ~10% lower). Observed ratios of mol C<sub>2</sub>H<sub>2</sub>:mol N<sub>2</sub> fixed ranged from 3 to 22 in this study (data not shown). On average, ratios were higher in the faster growing culture with the highest estimated N release rates. Because release rates appear to be rapid and there appears to be tight cou-

pling between <sup>15</sup>N<sub>2</sub> uptake and release, it may be impossible to derive ratios experimentally.

*N release*—On the basis of the difference between N<sub>2</sub> fixation estimates made by the acetylene reduction technique (gross N<sub>2</sub> fixation) compared with <sup>15</sup>N<sub>2</sub> uptake (net N<sub>2</sub> fixation), we determined that *Trichodesmium* release 80–90% of their recently fixed N<sub>2</sub> during growth at dilution rates ranging from 0.10 to 0.33 d<sup>-1</sup> (Table 4). Release rates appear to be higher when cells are growing faster. These rates are somewhat higher than rates of DON release observed in natural populations (Glibert and Bronk 1994) but within the range estimated during previous batch culture studies (Mulholland et al. 2004) and estimated on the basis of NH<sub>4</sub><sup>+</sup> uptake in batch cultures (Mulholland and Capone 1999, 2001; Mulholland et al. 1999). Consistent with these high release rates, NH<sub>4</sub><sup>+</sup> regeneration from isotope dilution was quite high, as in a previous study (Mulholland et al. 2004), suggesting ample recycling in these systems even though recovery of NH<sub>4</sub><sup>+</sup> from <sup>15</sup>N<sub>2</sub> was low. Uptake and regeneration of DON was not measured in these studies, which might account for the additional N release.

Although NH<sub>4</sub><sup>+</sup> was released into the culture medium during growth in the present study, uptake was generally much lower than that observed in batch culture studies (Mulhol-

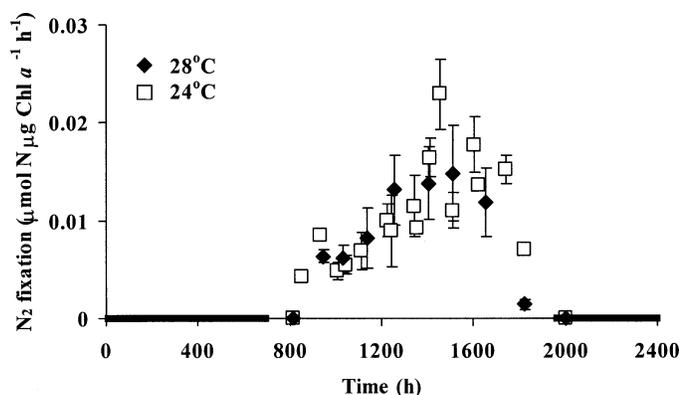


Fig. 4. Hourly gross N<sub>2</sub> fixation rates estimated by the acetylene reduction technique and a conversion factor of 3:1 (mol C<sub>2</sub>H<sub>2</sub> reduced: mol N<sub>2</sub> fixed) in steady state continuous cultures of *Trichodesmium* IMS101 growing on medium with 1 μmol L<sup>-1</sup> phosphate at dilution rates of 0.33 d<sup>-1</sup> and at temperatures of 24°C or 28°C. Error bars are standard deviations from replicate cultures.

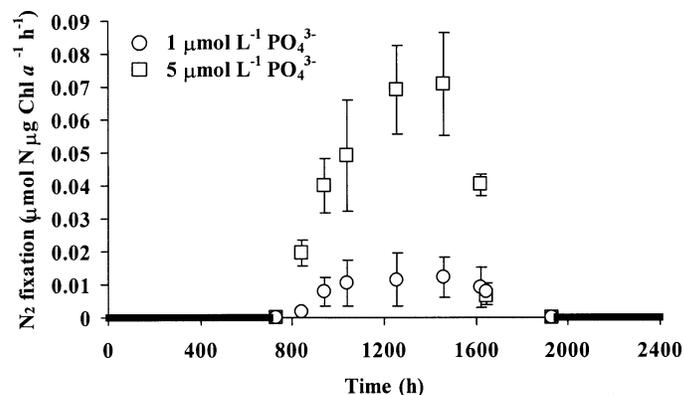


Fig. 5. Hourly gross N<sub>2</sub> fixation rates estimated by the acetylene reduction technique and a conversion factor of 3:1 (mol C<sub>2</sub>H<sub>2</sub> reduced: mol N<sub>2</sub> fixed) in steady state continuous cultures of *Trichodesmium* IMS101 growing on media containing 1 or 5 μmol L<sup>-1</sup> phosphate at dilution rates of 0.33 d<sup>-1</sup> and at temperatures of 28°C. Error bars are standard deviations from replicate cultures.

Table 6. Maximal hourly rates of N<sub>2</sub> fixation, carbon fixation, and growth rates reported for cultures of *Trichodesmium*.

<i>Trichodesmium</i> isolate	Growth conditions	N <sub>2</sub> fixation ( $\mu\text{mol N}$ [ $\mu\text{g Chl } a$ ] <sup>-1</sup> h <sup>-1</sup> )	Carbon fixation ( $\mu\text{mol C}$ [ $\mu\text{g Chl } a$ ] <sup>-1</sup> h <sup>-1</sup> )	Growth rate (d <sup>-1</sup> )	Reference
NIBB1067	Replete; batch			0.1–0.3	Ohki and Fujita 1982
NIBB1067*	Replete; exp. batch			0.07	Ohki et al. 1986
NIBB1067*	Replete; exp. batch	0.023		None reported	Ohki and Fujita 1988
	Linear batch	0.014–0.017			
	Stationary batch	0.002–0.003			
NIBB1067†	Replete; exp. batch	0.04–0.13		None reported	Ohki et al. 1992
NIBB1067	Replete; batch	0.03		0.25–0.33	Mulholland et al. 1999
	Long-term N response	0.006–0.01		0.25–0.03	
IMS101‡	SW; batch	0–0.008		~0.13	Prufert-Bebout et al. 1993
IMS101*	SW; batch	0.013	0.22	None reported	Paerl et al. 1994
	With Fe addition	0.025			
IMS101‡	Replete; batch	0.03–0.30		0.25–0.33	Chen et al. 1996
IMS101*	Replete; exp. batch 24°C and 28.5°C	Up to 0.012		None reported	Chen et al. 1998
IMS101†	Replete; exp. batch	0.022		None reported	Mulholland and Capone 1999
IMS101	Replete; exp. batch	0.015 (0.003)	0.25 (0.003)	0.12 0.2–0.25‡	Mulholland and Capone 2001
IMS101§	Replete; exp. batch	0.006		0.12	Berman-Frank et al. 2001a
	Low Fe	0.0001		0.04	
	Intermediate Fe	0.003			
IMS101‡	Replete; axenic batch; 40 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ 80 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$	0.011 0.02	Up to 1.33	None reported	Berman-Frank et al. 2001b
IMS101†	Axenic batch	0.24–0.27		0.54	Tuit et al. 2004
IMS101	Cont. cult.; P replete, 28°C	0.071 (0.016)		0.33	This study
	Cont. cult.; P deplete, 28°C	0.015 (0.005)		0.33	
	Cont. cult.; P deplete, 24°C	0.010 (0.0004)	0.15 (0.020)	0.10	
	Cont. cult.; P deplete, 24°C	0.011 (0.0003)	0.14 (0.01)	0.20	
	Cont. cult.; P deplete, 24°C	0.023 (0.004)	0.17 (0.03)	0.33	
GBRTRLI101	Replete; exp. batch	0.0083		None reported	Fu and Bell 2003a
	10 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$	0.0023			
	25–160 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$	0.0046–0.0083			
	0.1 $\mu\text{mol L}^{-1}$ DIP	0.0016			
	1.2–35.1 $\mu\text{mol L}^{-1}$ DIP	0.0051–0.0074			
	Short-term N response	0.0069			
	Long-term N response	0.0014–0.0023			
GBRTRLI101¶	Replete; exp. batch	0.0028		0.17–0.175	Fu and Bell 2003b

Cont. cult., continuous culture; SW, seawater; exp., exponential.

\* Use 3 : 1 ratio of moles C<sub>2</sub>H<sub>2</sub>:N<sub>2</sub> reduced.

† Use 2.1 pg Chl *a* cell<sup>-1</sup> (from Mulholland and Capone 2001).

‡ Chlorophyll-based turnover times.

§ Used 4.4 : 1 ratio of moles C<sub>2</sub>H<sub>2</sub>:N<sub>2</sub> reduced derived for this study; note that values in the text reported as nmol N  $\mu\text{g Chl } a^{-1} \text{ h}^{-1}$  should be units of nmol N<sub>2</sub>  $\mu\text{g Chl } a^{-1} \text{ h}^{-1}$  from rates of acetylene reduction (=ethylene production) reported in figures.

|| Used 1.55 pg Chl *a* cell<sup>-1</sup> (from Fu and Bell 2003b).

¶ Used values reported for cultures growing at salinities of 33–37.

land and Capone 1999, 2001; Mulholland et al. 1999). In previous studies, it was observed that *Trichodesmium* supplemented N acquisition from N<sub>2</sub> fixation by taking up NH<sub>4</sub><sup>+</sup> and that this could account for 70–90% of the total daily N turnover in culture systems (Mulholland and Capone 1999; Mulholland et al. 1999). It was speculated that variability in measured rates of NH<sub>4</sub><sup>+</sup> release and uptake might be because of the physiological status of cells at the time of sampling (Mulholland and Capone 2000). Because only a small fraction of *Trichodesmium* cells appears to contain nitrogenase, the enzyme complex mediating N<sub>2</sub> fixation, release, and uptake of fixed N (e.g., NH<sub>4</sub><sup>+</sup> and DON) could be

an important adaptation for extracellular distribution of N among cells and filaments (Mulholland and Capone 1999, 2000; Mulholland et al. 1999).

*C* fixation—Rates of C fixation by *Trichodesmium* also vary among field and culture studies, but there are far fewer measurements of C fixation by this genus (Table 6; Mulholland and Capone 2000; Orcutt et al. 2001). Estimates of biomass-specific C fixation by steady state continuous cultures are consistent with those measured in other culture studies that used *Trichodesmium* IMS101 (Table 6) and with results from some other field studies in the Pacific Ocean

(Mague et al. 1977), the Atlantic Ocean and Caribbean Sea (Carpenter et al. 1993; Orcutt et al. 2001), and the Gulf of Mexico (Mulholland et al. unpubl. data). As for N<sub>2</sub> fixation measurements, the range in estimated C fixation rates for this genus is large and results from some early studies (Carpenter and Price 1977; McCarthy and Carpenter 1979) are about two orders of magnitude lower than those reported in this study and the other field studies.

In steady state continuous cultures, we found that biomass-specific C fixation rates did not vary much among cultures growing at dilution rates of 0.10, 0.20, and 0.33 d<sup>-1</sup>. Volumetric rates did vary, however, so C-based estimates of cellular doubling times approximated the culture dilution rates (or the growth rates; see Table 3). This suggests that, in the field, growth rates can be better predicted from estimates of C-based doubling times. Variations in C fixation suggest that additional studies are necessary to determine how robust the relationship between C fixation and growth is under an ecologically relevant range of environmental and physiological conditions. For example, light was not examined in this study and is likely to be an important factor regulating C and N<sub>2</sub> fixation by *Trichodesmium*.

**C:N stoichiometry**—While a number of studies have examined N<sub>2</sub> fixation rates by *Trichodesmium*, few have simultaneously described C fixation under the same circumstances (e.g., for cultures, see Table 6, for natural environments, see Mulholland and Capone 2000; Orcutt et al. 2001). Recent work on *Trichodesmium* IMS101 suggests that the capacity for C and N<sub>2</sub> fixation, N uptake, and N recycling changes with physiological status over a growth cycle (Mulholland and Capone 2001; Mulholland et al. 2004), potentially explaining some of the observed natural variability in C and N<sub>2</sub> fixation and their stoichiometry.

C:N<sub>2</sub> fixation ratios often deviate from the Redfield ratio, the C:N ratio of *Trichodesmium* cellular material, or both. For example, C:N<sub>2</sub> fixation ratios ranged from 1.5 to 703 in studies in the North Atlantic Ocean and Sargasso and Caribbean Seas (Carpenter and Price 1977; McCarthy and Carpenter 1979; Orcutt et al. 2001). In simultaneous incubations examining C and N<sub>2</sub> fixation by individual colonies, ratios were seven times higher than the Redfield number, or about 46 (Orcutt et al. 2001). In the Gulf of Mexico, molar C:N<sub>2</sub> fixation ratios were between 4.75 and 17.2 by N<sub>2</sub> fixation rate estimates from acetylene reduction assays. Unlike the BATS study, molar C:N<sub>2</sub> fixation ratios were much higher if <sup>15</sup>N<sub>2</sub> uptake was used as an estimate of net N<sub>2</sub> fixation (Mulholland et al. unpubl. data). In contrast, Mague et al. (1977) estimated that molar C:N<sub>2</sub> incorporation rates in the North Pacific ranged only from 1.2 to 2.1.

In general, the high observed molar C:N<sub>2</sub> incorporation rates have led to large differences in the estimated C- and N-based doubling times such that carbon-based doubling times are usually faster than those based on N<sub>2</sub> fixation (Carpenter et al. 1993; Mulholland and Capone 2000; Orcutt et al. 2001), with some exceptions (e.g., Mague et al. 1977). On the basis of the measurements from simultaneous incubations examining C and N<sub>2</sub> fixation by *Trichodesmium*, C doubling time was estimated at 2 d, whereas N doubling times were 11 d (Orcutt et al. 2001).

In our steady state continuous cultures growing at dilution rates of 0.10, 0.20, or 0.33 d<sup>-1</sup>, molar C:N<sub>2</sub> fixation rates estimated with results from acetylene reduction assays ranged from 13.4 to 20 (Table 5), well within the reported range from field studies and in good agreement with those measured in the Gulf of Mexico. When we calculate C:N<sub>2</sub> fixation with <sup>15</sup>N<sub>2</sub> uptake as a measure of net N<sub>2</sub> fixation, ratios were much higher, ranging from 75 to 133, more consistent with the results observed at BATS (Orcutt et al. 2001). Much of the difference between molar calculations from acetylene reduction results compared with <sup>15</sup>N<sub>2</sub> uptake results can be attributed to N release (see the section “N Release” and Mulholland et al. 2004).

Some investigators have postulated that carbon ballasting might explain high C relative to N incorporation by *Trichodesmium* (Romans et al. 1994; Villareal and Carpenter 2003). Another potential reason for the observed stoichiometric imbalance between C and N<sub>2</sub> fixation might be the presence of alternative N sources. It is thought that N<sub>2</sub> fixation provides the bulk of the cellular N demand by *Trichodesmium* in natural populations. However, in exponentially growing cultures of *Trichodesmium* IMS101 and *Trichodesmium* NIBB1067 growing on medium without added N, N<sub>2</sub> fixation contributed only 23% and 14–16%, respectively, of the daily N demand, with NH<sub>4</sub><sup>+</sup> uptake accounting for the majority of the daily N turnover (Mulholland and Capone 1999; Mulholland et al. 1999). Additional N turnover from NH<sub>4</sub><sup>+</sup> regeneration and uptake within the culture vessels would not support net growth but could balance CO<sub>2</sub> fixation in excess of that necessary to support the observed C accumulation as biomass. In these steady state continuous cultures, rates of NH<sub>4</sub><sup>+</sup> uptake were comparable to N<sub>2</sub> fixation rates at our intermediate dilution rate (0.20 d<sup>-1</sup>). Including NH<sub>4</sub><sup>+</sup> uptake in our calculation of molar C:N uptake yields a ratio of 7.2, much closer to the C:N ratio of particulate material in the cultures.

Although most investigators consider N<sub>2</sub> fixation to be the primary source of N for *Trichodesmium* populations in nature, rates of incorporation of combined N sources by this diazotroph in nature are highly variable (e.g., see Mulholland and Capone 1999), with investigators finding negligible rates in some studies (Carpenter and McCarthy 1975; Glibert and Banahan 1988) and others finding high or moderate rates of N uptake by *Trichodesmium* populations (Mulholland and Capone 1999 and references therein; Orcutt et al. 2001). Significant uptake of combined N would result in underestimates of N-based doubling times on the basis of N<sub>2</sub> fixation.

Because the physiological status of natural populations is generally unknown at the time of sampling, it is difficult to assess their potential for N<sub>2</sub> fixation and the effect of new N inputs on community N dynamics. Our results suggest that continuous culture systems are excellent for physiological studies with *Trichodesmium*, and we report the first results from such studies.

Results from continuous culture studies suggest that biomass-specific N<sub>2</sub> fixation and N release rates vary with growth rate, whereas C fixation rates are more conservative and are a better predictor of population dilution (or growth) rates. Furthermore, N<sub>2</sub> fixation rates vary with the supply of limiting nutrient (in this case P). Although this is not sur-

prising, it begins to provide a framework for viewing highly variable field estimates of  $N_2$  and carbon fixation under ecologically relevant growth conditions.

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