Dinitrogen fixation and release of ammonium and dissolved organic nitrogen by *Trichodesmium* IMS101.

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ABSTRACT

Two methods used to measure dinitrogen (N_2) fixation, acetylene reduction and $^{15}N_2$ uptake, often result in different N_2 fixation rates. Part of the discrepancy may arise from the observation that *Trichodesmium* can release a fraction of their recently fixed N_2 as dissolved organic N (DON) and/or ammonium (NH_4^+). To resolve outstanding issues regarding N_2 fixation and the production of dissolved combined nitrogen (N) by *Trichodesmium*, we conducted a comprehensive analysis of N_2 fixation and the production of DON and NH_4^+ in cultures of *Trichodesmium* IMS101. We performed $^{15}N_2$ uptake experiments in parallel with acetylene (C_2H_2) reduction assays, measured production of $^{15}NH_4^+$ and DO^{15}N from $^{15}N_2$, and $^{15}NH_4^+$ uptake and regeneration by isotope dilution. Four main results are highlighted. First, $^{15}N_2$ uptake appears to provide a better approximation of net, N-specific growth rates than does N_2 fixation estimates made using C_2H_2 reduction. Second, the C_2H_2 reduction method provides a closer approximation of gross N_2 fixation. Third, simultaneous measurements of relevant N pools and pathways by several methods enabled us to rigorously evaluate deviations from theoretical conversion factors and to interpret the basis for those deviations. Our results suggest that a conversion ratio (moles C_2H_2 reduced: moles N_2 reduced to PON, ammonium and DON) of 4:1 may be more appropriate for total N_2 fixation. Fourth, the difference between estimates of gross N_2 fixation, made using the C_2H_2 reduction technique, and net $^{15}N_2$ uptake into particulate N may be a good indicator of N release from N_2 fixation.

Key words: Nitrogen fixation, nitrogen regeneration, *Trichodesmium*, ammonium uptake and regeneration

INTRODUCTION

Trichodesmium spp. fix dinitrogen (N_2), and thereby introduce new nitrogen (N) in regions where they occur. While this capability precludes N limitation of *Trichodesmium* growth and biomass accumulation, it is unclear how inputs of new N from N_2 fixation affect nutrient cycling and productivity in the oligotrophic ocean in general. It has been reported that *Trichodesmium* spp. release upwards of 50% of recently fixed N_2 as dissolved organic N (DON) (Glibert & Bronk 1994), largely, it appears, as amino acids (Capone et al. 1994). In natural systems, this recently fixed-N may provide combined N to support production by associated auto- and heterotrophs.

In culture systems, ammonium (NH_4^+) appears to be the primary recycling intermediate for recently fixed N_2 (Mulholland & Capone 2001). While N_2 fixation accounted for the net production of new biomass, release and uptake of NH_4^+ fueled additional and rapid turnover of this pool. Previous research demonstrated that both dissolved free amino acids (DFAA) and NH_4^+ accumulate in the culture medium during growth (Mulholland et al. 1999, Mulholland & Capone 2001), but only NH_4^+ is simultaneously taken up under these conditions (see also Mulholland & Capone 1999). Subsequent results from kinetic experiments that examined NH_4^+ uptake using incubation times of various lengths diverged widely; *Trichodesmium* showed a high affinity for NH_4^+ but, the longer the incubation time, the lower the apparent V_{max} (Mulholland et al. 1999 and Fig. 1). These results suggest that for NH_4^+, isotope dilution could be substantial (e.g., King and Berman 1984).

Though these earlier results indicated the potential importance of rapid NH_4^+ release and uptake by *Trichodesmium*, not all relevant pools and processes were measured, e.g., direct release of DON and NH_4^+. The objective of this study was to obtain a more comprehensive picture of the fate of recently fixed N_2. To accomplish this, another series of culture experiments were conducted in which we directly measured NH_4^+ uptake as well as the production of dissolved NH_4^+ and DON from recently fixed N_2 and NH_4^+ regeneration by isotope dilution.

While culture systems do not mimic the complexity of population interactions observed in nature, we chose them as an effective tool to better understand and complement field estimates and to identify and isolate relevant pathways affecting the cycling of nutrients under defined physiological conditions. In nature, the prior physiological history and status of freshly collected *Trichodesmium* colonies or cells is generally unknown and biomass constraints often prevent the simultaneous measurement of relevant N cycling pathways.

METHODS

Rates of N_2 fixation were measured by two methods: 1) using ^{15}N-labeled N_2, which estimates net N accumulation into particulate organic nitrogen (PON), and 2) using the acetylene (C_2H_2) reduction method, which estimates total N_2 fixation. Likewise, NH_4^+ regeneration was measured in two ways: 1) by adding ^{15}N-labeled N_2 gas and quantifying the appearance of ^{15}N in the NH_4^+ pool and 2) by adding ^{15}N-labeled NH_4^+ and measuring the degree of isotope dilution over time (Glibert et al. 1982). DON was also isolated at the end of the $^{15}N_2$ incubations so that rates of DON release could be measured

directly. These measurements were made periodically over an entire growth cycle to quantify the effect of the population's changing physiological state.

Batch cultures of *Trichodesmium* IMS101 were grown on an artificial seawater medium without added N (Chen et al. 1996). While cultures were not entirely free of contaminating bacteria, their numbers were kept low by maintaining cultures in exponential phase growth and performing transfers using sterile techniques. Cultures were grown at 27°C on a 12:12 hr L:D cycle under cool, white fluorescent lighting, supplied at between 55 and 65 µmol quanta $m^{-2}s^{-1}$ PAR. Cells were routinely mixed to prevent their adhesion to the sides of the culture vessels.

Experiments were initiated by inoculating 34 replicate culture vessels containing N-free medium with equal volumes of an exponentially growing *Trichodesmium* parent culture. *Trichodesmium* filament counts, particulate organic nitrogen (PON) and chlorophyll *a* (chl *a*) biomass were used to establish growth rates of the culture during the 18-day experiment. At each sampling point (about every 2 days), samples were preserved with Lugol's solution and the number of filaments (or trichomes) enumerated microscopically. Concentrations of PON were measured on an ANCA GSL interfaced with a Europa GEO 20/20 isotope ratio mass spectrometer (IRMS) at the end of ^{15}N experiments. Another set of samples were filtered onto pre-combusted (450°C for 2 hr) GF/F filters and frozen for chl *a* analysis (spectrophotometric determination after extraction in methanol; Mackinney 1941); the filtrates were frozen for analysis of NH_4^+ concentrations (autoanalyzer, Friederich & Whitledge 1972), dissolved free amino acids (DFAA) (high performance liquid chromatography [HPLC]; Cowie & Hedges 1992) and total dissolved nitrogen (TDN) (persulfate oxidation; Bronk et al. 2000). DON was

calculated as the difference between TDN and NH_4^+. Instrument error based on repeat injection was less than 10% for NH_4^+ and DFAA analyses. Nitrate concentrations were always undetectable in previous culture experiments using media without added N, and so it was not measured during this study. Prufert-Bebout et al. (1993) observed nitrate concentrations of about 0.5 μM in cultures, however, these were grown on seawater-based media rather than the defined medium used for this study.

At each time point, samples for intracellular pools of NH_4^+ and DFAA were also collected. For these measurements, an aliquot of culture was filtered through a 3.0 μm filter and the retained *Trichodesmium* filaments and filters were rinsed with fresh medium. The filter tower was then placed onto an acid-cleaned filter flask and 25 ml of boiling deionized and distilled water was added to the filter tower (combined heat and osmotic shock; see Thoresen et al. 1982). The resulting filtrate was collected and frozen for analysis of dissolved NH_4^+ and DFAA using the methods described above.

Over the course of the 18-day experiment, replicate cultures were sacrificed for rate measurements. All measurements were made simultaneously at mid-day because rates of N_2 fixation are restricted to the light cycle and are maximal at or near mid-day. Rates of N_2 fixation, NH_4^+ uptake, and $^{15}NH_4^+$ and DO^{15}N production from $^{15}N_2$ were measured using highly enriched (96-99%) $^{15}N_2$ and $^{15}NH_4^+$ substrates as described below (Mulholland et al. 1999, Mulholland & Capone 2001). A previous study indicated that *Trichodesmium* might have significant intracellular pools of NH_4^+ and DFAA (Mulholland et al. 1999). If this is the case, then NH_4^+ and DON production from $^{15}N_2$ might be underestimated. So, in addition, independent estimates of $^{15}NH_4^+$ uptake and

regeneration were made using the isotope dilution technique (Glibert et al. 1982). Because of culture volume constraints, isotope dilution experiments were not replicated.

Rates of N_2 and NH_4^+ uptake were measured using tracer additions (< 10%) of highly enriched (99%) $^{15}N_2$ and $^{15}NH_4^+$ (Montoya et al. 1996, Mulholland et al. 1999, Mulholland & Capone 1999 & 2001). For $^{15}N_2$ 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). A gas-tight syringe was used to inject 160 µl of $^{15}N_2$ (Cambridge Isotopes Laboratories) into each incubation bottle as described by Montoya et al. (1996). Sample bottles were then replaced in the incubator. Ambient N_2 concentrations in the culture bottles were calculated using the equations of Weiss (1970), assuming that cultures were at equilibrium with the atmosphere at the start of incubations. The resulting $^{15}N_2$ additions were about 10%. $^{15}NH_4^+$ uptake experiments were done in combusted 25 ml glass scintillation vials. Twenty ml of culture was placed in each vial and 0.03 µM $^{15}NH_4^+$ (< 10% of the ambient pool) was added to initiate incubations.

An advantage to measuring N_2 fixation using $^{15}N_2$ is that dissolved NH_4^+ and DON pools can be isolated and the production of dissolved $^{15}NH_4^+$ and $DO^{15}N$ can be measured in the sample filtrate from uptake experiments. The DON pool was isolated using ion retardation resin (Bronk & Glibert 1993, Bronk et al. 1998). The manufacturing process of the resin formally used in this isolation, BioRad AG 11 A8 (Bronk & Glibert 1991), changed in the early 1990s. As a result of the change, the resin now retains a variable amount of DON (Bronk 2000). The resin used in this study was manufactured in the Bronk lab by chemically altering another resin (Dowex anion exchange resin, BioRad

AG1-X8) to produce AG 11 A8 using the method of Hatch et al. (1957). The resin produced in the lab did not retain DON, but had an isolation efficiency comparable to the original BioRad AG 11 A8 resin as described in Bronk & Glibert (1991). The NH_4^+ pool was isolated with solid phase extraction (Dudek et al. 1986). The recovery from solid phase extraction was, on average, 35%. The low recovery is a result of the inefficiency in transferring the column eluate to a glass fiber filter prior to mass spectrometric analysis. Because the loss of sample does not result from a chemical reaction, there is no discernible isotopic fractionation. As in previous studies, rates of $^{15}NH_4^+$ and $DO^{15}N$ production from $^{15}N_2$ were calculated using N_2 as the source pool (Equation 1; Glibert & Bronk 1994). This assumes that intracellular pools of NH_4^+ and DON are minimal and that release of these compounds occurs prior to their assimilation into particulate N.

$$\text{Production of } NH_4^+ \text{ or DON} = \frac{\text{atom \% excess } NH_4^+ \text{ or DON}}{(\text{atom \% enrichment } N_2 * \text{incubation time})} \times [NH_4^+] \text{ or [DON]}, \quad (1)$$

Uptake of $^{15}N_2$ and production of $^{15}NH_4^+$ and $DO^{15}N$ were measured in 2 hr incubations that were initiated with the addition of $^{15}N_2$ gas (99% enriched) and terminated by gentle filtration through pre-combusted (450^{o}C for 2 hr) GF/F filters. NH_4^+ uptake and isotope dilution incubations were 1 hr. Both were in the linear range of uptake during time courses conducted separately (data not shown). All ^{15}N rate samples were analyzed on a Europa Geo 20/20 mass spectrophotometer as described above.

N_2 fixation rates were also estimated using the C_2H_2 reduction technique (Capone 1993). Assays were initiated by adding 1 ml of C_2H_2 to the headspace of serum vials containing 10 ml of culture. Immediately after the C_2H_2 addition and at 30 min increments over 2 hr, 500 µl of headspace was removed and the production of ethylene

was measured using a Shimadzu gas chromatograph. Ratios of 3:1 and 4:1 were used to convert rates of ethylene production (C_2H_2 reduction) to N_2 fixation (Montoya et al. 1996). Both of these ratios have been used in previous studies. While 3:1 is the theoretical ratio of mol C_2H_2 reduced per mol N_2, the 4:1 ratio is often considered more appropriate (see Capone 1988 & 1993, Montoya et al. 1996). For *Trichodesmium*, average deviations in the C_2H_2:N_2 reduction ratio range from 3:1 to 9.3:1 in 3 to 6 hour incubations of natural populations (Montoya et al. 1996). Deviations between C_2H_2 reduction and $^{15}N_2$ uptake-based estimates of N_2 fixation were assessed relative to measured rates of N release and N regeneration by isotope dilution.

Recovery of $^{15}N_2$ in particulate and dissolved pools (NH_4^+ and DON) was measured to determine whether the ratio of C_2H_2 reduction to total $^{15}N_2$ uptake could serve as a secondary measure of the release of recently fixed N_2 (see Fig. 2 for measured pools/pathways). If there were significant short-term release of recently fixed N_2, $^{15}N_2$ uptake to PON would underestimate total N_2 fixation.

RESULTS

Biomass and cellular pools. The doubling time for these cultures was about 5 days, which is similar to growth rates reported in previous studies (see Mulholland & Capone 2001 for a summary). Growth rates were estimated using three indices of biomass: chlorophyll *a*, number of filaments and PON (Fig. 3). All the indices had the same temporal pattern and cultures achieved their peak biomass at 15 days. NH_4^+ accumulated to concentrations of up to 1.5 μmol l^{-1} in the medium during the first 5 days of growth but by day 15, concentrations were comparable to those measured at the outset of the

experiment (0.6 μmol l^{-1}) (Fig. 4). DFAA concentrations remained low (< 0.2 μmol l^{-1}) during the 15 days that culture biomass increased. DON concentrations, however, increased after day 11.

Intracellular concentrations of NH_4^+ and DFAA ranged from 0.07 – 0.74 nmol filament^{-1} (= about 0.7 to 7.4 pmol cell^{-1}, based on an average of 100 cells filament^{-1}) and 0.13 to 1.5 nmol filament^{-1} (= about 1.3 to 15 pmol cell^{-1}, based on an average of 100 cells filament^{-1}), respectively (Fig. 4B). There was a declining trend in intracellular NH_4^+ concentrations over the growth period while intracellular DFAA pools were higher during early and mid-exponential growth phases.

N_2 fixation and release of recently fixed N_2. Rates of N_2 fixation estimated using the C_2H_2 reduction assay and the conventional conversion factor of 3:1, exceeded rates of net $^{15}N_2$ uptake during most of the growth cycle (Table 1, Fig. 5). During the outset of the experiment, when biomass was very low, C_2H_2 reduction estimates were slightly less than or about equal to rates of net $^{15}N_2$ uptake into particulate matter retained on the GF/F filter (and presumably in cells) at the end of the incubation. These estimates diverged later in the growth cycle with C_2H_2 reduction, based on a constant 3:1 ratio, exceeding net $^{15}N_2$ uptake by a factor of two to three.

Divergences between estimates of nitrogenase activity measured by C_2H_2 reduction and $^{15}N_2$ uptake have been previously related to the fact that the natural hydrogenase activity of nitrogenase while fixing N_2 is greatly reduced in the presence of C_2H_2, resulting in reducing equivalents being shunted to C_2H_2 reduction (Scranton 1984, Scranton et al. 1987, Postgate 1998). The divergence in rate estimates for N_2 fixation estimated using C_2H_2 reduction versus $^{15}N_2$ uptake methods might also be due to the release of recently

fixed N_2 as DON or NH_4^+. However, estimated rates of NH_4^+ and DON production from $^{15}N_2$ were also low compared with net $^{15}N_2$ uptake and C_2H_2 reduction throughout the growth cycle (Table 1) and relative to previous field estimates (Capone et al. 1994, Glibert & Bronk 1994). When rates of NH_4^+ and DON production were added to net $^{15}N_2$ uptake, to estimate total $^{15}N_2$ uptake, this value was generally still much lower than estimates of C_2H_2 reduction when assuming a 3:1 ratio throughout the experiment (Fig. 5). However, using a 4:1 ratio brings the C_2H_2 reduction and $^{15}N_2$ based estimates much closer in line with total $^{15}N_2$ fixation for days 3 through 18 (Fig. 5, Table 1).

We derived an empirical conversion ratio for each time point by comparing the C_2H_2 reduction rate directly to the net $^{15}N_2$ uptake and total $^{15}N_2$ uptake (the sum of net $^{15}N_2$ uptake and $^{15}NH_4^+$ and $DO^{15}N$ production) (Table 1). In general, estimates were relatively close to the theoretical 3:1 ratio only on day 1. In late exponential and early stationary phase, the two estimates likely diverge because of considerable release of NH_4^+ and DON.

A summation of total N accumulated in the particulate and dissolved pools was also calculated for the cultures. There was an accumulation of 162 μmol L^{-1} total N (PON, DON and NH_4^+) over the 18-day experiment based on changes in these concentrations. Rates of net $^{15}N_2$ uptake and $^{15}NH_4^+$ and $DO^{15}N$ production were integrated over the growth curve, interpolating between days where no measurements were made and assuming these rates occurred over the 12 hr light period (Fig. 6). Total $^{15}N_2$ uptake (into particulate matter and recovered in the dissolved NH_4^+ and DON pools) could account for 125 μmol L^{-1} N of this accumulation. The rate measurements likely underestimate net N accumulation, but they were within the combined error of measurements made on

replicate cultures, suggesting that $^{15}N_2$ uptake and release of recently fixed N_2 could account for N dynamics in cultures. Integrated N_2 fixation, estimated using C_2H_2 reduction with a 3:1 ratio, introduced 225 µmol N L^{-1} new N to the culture system during this experiment, whereas assuming a 4:1 ratio yielded a value of 169 µmol N L^{-1}, very close to that observed (Fig. 6).

Accumulation of PON estimated using total $^{15}N_2$ uptake closely paralleled the observed growth rates in the cultures while C_2H_2 reduction-based estimates of PON accumulation were faster than the observed growth rates during most of the experiment (after day 3).

NH_4^+ uptake, regeneration and isotope dilution. Despite the low estimates of NH_4^+ production from $^{15}N_2$, rates of NH_4^+ uptake were comparable to or higher than rates of $^{15}N_2$ uptake during exponential growth (Table 1). When corrected for isotope dilution (see Glibert et al. 1982, Glibert & Capone 1993), uptake of NH_4^+ exceeded rates of $^{15}N_2$ uptake and were often comparable to rates of N_2 fixation estimated by C_2H_2 reduction (Table 1). Unlike estimates of $^{15}NH_4^+$ release from $^{15}N_2$ uptake, rates of NH_4^+ regeneration estimated using the isotope dilution method were substantial and exceeded rates of $^{15}NH_4^+$ production from recently fixed $^{15}N_2$ by up to two orders of magnitude. Rates of NH_4^+ uptake corrected for isotope dilution were comparable to rates of regeneration of NH_4^+ from isotope dilution (Table 1), indicating rapid turnover and a tight coupling between NH_4^+ uptake and release and consistent with the observation that NH_4^+ did not accumulate in the culture medium (Fig. 4).

DISCUSSION

Trichodesmium *uptake of NH_4^+ and DON.* Early tracer studies suggested that *Trichodesmium* spp. had a relatively low capacity for uptake of combined N (Carpenter & McCarthy 1975, Glibert & Banahan 1988) and were primarily dependent upon N_2 fixation to meet their N nutritional needs. Subsequent work, however, has found a relatively high capacity for NH_4^+ assimilation in field populations and cultures (Mulholland et al. 1999; Mulholland & Capone 1999, 2000) although stable isotope (Carpenter et al. 1997) and culture studies (Mulholland & Capone 2001) still indicate that net growth is largely supported by N_2 fixation. Adding to the complexity, high rates of DON release from recently fixed N_2 have been observed in field studies (Capone et al. 1994, Glibert & Bronk 1994) and release of NH_4^+ has been inferred, but not directly measured, in culture studies (Prufert-Bebout et al. 1993, Mulholland & Capone 2001). In order to obtain a broader understanding of N dynamics and metabolism by these organisms, we examined rates of NH_4^+ and DON release from $^{15}N_2$ uptake experiments, in parallel with estimates of N_2 fixation using the C_2H_2 reduction technique, and NH_4^+ uptake and regeneration from isotope dilution in *Trichodesmium* cultures.

Like previous culture and field studies (Mulholland et al. 1999, Mulholland & Capone 1999 & 2001), we observed rates of NH_4^+ uptake that were comparable to or higher than rates of N_2 fixation estimated by C_2H_2 reduction. These high uptake rates are consistent with the stoichiometric imbalance between CO_2 fixation and N_2 fixation over a growth cycle (Mulholland & Capone 2001). Additional N turnover from NH_4^+ regeneration and uptake within the culture vessels would not support net growth but could balance CO_2 fixation in excess of that necessary to support the observed C

accumulation as biomass. Alternatively, as we have previously speculated, the release and subsequent uptake of NH_4^+ or other fixed N compounds may be a mechanism whereby fixed N is transferred between cells capable of fixing N_2 and those that are not (Mulholland & Capone 1999 & 2000), as might be required by the cyanocyte model which argues that only a subset of the cells of a trichome are induced for N_2 fixation (Berman-Frank et al. 2001).

Based on our results from $^{15}N_2$ uptake experiments, the high rates of NH_4^+ uptake observed in cultures of *Trichodesmium* cannot be supported by the measured release of recently fixed $^{15}N_2$ as $^{15}NH_4^+$. In contrast, high rates of NH_4^+ regeneration from isotope dilution suggest that in fact, release rates are substantial and that release and uptake are tightly coupled in these culture systems. The lack of NH_4^+ accumulation in the growth medium over most of the growth cycle supports this and the tight coupling precludes accurate estimates of gross NH_4^+ release based on quantifying the accumulation of ^{15}N label in the NH_4^+ pool during $^{15}N_2$ uptake studies.

Release of NH_4^+ and DON. In contrast to two field studies (Capone et al. 1994, Glibert & Bronk 1994, O'Neil et al. 1996), observed rates of $^{15}NH_4^+$ and $DO^{15}N$ production from recently fixed $^{15}N_2$ were low in the cultures. We discuss two potential reasons for these low rates: 1) the absence of grazers in the cultures and 2) the presence of large intracellular pools of NH_4^+ and DON in cultured *Trichodesmium*.

The presence of grazers and associated sloppy feeding is an important mechanism for the release of regenerated N (Bronk 2002). Rates of DON release were found to be significantly higher in the presence of grazers in California coastal waters and DON release rates were closely correlated to NH_4^+ regeneration (Ward & Bronk 2001). One

grazer, the harpacticoid copepod, *Macrosetella gracilis*, has been shown to feed on *Trichodesmium colonies* (O'Neil & Roman 1992, O'Neil 1998). These copepods do not appear to make solid fecal pellets such that most of the N they release remains in the dissolved fraction (O'Neil et al. 1996). Therefore, one likely reason for the lower rates of N release in this culture study was the absence of grazers.

Another contributing factor to the low rates of N release in the cultures may have been the presence of large intracellular pools of unlabeled NH_4^+ and DON compounds (e.g., DFAA). Initial NH_4^+ and DON release may have been isotopically light material that was present in cells prior to the $^{15}N_2$ addition. Based on rates of total N_2 uptake, it would have taken less than one to several hours to turn over the intracellular NH_4^+ pool (Table 2) and, in most cases, even longer to turn over the intracellular DFAA pool. Hence, 2 hr incubations may have been insufficient for the intermediate internal NH_4^+ to reach isotopic equilibrium with the initial $^{15}N_2$ tracer pool thereby precluding accurate estimation of $^{15}NH_4^+$ or $DO^{15}N$ release rate. Similarly, NH_4^+ regeneration based on isotope dilution can also be underestimated if the intracellular pools are emptied in less than the 1 hr incubation period because $^{15}NH_4^+$ taken up might be released. The total intracellular DON pools were not measured but, intracellular DFAA pools were often much larger than intracellular NH_4^+ pools (e.g., Table 2) and so similar problems could have resulted in underestimates of $DO^{15}N$ release from $^{15}N_2$ uptake.

While these observations were made in cultured populations, we previously measured intracellular pools of DFAA and NH_4^+ in natural populations of *Trichodesmium* (Mulholland et al. 1999). During those studies, intracellular pool concentrations were much lower (maximum of about 0.03 nmol NH_4^+ $filament^{-1}$ and 0.06

nmol DFAA filament^{-1}) than in this culture study where the medium was nutrient replete (with respect to P and trace elements). However, biomass-specific N_2 fixation rates are often lower and N-based turnover times longer in field studies (Mulholland & Capone 2000) and so these pools might still be sufficiently large to cause underestimates in N release from $^{15}N_2$ uptake experiments for the reasons discussed above.

Simultaneous release and uptake of NH_4^+ or DON on time-scales shorter than the incubation period would also bias both the measurements of $^{15}NH_4^+$ or DO^{15}N production from $^{15}N_2$ uptake and NH_4^+ regeneration by the isotope dilution method because released material would not accumulate in the growth media. Correcting uptake calculations for isotope dilution yields much higher uptake rates than those derived without this correction (Table 1). NH_4^+ uptake was often higher than total N_2 fixation, particularly in the initial growth phases. Tightly coupled release and uptake of NH_4^+ is supported by the observed NH_4^+ concentrations, the high rates of NH_4^+ regeneration from isotope dilution, and the absence of sustained NH_4^+ accumulation over the growth cycle.

Tightly coupled release and uptake was likely the case for DON as well, however, no independent measure of DON regeneration was made. For DON, there is the additional problem of identifying the relevant production pool. For example, an earlier study suggested that the primary organic compounds released by *Trichodesmium* were DFAA (Capone et al. 1994).

Comparing $^{15}N_2$ uptake with C_2H_2 reduction. One important component of the present study is the comprehensive analysis of ^{15}N products from $^{15}N_2$ uptake done in parallel with C_2H_2 reduction determinations. As has been previously suggested (Carpenter 1973, Karl et al. 2002), we submit, and provide evidence to support, that C_2H_2

reduction provides an estimate of gross N_2 fixation, as it should assay all nitrogenase activity, whereas $^{15}N_2$ uptake into particulate matter provides an estimate of net N_2 fixation. Having simultaneous determination of $^{15}NH_4^+$ and $DO^{15}N$ production, we can rigorously evaluate deviations from theoretical conversion factors and interpret the basis for those deviations.

The relationship between $^{15}N_2$ fixation and C_2H_2 reduction is dependent upon a number of factors. For one, nitrogenase-dependent H_2 release, which is inhibited by C_2H_2, results in a theoretical stoichiometric ratio of C_2H_2 reduction to $^{15}N_2$ fixation of 3:1 (mol:mol) (Postgate 1998). However, many cyanobacteria, including *Trichodesmium* (Saino & Hattori 1982, Scranton 1984, Scranton et al. 1987), have efficient uptake hydrogenases to recoup H_2 lost during natural N_2 fixation. This would drive the ratio closer to the theoretical 4:1 ratio.

Ratios of C_2H_2 reduction to net $^{15}N_2$ uptake greater than that theoretically predicted may also be indicative of substantial N release from N_2 fixation. High rates of NH_4^+ regeneration from isotope dilution in this study suggests that the release of recently fixed N_2 is more substantial than production of $^{15}NH_4^+$ from N_2 would predict (for the reasons discussed above). Therefore, divergence of the C_2H_2 reduction:net $^{15}N_2$ uptake ratio from the theoretical ratio of 3:1 in the field studies may indicate that there was release of recently fixed N_2, especially where incubations were long. Extensive field studies (191 paired comparisons) suggest a mean ratio of C_2H_2 reduction to net $^{15}N_2$ uptake of about 3.6:1 (Capone et al., submitted). This is consistent with the observations that recently fixed N_2 is released as DON (e.g., Capone et al. 1994, Glibert & Bronk 1994). In contrast, in an earlier culture study, N_2 fixation estimated using C_2H_2 reduction and a

ratio of 3:1, more closely predicted the increase in PON (Mulholland & Capone 2001). Similarly, Orcutt et al. (2001) reported an average ratio of about 3:1, with considerable variance around that mean, for a multi-year study at the Bermuda Atlantic Time Series station.

Conclusions. The current findings suggest that $^{15}N_2$ uptake approximates net N specific growth rates (Table 2; Fig. 6) while the C_2H_2 reduction technique is a good estimator of gross N_2 fixation. When considering total $^{15}N_2$ fixation (sum of PON plus released DON and NH_4^+), a conversion ratio of 4:1 is more appropriate for quantification of total N_2 fixation when measured by C_2H_2 reduction than that derived using the theoretical 3:1 conversion factor. Indeed, the difference between estimates of gross N_2 fixation, made using the C_2H_2 reduction technique and the theoretical 3:1 conversion factor, and net N_2 fixation, made using $^{15}N_2$, is a good indicator of N release from N_2 fixation.

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Table 1. Comparison of rates of N_2 fixation estimated using acetylene (C_2H_2) reduction, and conversion factors of 3 or 4, with rates of $^{15}N_2$ uptake, $^{15}NH_4^+$ and $DO^{15}N$ production, and $^{15}NH_4^+$ uptake with estimates of NH_4^+ regeneration from isotope dilution corrected (IDC). Units are nmol N l^{-1} h^{-1}. The ratios of N_2 fixation estimated by the acetylene reduction (AR) method and net or total $^{15}N_2$ uptake are also compared. The standard deviation of replicate measurements is reported in parentheses and "na" indicates that no standard deviation was available.

Day	C_2H_2 reduction 3:1 ratio	C_2H_2 reduction 4:1 ratio	Net $^{15}N_2$ uptake (A)	$^{15}NH_4^+$ production (B)	$DO^{15}N$ production (C)	Total $^{15}N_2$ uptake (A+B+C)	$^{15}NH_4^+$ uptake	IDC $^{15}NH_4^+$ uptake	IDC $^{15}NH_4^+$ regeneration	Ratio of AR to net N_2[a]	Ratio of AR to total N_2[a]
0	48.7 (17)	36.5 (13)	84 (4.6)	5.3 (1.1)	6.4 (3.1)	95.7	489 (65)			1.74	1.53
1	249 (7.4)	187 (5.6)	246 (117)	7.0 (0.8)	6.2 (1.5)	259.2	561 (336)	708 (na)	1564	3.04	2.88
3	479 (36)	359 (27)	319 (22)	25.7 (25)	10.9 (5.1)	355.6	402 (56)	457 (63)	479	4.50	4.04
5	672 (101)	504 (76)	401 (72)	21.3 (4.5)	6.9 (0.9)	429.2	925 (381)	1120 (462)	1937	5.03	4.70
9	2087 (267)	1565 (200)	875 (246)	14.0 (5.7)	13.2 (0.5)	902.2	817 (535)	2982 (1950)	3015	7.16	6.94
11	1356 (97)	1017 (73)	620 (51)	10.7 (1.2)	11.8 (6.5)	642.5	641 (566)	1642 (1450)	3335	6.56	6.33
12	1572 (298)	1179 (224)	624 (278)	9.2 (0.2)	99.7 (1.4)	732.9	399 (na)	756 (na)	2048	7.56	6.43
14	918 (13)	689 (10)	786 (335)	8.2 (0.1)	57.5 (19.4)	851.7	497 (141)	647 (184)	841	3.50	3.23
15	791 (42)	593 (32)	349 (25)	9.3 (3.3)	198 (86)	556.3	436 (114)	595 (155)	695	6.80	4.27
18	879 (213)	659 (160)	269 (237)	72.9 (na)	42.2 (na)	384.1	1315 (69)	3584 (187)	7428	9.80	6.87

[a]mol C_2H_4 formed:mol N_2 fixed

Table 2. Comparison of trichome-specific N_2 fixation rates, intracellular NH_4^+ and PON pools and turnover of intracellular pools of NH_4^+ ($IN\text{-}NH_4^+$) and PON based on N_2 fixation estimates. The standard deviations of replicate measurements are in parentheses.

Day	C_2H_2 Reduction (nmol N trichome^{-1} h^{-1}) 3:1	C_2H_2 Reduction (nmol N trichome^{-1} h^{-1}) 4:1	Total $^{15}N_2$ Uptake (nmol N trichome^{-1} h^{-1})	$IN\text{-}NH_4^+$ pool (nmol N trichome^{-1})	IN-DFAA pool (nmol N trichome^{-1})	PON pool (nmol N trichome^{-1})	Turnover of $IN\text{-}NH_4^+$ pool[1] (h)	Turnover of $IN\text{-}NH_4^+$ pool[2] (h)	Turnover of PON pool[1] (d)	Turnover of PON pool[2] (d)
0	0.117 (0.040)	0.088 (0.030)	0.229	0.48		52.2 (3.6)	4.1	2.1		
1	0.359 (0.011)	0.269 (0.008)	0.373	0.74		42.6 (14.9)	2.1	2.0	4.9	4.8
3	0.492 (0.037)	0.369 (0.028)	0.366	0.47	0.74	31.9 (4.4)	1.0	1.3	2.7	3.6
5	0.404 (0.060)	0.303 (0.045)	0.258	0.45	0.44	33.8 (1.9)	1.1	1.7	3.5	5.5
9	1.37 (0.175)	1.028 (0.131)	0.590	0.36	1.50	54.8 (11.0)	0.3	0.6	1.7	3.9
11	0.425 (0.030)	0.319 (0.023)	0.201	0.15	0.63	33.1 (6.8)	0.4	0.7	3.2	6.9
12	0.435 (0.082)	0.326 (0.062)	0.203	0.41	0.49	31.9 (9.8)	0.9	2.0	3.1	6.5
14	0.138 (0.002)	0.104 (0.002)	0.128	0.27	0.46	22.9 (6.5)	2.0	2.1	6.9	7.5
15	0.119 (0.006)	0.089 (0.005)	0.083	0.07	0.13	26.7 (8.3)	0.6	0.8	9.4	13.4
18	0.288 (0.070)		0.126	0.08	0.13	57.8 (15.5)	0.3	0.6	8.4	19.1

[1]Based on C_2H_2 reduction.

[2]Based on total $^{15}N_2$ uptake

Figure Legends:

Figure 1. Saturation kinetics for NH_4^+ uptake in *Trichodesmium* NIBB1067 grown on medium without added N substrates during 0.5 h, 1.0 h, 2.0 h, 4.0 h and 8.0 h incubations. Error bars represent the standard deviations from replicate uptake measurements.

Figure 2. Pools and pathways measured using ^{15}N tracers.

Figure 3. Accumulation of chlorophyll *a* (Chl *a*), filaments and particulate organic N (PON) in batch culture of *Trichodesmium* IMS101 growing on medium without added N sources over an 18-day growth period. Error bars represent standard deviations of from replicate cultures.

Figure 4. Accumulation of NH_4^+, DFAA and DON in the culture medium of *Trichodesmium* IMS101 growing in batch culture on medium without added N sources, over the course of an 18-day growth cycle. Replicate samples were not collected. Standard deviations from replicate injections were < 10% so not shown.

Figure 5. Rates of N_2 fixation estimated from net $^{15}N_2$ uptake (uptake of $^{15}N_2$ into PON collected on a GF/F filter at the end of the incubation); acetylene (C_2H_2) reduction (conversion factor of 3:1); and total $^{15}N_2$ uptake estimated by adding the rates of net $^{15}N_2$ uptake, DO^{15}N production and $^{15}NH_4^+$ production in batch cultures of *Trichodesmium*

IMS101 growing on medium without added combined N. Error bars indicate standard deviations from replicate cultures.

Figure 6. Total accumulation of N in culture vessels over the course of the growth cycle estimated from acetylene reduction (using conversion factors of 3 and 4), total $^{15}N_2$ uptake (estimated by adding the rates of net $^{15}N_2$ uptake, $DO^{15}N$ production and $^{15}NH_4^+$ production), and the observed accumulation of total N (estimated by adding PON plus total dissolved N (TDN)) in batch cultures of *Trichodesmium* IMS101 growing on medium without added combined N. Error bars indicate standard deviations from replicate cultures.

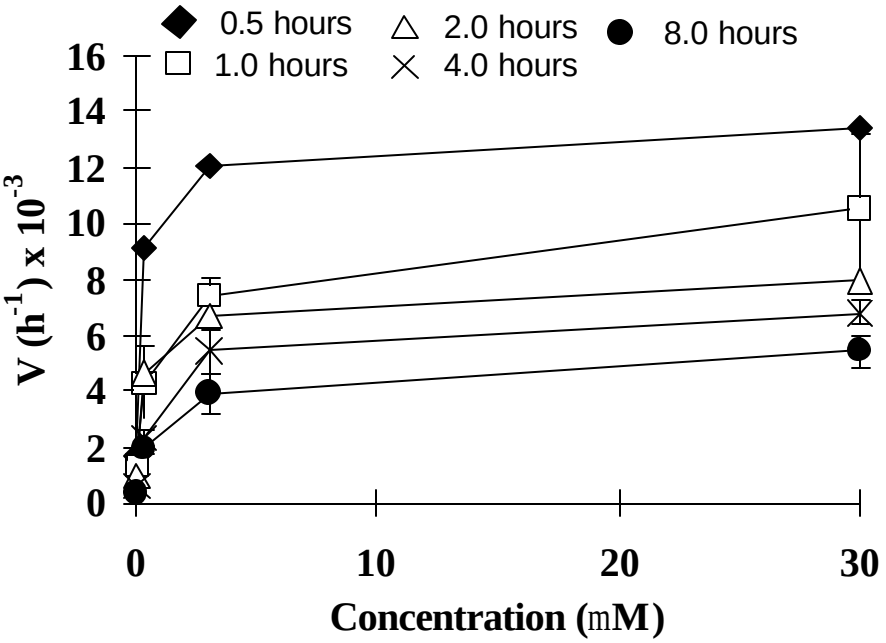
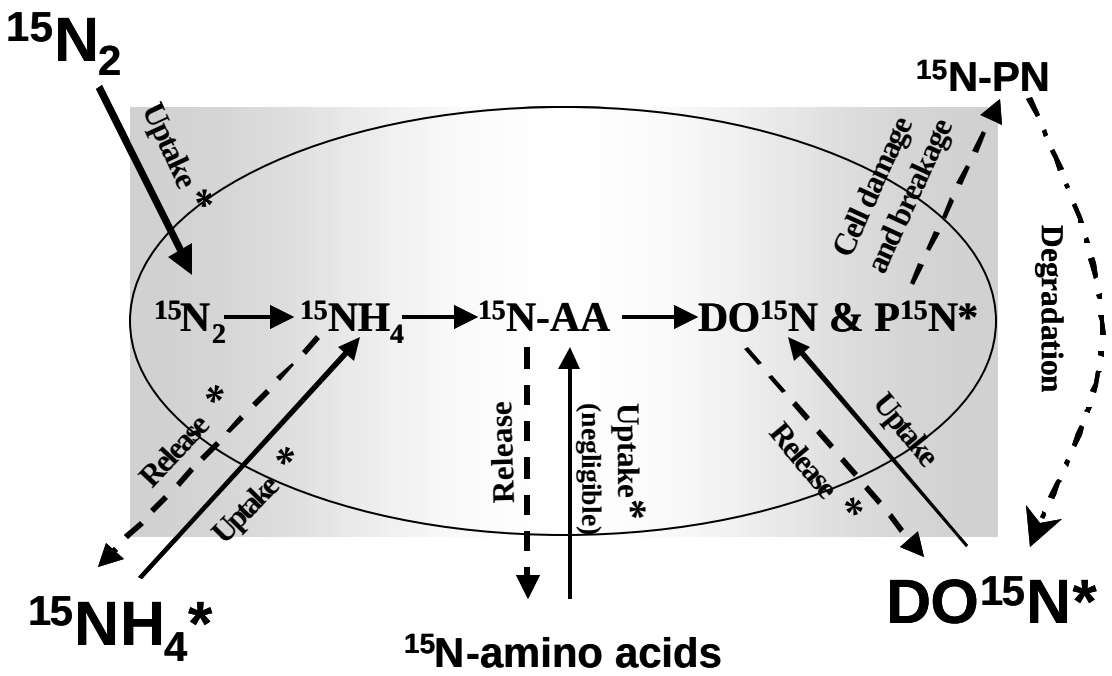


Figure 1. Mulholland et al.



*Indicates pools and pathways being measured.

Figure 2. Mulholland et al.

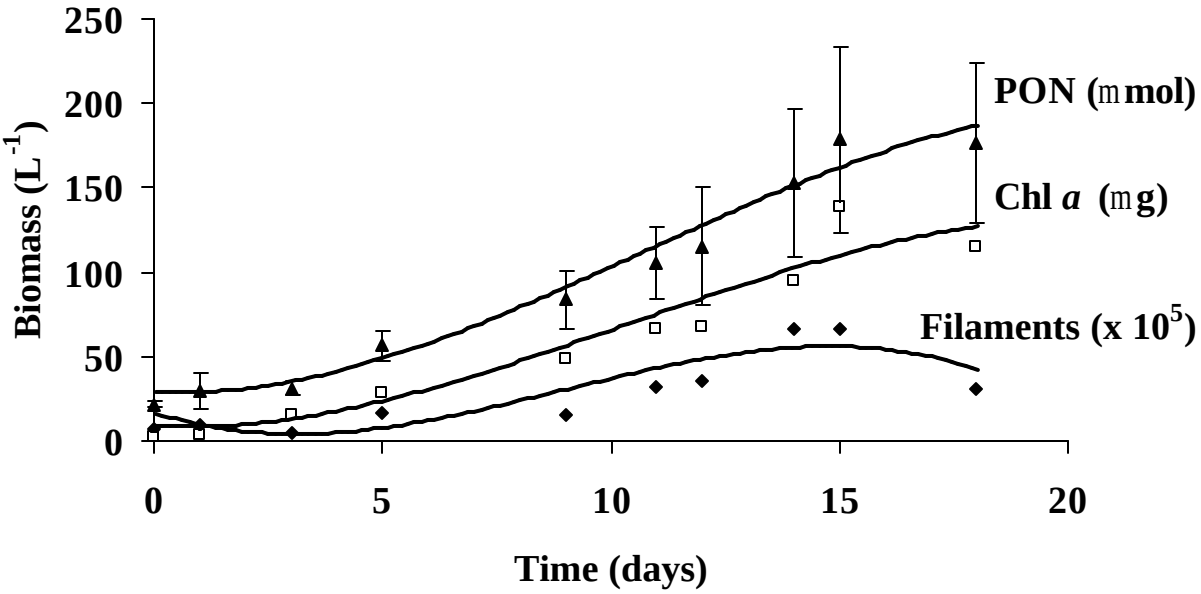


Figure 3. Mulholland et al.

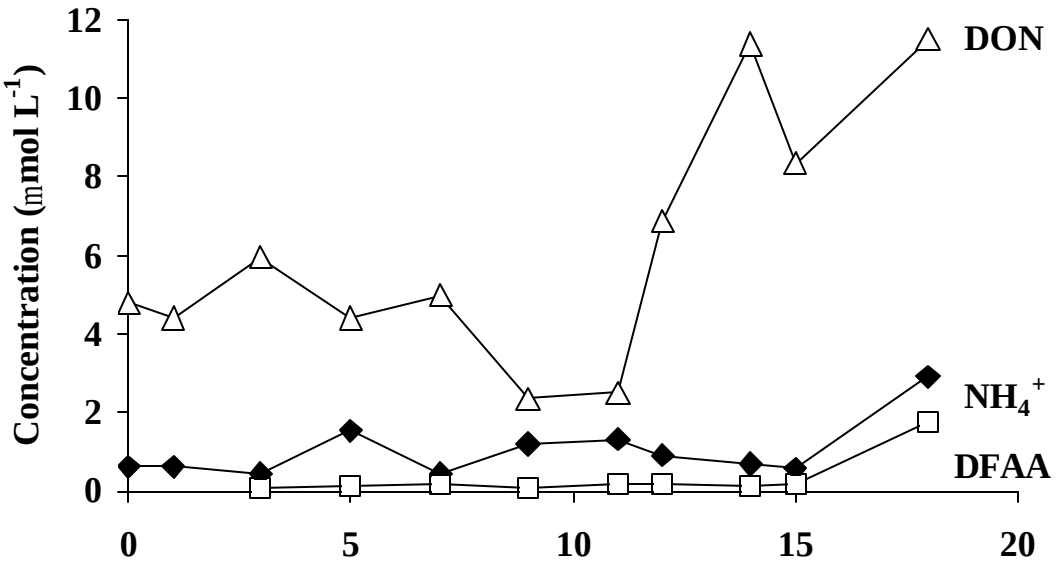


Figure 4. Mulholland et al.

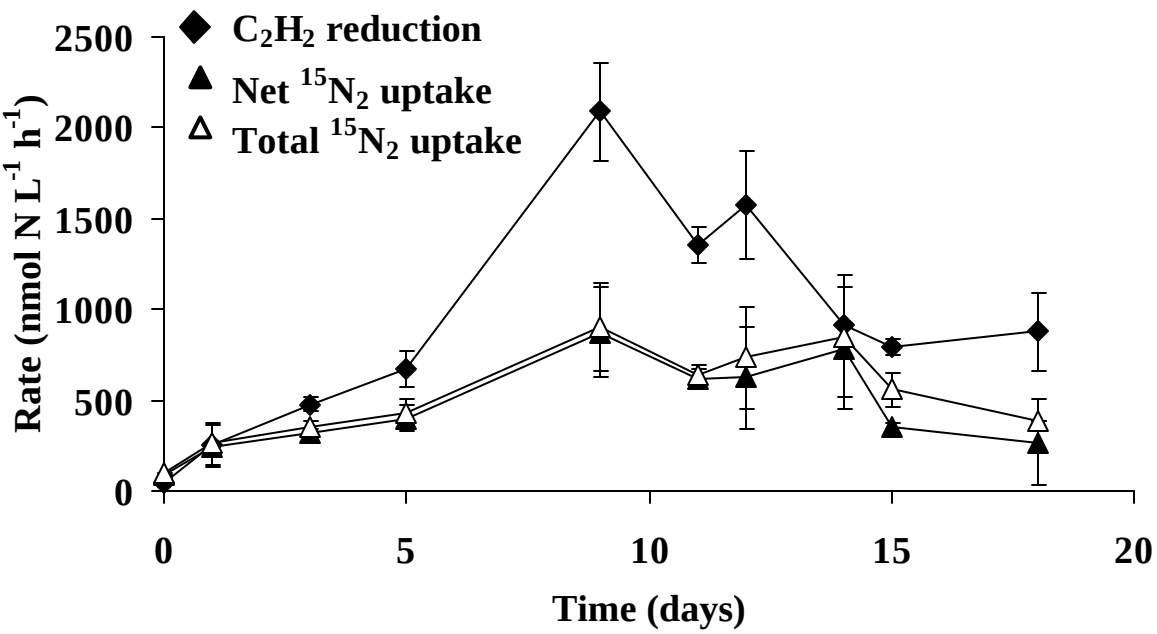


Figure 5. Mulholland et al.

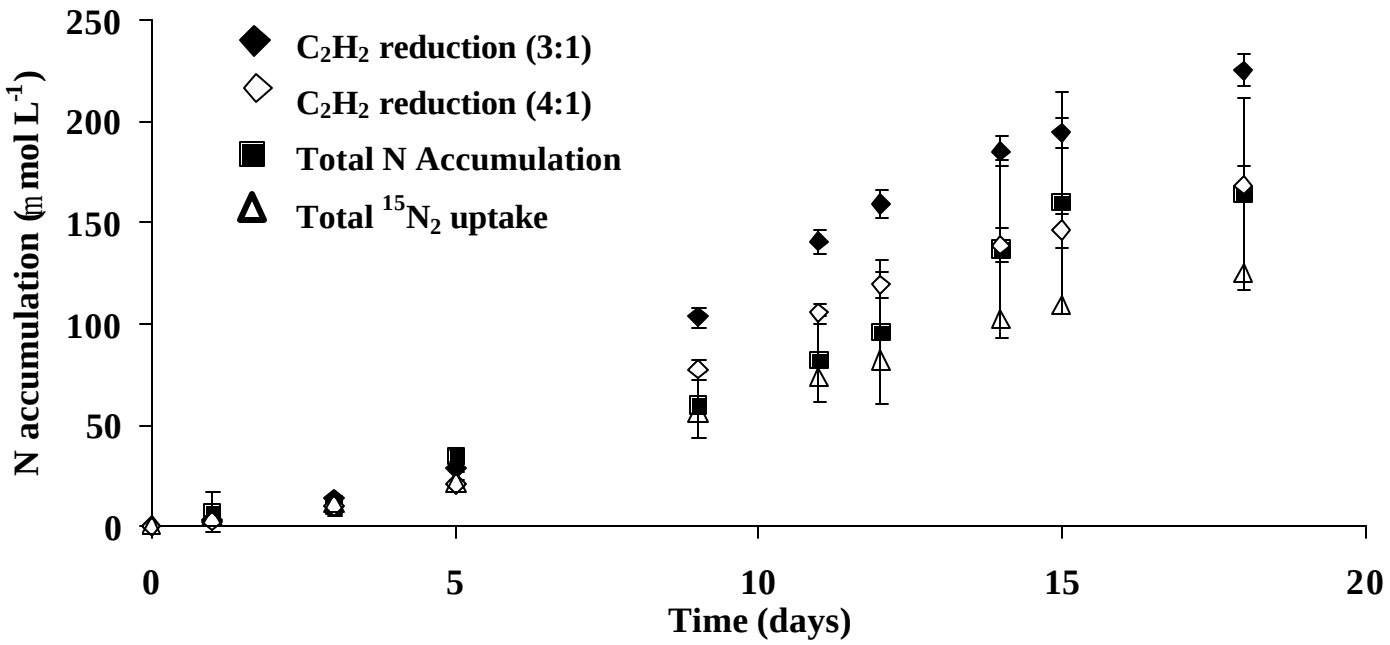


Figure 6. Mulholland et al.