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ORIGINAL ARTICLE

Modeled contributions of three types of diazotrophs to nitrogen fixation at Station ALOHA

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A diagnostic model based on biomass and growth was used to assess the relative contributions of filamentous nonheterocystous *Trichodesmium* and unicellular cyanobacteria, termed Groups A and B, to nitrogen fixation at the North Pacific Station ALOHA over a 2-year period. Average (and 95% confidence interval, CI) annual rates of modeled monthly values for *Trichodesmium*, Group B and Group A were 92 (52), 14 (4) and 12 (8) mmol N per m^2 per year, respectively. The fractional contribution to modeled instantaneous nitrogen fixation by each diazotroph fluctuated on interannual, seasonal and shorter time scales. *Trichodesmium* fixed substantially more nitrogen in year 1 (162) than year 2 (12). Group B fixed almost two times more nitrogen in year 1 (17) than year 2 (9). In contrast, Group A fixed two times more nitrogen in year 2 (16) than year 1 (8). When including uncertainties in our estimates using the bootstrap approach, the range of unicellular nitrogen fixation extended from 10% to 68% of the total annual rate of nitrogen fixation for all three diazotrophs. Furthermore, on a seasonal basis, the model demonstrated that unicellular diazotrophs fixed the majority (51%–97%) of nitrogen during winter and spring, whereas *Trichodesmium* dominated nitrogen fixation during summer and autumn (60%–96%). Sensitivity of the modeled rates to some parameters suggests that this unique attempt to quantify relative rates of nitrogen fixation by different diazotrophs may need to be reevaluated as additional information on cell size, variability in biomass and C:N, and growth characteristics of the different cyanobacterial diazotrophs become available.
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Introduction

Dinitrogen (N_2)-fixing microorganisms contribute substantially to plankton productivity and have an important impact on ocean biogeochemistry (Galloway *et al.*, 1996; Karl *et al.*, 2002). In the subtropical North Pacific, Karl *et al.* (1997) demonstrated that N_2 fixation by diazotrophs can support up to half of new production, while Dore *et al.* (2002) estimated that N_2 fixation may contribute 36%–69% of particulate nitrogen export.

The filamentous, nonheterocyst-forming cyanobacterium *Trichodesmium* is thought to be responsible for the majority of N_2 fixation, and therefore a major source of nitrogen, in the photic zone of oligotrophic marine ecosystems (Capone *et al.*, 1997, 2005; Letelier and Karl, 1998; Capone and Carpenter, 1999). These relatively large microorganisms can form colonies that are visible to the unaided eye. More recently, the potential of unicellular cyanobacteria to contribute substantially to N_2 fixation in tropical North Atlantic and the subtropical North Pacific has been attributed to their high abundances and estimated rates of N_2 fixation (Zehr *et al.*, 2001, 2007; Falcon *et al.*, 2002, 2004; Montoya *et al.*, 2004). High abundances of these single-celled microorganisms should make it easier to measure their rates of N_2 fixation, compared to measurements on the less abundant, colony-forming *Trichodesmium* cells. However, the smaller size of unicellular cyanobacteria causes difficulties in assessing their potential contribution to N_2 fixation, since they are more difficult to isolate and concentrate compared to larger diazotrophs. Size-fractionation experiments can be used to determine the contribution of

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large and small N_2-fixing microorganisms (Montoya *et al.*, 2004; Zehr *et al.*, 2007), but these experiments can be challenging to interpret.

Physiological adaptations among the various cyanobacterial diazotrophs may permit them to occupy different ecological niches in oligotrophic oceans (Church *et al.*, 2005b). Differences in biomass and growth characteristics of microorganisms that vary in size (Goebel *et al.*, in revision) may be the most important factors determining the potential contribution of different cyanobacterial diazotrophs to ecosystem N_2 fixation. More specifically, rates of N_2 fixation are likely to vary with diazotroph-specific differences in cell size and abundance, cellular carbon and nitrogen requirements, and the relationship of growth rate to light quality and quantity.

In this study, we explored the relative potential contributions of the cyanobacteria *Trichodesmium*, unicellular Group B (represented by cultivated isolates of *Crocosphaera*) and the uncultivated unicellular Group A to N_2 fixation in the subtropical North Pacific Ocean using a modeling approach. The potential contributions of these three diazotrophs to N_2 fixation were modeled as a function of field-measured cell abundances as well as measured and derived growth and biomass characteristics (Goebel *et al.*, in revision). Consistent with laboratory experiments (Staal *et al.*, 2007) and other published models of the cyanobacteria *Trichodesmium* (for example, Hood *et al.*, 2001, 2002, 2004), we set N_2 fixation to be a function of light-dependent growth for all diazotrophs modeled. In addition, N_2 fixation was assumed to follow elemental stoichiometries (C:N) of cyanobacterial biomass. This approach is a first attempt at assessing the potential contributions of different diazotrophs to N_2 fixation.

Methods

Model overview

For each cyanobacterial diazotroph, N_2 fixation rates (N; μmol N m^{-3} day^{-1}) that varied with time (t) and depth (z) were modeled as a function of volumetric biomass (B; μmol C m^{-3}) and growth (μ; day^{-1}), which was converted to units for N_2 fixation with a carbon to nitrogen ratio (r = C:N; μmol C μmol N^{-1}):

$$\mathrm{N}\,(z,t) = \mathrm{B}\,(z,t)\;\mu\,(z,t)/r \qquad (1)$$

Biomass of each diazotroph was dependent on cell concentration (c; cells m^3), average volume per cell (V; μm^3 $cell^{-1}$) and a volumetric measure of carbon content (C; fg C μm^{-3}) that was converted to molar units:

$$\mathrm{B}\,(z,t) = \mathrm{c}\,(z,t)\;\mathrm{v}\;\mathrm{C} \qquad (2)$$

Growth responses to seasonal and depth-dependent changes in irradiance (E; μmol quanta m^{-2} s^{-1}) were modeled as a function of diazotroph-specific parameters for maximum growth rate (μ_{max}; day^{-1}), half-light saturation (K_e; μmol quanta m^{-2} s^{-1}) and reduction in growth at high light or photoinhibition (β; (μmol quanta m^{-2} s^{-1})$^{-1}$), as described by the following classical Poisson function (see in MacIntyre *et al.*, 2002, and Equation 6 in Jassby and Platt, 1976, attributed to Webb *et al.*, 1974):

$$\mu\,(z,t) = \mu_{\max}\,[1 - \exp\,(-E(z,t)/K_e)]\;\exp(\beta\,E(z,t)) \qquad (3)$$

Depth changes in E were obtained from a standard light extinction expression:

$$E\,(z,t) = E_0(t)\;\exp(k_d\,z) \qquad (4)$$

In this model, variations in N_2 fixation in depth and time are driven by variations in cell concentrations, which were measured in the field, and by changes in irradiance. Monthly averaged irradiance levels, $E_0(t)$, were determined from daily measurements of incident irradiance at Station ALOHA (A Long-term Oligotrophic Habitat Assessment), 22°,45.0′N, 158°,00.0′W, from the Hawaii Ocean Time-series buoy HALE ALOHA (Hawaii Air-sea Logging Experiment) (<http://www.satlab.hawaii.edu/hots/buoydata/data.mat>). A constant light attenuation, $k_d = 0.046\ m^{-1}$, was used to approximate the annual average photic depth (105 ± s.d. 9 m) observed at Station ALOHA (Letelier *et al.*, 2004; <http://hahana.soest.hawaii.edu/hot/hot-dogs/prrseries.html>).

Our model neglects some factors that likely affect diazotroph growth and N_2 fixation, such as differences in nutrient uptake capacities and temperature-dependent metabolism, for which limited physiological information is available. However, it is driven by observations of cell abundance, which is inherently a function of abiotic factors, such as temperature and nutrients, and loss processes, such as grazing (that is, cell abundance or stock is not modeled as a prognostic variable that is a direct function of abiotic factors and loss processes). The model also includes irradiance-dependent growth both vertically and in time. N_2 fixation for *Trichodesmium* has been shown to vary with irradiance in laboratory experiments (Staal *et al.*, 2007) and this relationship has been parameterized in numerical models (Fennel *et al.*, 2002; Hood *et al.*, 2001, 2002, 2004). Fundamentally, it assumes that photosynthesis supplies the energy necessary to fix N_2 on a daily basis.

Field observations

Cell abundances of *Trichodesmium* and unicellular cyanobacterial Groups A and B were estimated from measurements of the numbers of diazotroph-specific nitrogenase *nifH* genes (*nifH* copies l^{-1}) analyzed by quantitative polymerase chain reaction (qPCR). To carry out statistical analyses, abundance measurements below detection required resolution. The detection limit of the qPCR data was, on average, 50 gene copies l^{-1}. Values below this limit of detection were given a value of one-half the detection

limit, except in instances where there was a higher efficiency of amplification, and therefore a lower number of gene copies l^{-1} could be discerned. No amplification product was observed in the no template control reactions. DNA samples for *nifH* gene abundance determination were taken approximately monthly at eight depths (5, 25, 45, 75, 100, 125, 150 and 175 m) throughout the water column over the period of 2 years (30 October 2004 to 19 October 2006) at Station ALOHA. The genome sequences of *Trichodesmium* and *Crocosphaera* (http://www.jgi.doe.gov) contain a single *nifH* gene, therefore we assumed equivalence between *nifH* genes and cellular abundances for *Trichodesmium* and Group B, respectively. We assumed that this was also the case for the uncultivated unicellular Group A cyanobacterium. Assuming that one gene copy represents one cell, maximum observed cell abundance for *Trichodesmium* in this study ($\sim 1e^5$ *nifH* copies $l^{-1} \approx 1e^5$ cells l^{-1}) exceeded maximum observed microscopic counts of *Trichodesmium* measured in the North Pacific over a 3-year period by Letelier and Karl (1996) ($\sim 1e^5$ trichomes $m^{-3} \approx 1e^4$ cells l^{-1}, assuming an average of 100 cells per trichome). This difference in observed maxima suggests that either the abundances of our data set extend beyond previous reports of maximum abundance in *Trichodesmium*, or that there could be more than one gene copy per cell for *Trichodesmium* (which may be true for all cyanobacterial diazotrophs).

Modeled rates of N_2 fixation

Rates of N_2 fixation were modeled as a function of biomass and light (Equation (1)), and were based on a monthly data set of cell abundance profiles for each diazotroph that spanned a 2-year period from 30 October 2004 to 19 October 2006 ($n = 23$). The log of carbon biomass of each diazotroph was calculated and plotted to illustrate variations of biomass of each diazotroph in time and space. The diagnostic model was applied to the entire data set to calculate discrete, temporally and spatially varying rates of N_2 fixation for each diazotroph. Modeled rates of N_2 fixation were binned by month to calculate an average annual rate of N_2 fixation for each diazotroph. Discrete rates were integrated over time and depth for each year of the study to calculate interannual differences of annual N_2 fixation rates. Interannual differences in modeled N_2 fixation rates were represented by the months October 2004 through September 2005 (year 1) and months October 2005 through September 2006 (year 2). Depth-integrated rates for each month provide a range in the percent contribution of *Trichodesmium* and unicellular diazotrophs to total N_2 fixation by these three groups over the 2-year period, and we plot average values for each month of the year for each diazotroph.

Uncertainty estimates of N_2 fixation

Ideally, uncertainty estimates should be determined directly from the distribution of annual N_2 fixation estimates over multiple years. However, estimation of the uncertainty in annual N_2 fixation rates in the present study was made difficult by the sample size of the current data set. We apply a nonparametric Monte Carlo method bootstrap analysis to the 2-year data set to gain a more robust estimate of uncertainty in the annual rate of N_2 fixation for each diazotroph.

For the application of the bootstrap with replacement method (Efron and Tibshirani, 1993), 10 000 sets of 12 profiles were sampled with replacement from the appropriate data set for each diazotroph. For each depth, a pseudorandom number determined which particular measured data value was selected for the synthetic profile, and any particular value could be drawn repeatedly. For *Trichodesmium*, two summer months (July and August 2004) exhibited a well-known seasonal increase in abundance (Mague *et al.*, 1977; Letelier *et al.*, 1996; Dore *et al.*, 2002). To enforce this known seasonality in *Trichodesmium* concentrations, July and August values for *Trichodesmium* were drawn only from the profiles measured during these summer months. Results for *Trichodesmium* with and without imposed seasonality are presented. One benefit of this bootstrap approach was that the final distributions of synthetic profiles were identical to the available data set, with no prior assumptions that the statistics follow a simple form represented by a few parameters. One drawback was that the limited data from which the synthetic profiles were drawn may not have been representative of the full range of values that could have been observed in the field.

Parametric error estimation was attempted. The small size of the data set ($n = 21$–23), however, resulted in inconclusive tests for normality at several depths, even for log-transformed data. Therefore, we discounted the results of the parametric analysis and report only the results of the nonparametric bootstrap approach.

Model parameters

Our baseline parameters are described in detail in this section and outlined in Table 1. We note that model parameterizations for biomass, growth and N_2 fixation in cyanobacteria were based on assumptions and approximations (for example, laboratory conditions) that are reported to and likely vary in natural environmental conditions. As a result, we evaluated the sensitivity of our results to parameters controlling biomass, controlling growth, and the C:N ratio. Specifically, we tested a range of values for cell volume (V), carbon content (C), maximum growth rate (μ_{max}), the half saturation constant (K_e) and photoinhibition (β), and the C:N ratio. The baseline values and the ranges tested in the sensitivity analyses for each parameter are discussed below.

Table 1 Model parameters used in for calculations of N_2 fixation for *Trichodesmium* and unicellular Groups A and B

Parameter	*Trichodesmium*	*Group B*	*Group A*
Cell size (μm)	7 *(l)* × 6.5 *(d)*[a]	5 *(d)*[a]	1 *(d)*[a]
Cell shape	Cylindrical	Spherical[a]	Spherical[a]
μ_{max} (per day)	0.51[a]	0.49[a]	0.8[b]
K_e (μmol quanta per m^2 per s)	66[a]	57[a]	60[c]
β (μmol quanta per m^2 per s)$^{-1}$	0[a]	0[a]	0[b]
C content (fg C per μm^3)	180[d]	450[d]	550[b]
C:N	6.3[e]	6.3[c] (8.7[d])	6.3[c]

[a]Laboratory measurements of Goebel *et al.* (in revision).
[b]Estimated from allometric relationships of Goebel *et al.* (in revision).
[c]Estimated as an average of values for *Trichodesmium* and *Crocosphaera*.
[d]Tuit *et al.* (2004).
[e]LaRoche and Breitbarth (2005).

Cell volume. In the baseline model, we used the cell sizes of each diazotroph reported for the experiments of Goebel *et al.* (in revision) (Table 1). The sizes of the investigated cyanobacteria, particularly for Group A, were poorly constrained; therefore we tested the sensitivity of the model to different cell sizes. Reported ranges in cell size are 5–21 μm (*d*) and 8–10 μm (*l*) for *Trichodesmium* (LaRoche and Breitbarth, 2005) and 2–8 μm (*d*) for *Crocosphaera* (J Waterbury, personal communication). Present estimates for Group A are 0.7–1.0 (*d*) μm (B Carter and J Zehr, unpublished data), although we expanded this range to 2 μm to span the gap within the size spectrum tested. We tested the sensitivities of annual rates of N_2 fixation to diazotroph-specific ranges in cell volume (μm^3) for *Trichodesmium* (200–1000), Group B (4–270) and Group A (0.2–4.0).

Carbon content. A volumetric carbon content for *Trichodesmium* of ~180 fg C μm^{-3} (Tuit *et al.*, 2004) was used as the baseline for the modeled fixation rates (Table 1). This value, however, was lower than that reported by Letelier and Karl (1996) for North Pacific Station ALOHA samples of *Trichodesmium thiebautii* and *T. erythraeum* (~250 fg C μm^{-3}) and Carpenter *et al.* (1997) for field samples of *T. thiebautii* from the tropical North Atlantic (424 fg C m^{-3}). While these higher values suggest that the chosen carbon content parameter could underestimate modeled *Trichodesmium* biomass, the chosen value from Tuit *et al.* (2004) was more representative of the species, strain and size of the representative for *Trichodesmium* considered here and, more importantly, was cultured and measured under controlled conditions. The carbon content for *Crocosphaera* (450 fg C μm^{-3}) was obtained from Tuit *et al.* (2004) and that for Group A (550 fg C μm^{-3}) was estimated from an allometric relationship by Goebel *et al.* (in revision). We evaluated changes in modeled annual rates of N_2 fixation across a range in carbon content of 150–550 fg C μm^{-3} for all diazotrophs.

Photophysiological parameters (μ_{max}, K_e, β). Growth-irradiance parameters for *T. erythraeum* strain IMS101 and *Crocosphaera watsonii* strain WH8501 measured in laboratory incubation experiments of Goebel *et al.* (in revision) were used to constrain growth characteristics, hence modeled rates of N_2 fixation, of *Trichodesmium* and Group B populations measured at Station ALOHA (Table 1). We refer specifically to *Crocosphaera* when discussing experimental results of the isolated strain, while Group B refers to field observations and modeled rates of N_2 fixation of this phylotype of cyanobacteria. Goebel *et al.* (in revision) used published allometric relationships (1) to test the consistency of growth-irradiance (and carbon content) characteristics of *Trichodesmium* and *Crocosphaera* and (2) to estimate these characteristics for Group A. While laboratory-measured parameters for *Trichodesmium* and *Crocosphaera* were consistent with the published allometric models tested (Goebel *et al.*, in revision), it is important to note that the growth-irradiance (and carbon content) parameters for the uncultivated Group A were estimates and require direct measurements upon the availability of an isolated representative culture. Therefore, until more specific, direct measurements of photophysiological properties can be made on an isolated strain of Group A, we make our best estimates of N_2 fixation using these model parameterizations, as listed in Table 1.

Growth parameters have been shown to vary widely over small ranges in cell size (for example, Nielsen, 2006). The sensitivity of annual N_2 fixation rates of each diazotroph was tested across a range in μ_{max} (0.1–0.9 day^{-1}) observed for natural plankton populations and the range of β values (0–0.0004 μmol quanta m^{-2} s^{-1}) observed in the experiments of Goebel *et al.* (in revision). Measurements of half-light saturation (K_e) in laboratory experiments yielded similar values for *Trichodesmium* and *Crocosphaera;* no estimate of this parameter yet exists for Group A. Therefore, the sensitivity of this parameter was not explored further.

These parameters, together with the irradiance, determine the diazotroph growth rate, μ. While some evidence suggests that all *Trichodesmium* cells of a trichome do not necessarily fix N_2 simultaneously (Bergman and Carpenter, 1991; Fredriksson and Bergman, 1995; Lin *et al.*, 1998), this model does not require such differentiation as μ represents an average value for all cells within a filament or colony.

C:N ratio. LaRoche and Breitbarth (2005) reported *Trichodesmium* molar C:N ratios ranged between 4.7 and 7.3, averaging 6.3. This range encompassed C:N ratio measurements of natural (5.6–7.3; Carpenter *et al.*, 2004 and 6.6; see Orcutt *et al.*, 2001) and cultivated (6.2 ± s.d. 0.7; Mulholland and Capone,

2001) *Trichodesmium* cells. Recent culture measurements by White *et al*. (2006) expanded upon this range ($\sim$6.3–8.6).

We recognize the apparent non-Redfield–stoichiometric relationship between carbon fixation and N_2 fixation (13–437 mol C mol^{-1} N) in *Trichodesmium* as demonstrated in field (Orcutt *et al*., 2001) and laboratory (Mulholland and Capone, 2001) studies. These fixation ratios were highly variable and substantially larger than molar C:N mass ratios reported throughout the literature (4.7–8.6). Excess fixation of carbon relative to that incorporated into biomass may be attributed to the multiple cell processes that require carbon, including respiration (Kana, 1993), carbohydrate ballasting/buoyancy regulation (Villareal and Carpenter, 1990; Romans *et al*., 1994) and N_2 fixation. High C:N fixation rates may also be attributed to rapid excretion of fixed N_2 (Prufert-Bebout *et al*., 1993; Glibert and Bronk, 1994; Mulholland *et al*., 2004) and/or the uptake of combined nitrogen forms (Mulholland and Capone, 1999, 2001). Our model is based on biomass and growth, and thus the biomass-based elemental C:N ratio is appropriate for this study. This ratio serves as a proxy for the amount of carbon-supported nitrogen incorporated into biomass. Laboratory studies show that *Trichodesmium* utilizes combined forms of nitrogen at high concentrations (Ohki *et al*., 1991; Mulholland and Capone, 1999, 2001), however measured uptake rates of other forms of nitrogen can vary from negligible (Carpenter and McCarthy, 1975; Glibert and Banahan, 1988) to considerable (Mulholland and Capone, 1999). For the model, we assume that nitrogen incorporated into biomass is derived entirely from N_2 fixation. This follows the model assumption of Fennel *et al*. (2002), and is supported by evidence of obligate diazotrophy in *Trichodesmium* (Wada and Hattori, 1991), as well as evidence for negligible uptake of combined forms of nitrogen based on *in situ* measurements in the North Atlantic (4.0$\pm$s.d. 7.7% of total N_2 fixed; Orcutt *et al*. 2001).

For *Crocosphaera*, Tuit *et al*. (2004) reported a range (5.90–11.37) and average C:N mass ratios that did not differ significantly during (8.6$\pm$s.d. 1.8) and outside (8.8$\pm$s.d. 1.5) periods of N_2 fixation. This agreement, as well as the agreement between measured C:N fixation ratios and elemental ratios in other cyanobacteria (Stal and Walsby, 1998), provides support for our use of a stoichiometric C:N ratio.

Since the average value reported for *Trichodesmium* (6.3) was well within the range of values recorded for *Crocosphaera*, and no estimates of C:N for the uncultivated Group A yet exist, we used a value of 6.3 for the C:N ratio for all three diazotrophs in the base model (Table 1). In addition, we report results for *Crocosphaera* using a ratio of 8.7 and evaluate the sensitivity to the reported range of values (4.7–8.6 for *Trichodesmium* and 5.9–11.4 for *Crocosphaera*). For Group A, we considered a range of C:N identical to that for *Crocosphaera*.

Results

Observed data

Figure 1 shows contours of the log of volumetric carbon biomass for each diazotroph with depth over the 2-year period as calculated using Equation (2). Using the logarithm highlights the substantial variability both in space and in time for each diazotroph. Accumulated biomass over the period of 1 year (mmol C m^{-2}) was greater in year 1 than year 2 for *Trichodesmium* (2775 versus 464) and Group B (333 versus 149), but for Group A it was larger in year 2 (190) than year 1 (69).

Modeled volumetric rates of N_2 fixation for each diazotroph are shown in Figure 2. The linear scale of this plot emphasizes individual periods of large fixation rates for each organism. Average annual modeled rates for *Trichodesmium*, Group B and Group A were 85, 14 and 13 mmol N m^{-2} year^{-1}, respectively (Table 2). When each year was examined independently, *Trichodesmium* fixed substantially more N_2 in year 1 (162) than year 2 (12). Group B fixed almost two times more N_2 in year 1 (17) than year 2 (9). In contrast, Group A fixed two times more N_2 in year 2 (16) than year 1 (8).

Depth-integrated daily rates of N_2 fixation (μmol N m^{-2} day^{-1}) were used to calculate potential ranges and averages for *Trichodesmium* (0–2110 and 212$\pm$s.d. 517), Group B (0–189 and 29$\pm$s.d. 46) and Group A (0–118 and 27$\pm$s.d. 30). Modeled rates were dominated by the unicellular diazotrophs during winter and spring (January–June), fixing an average of 74$\pm$s.d. 21% and range of 42%–99% of the total N_2 fixation by the three diazotrophs during this period (not shown). In addition, modeled rates by unicells surpassed that of *Trichodesmium* during some autumn (November 2005 and October 2006) and summer (September and July 2006) months (not shown). *Trichodesmium* dominated total N_2 fixation of the three diazotrophs during the remaining summer and autumn months, fixing an average of 56$\pm$s.d. 37% and a range of 3%–99% of the total N_2 during these months (not shown). When years are combined (Figure 3), total modeled N_2 fixation by the three diazotrophs was largest in July and August, reflecting the large *Trichodesmium* abundance during that time (Figure 3a). However, unicells fixed the majority of total modeled N_2 fixed from February to June (51%–97%), whereas *Trichodesmium* fixed the majority of N_2 from July to November (60%–96%) (Figure 3b). Seasonal opposition between *Trichodesmium* and the unicellular diazotrophs was attributed primarily to Group A rather than Group B. Group A contributed 45%–94% of the total unicellular N_2 fixation on a monthly bin-averaged (seasonal) basis (Figure 3c).

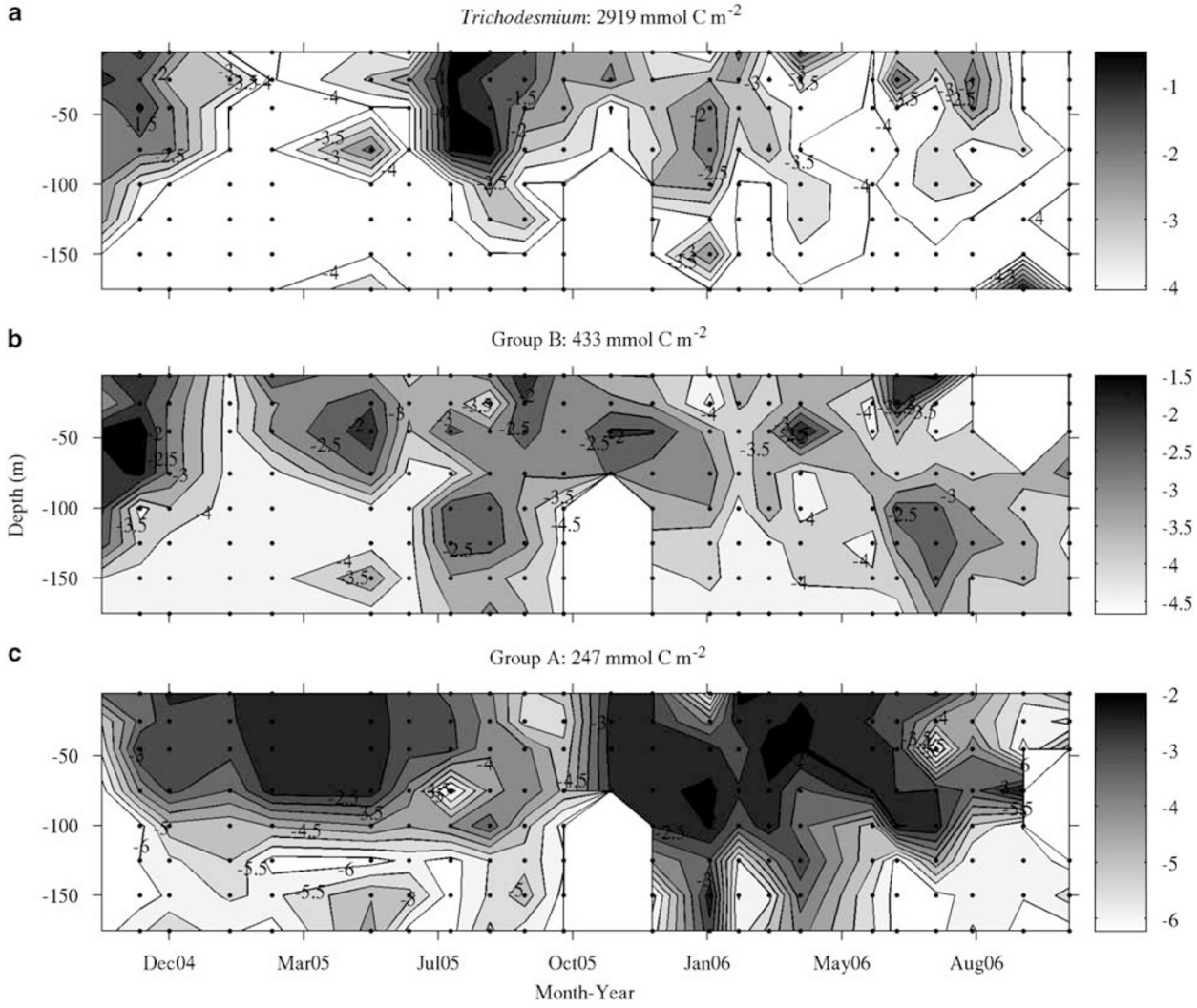

Figure 1 Log_{10}-transformed modeled carbon biomass (μmol C m^{-3}) for (**a**) *Trichodesmium*, (**b**) Group B and (**c**) Group A, based on monthly observations of cell abundance at depths 5, 25, 45, 75, 100, 125, 150 and 175 m at Station ALOHA, collected over a 2-year period ($n = 23$ months). Observed data points are posted. The interval between contour lines is 0.5 for all three plots. Accumulated, modeled carbon biomass (mmol C m^{-2}) for the observed period is displayed in the title of each plot. Note different scale bars.

Uncertainty estimates of N_2 fixation

Figure 4 shows modeled annual rates of N_2 fixation (mmol N m^{-2} year) for 10 000 synthetic data sets for each diazotroph using the nonparametric bootstrap approach, and Table 2 compares the results with those obtained using the original data set. For *Trichodesmium*, the mean (and 95% CI range) was 92 (40–150) with imposed seasonality of summertime values and 87 (32–159) without this constraint; for Group B and Group A, the results were 14 (6–23) and 12 (8–17), respectively. These results compared well with the results of the modeled rates of N_2 fixation based on the observed data. The relative contribution of each diazotroph to a total annual N_2 fixation for the three diazotrophs represented, based on the bootstrap analysis, ranged from 32% to 90% for *Trichodesmium*, 3% to 32% for Group B and 4% to 38% for Group A, and 10% to 68% for unicellulars as a whole.

Sensitivity analyses

Biomass. Figure 5 presents modeled annual N_2 fixation rates for each diazotroph as a function of cell volume and carbon content. Across the given ranges, fixation by *Trichodesmium* was an order of magnitude greater than that for Groups A and B. Though linearly dependent on cell volume, N_2 fixation rates of spherical unicellular diazotrophs increase as the cube of the cell radius, in contrast to cylindrical *Trichodesmium* cells that increase with the square of the cell radius, and therefore estimates are particularly sensitive to the cell size of unicellulars. The tested range in cell volume for Group A is fourfold, resulting in nearly two orders of magnitude variability in N_2 fixation. For Group B, cell diameters vary by nearly a factor of three (J Waterbury, personal communication), leading to a possible 30-fold variation in N_2 fixation. *Trichodesmium* diameters vary by a factor of four, but

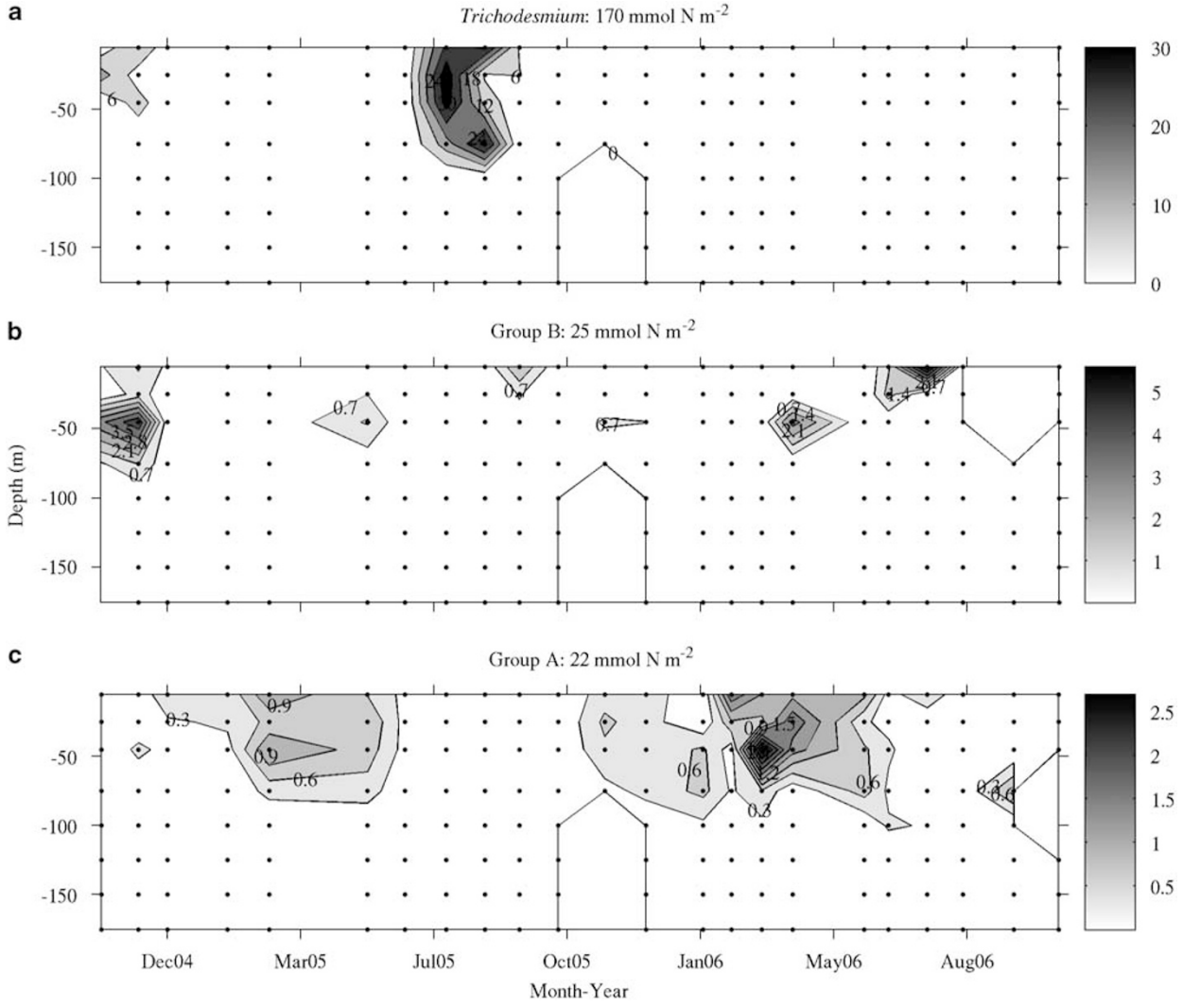

Figure 2 Modeled rates of potential N_2 fixation (μmol N m^{-3} day^{-1}) for (**a**) *Trichodesmium*, (**b**) Group B and (**c**) Group A, based on monthly observations of cell abundance at depths 5, 25, 45, 75, 100, 125, 150 and 175 m at Station ALOHA, collected over a 2-year period ($n = 23$ months). Observed data points are posted. The interval between contour lines (μmol N m^{-3} day^{-1}) is 6 for *Trichodesmium*, 0.7 for Group B and 0.3 for Group A. Total modeled rate of N_2 fixation (mmol N m^{-2}) by the three diazotrophs for the observed period is displayed in the title of each plot. Note different scale bars.

Table 2 Model output of annual rates of N_2 fixation (mmol N m^{-2} per year) of observed and nonparametric bootstrap analysis, for each diazotroph investigated

Data source	*Trichodesmium*	*Group B*	*Group A*
Observed			
Year 1	162	17	8
Year 2	12	9	16
Mean of monthly bins	85	14	13
Nonparametric			
Mean	92 (87)	14	12
95% CI	40–150 (32–159)	6–23	8–17

In the statistical analyses for *Trichodesmium* data, we show results for the analyses with and without (in parentheses) imposed seasonality (see Methods on the 'Uncertainty estimates of N_2 fixation').

reported lengths are less variable and its cylindrical shape depends upon the square of the radius, resulting in a 16-fold variation in modeled N_2 fixation rates. Modeled annual rates of N_2 fixation and 95% CI for Group A were 4.6 ± 1.8, 44.6 ± 16.0 and 92.5 ± 34.1 mmol N m^{-2} year for cell diameters of 0.7, 1.5 and 2 μm, assuming a carbon content of 550 fg C μm^{-3}. Similarly, a range in cell diameter of 3–8 μm for Group B resulted in fixation rates of 4.8 ± 2.2–57.0 ± 35.7 mmol N m^{-2} year^{-1}, for an assumed carbon content of 450 fg C μm^{-3}.

The linear relation between volumetric carbon content and N_2 fixation in our model indicated that potential N_2 fixation rates could vary 3.7-fold across the range in volumetric carbon content values tested for each diazotroph. However, best measures of this

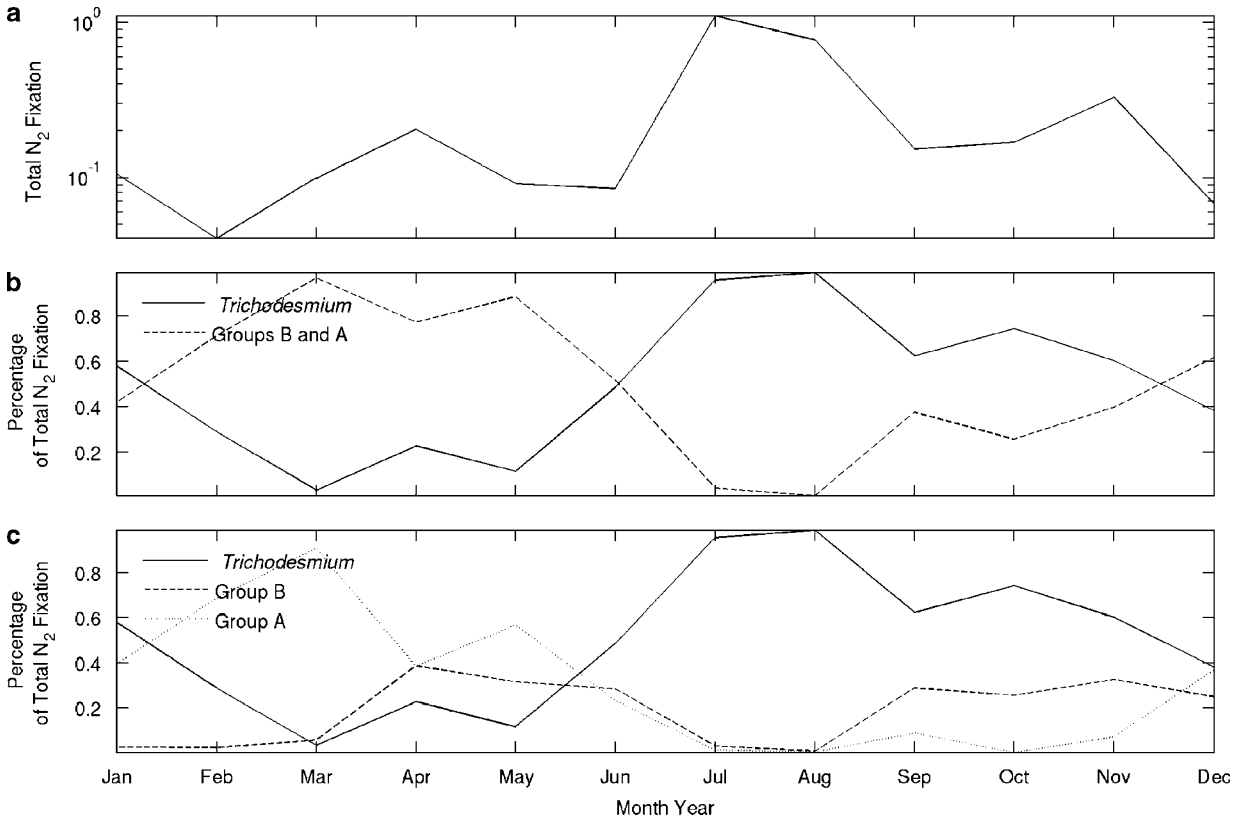

Figure 3 (**a**) Total daily, depth-integrated modeled rates of N_2 fixation (mmol N m^{-2} day^{-1}) for *Trichodesmium* and unicellular Groups A and B. The percentage contributions of *Trichodesmium* and unicellular diazotrophs (**b**) as a whole or (**c**) individually to the total daily, depth-integrated modeled rates of N_2 fixation shown in (**a**). Modeled output was bin-averaged by month at depths 5, 25, 45, 75, 100, 125, 150 and 175 m and depth-integrated over the 175 m sampled water column.

value placed *Trichodesmium* at the lower end of the range and unicellular organisms at the upper end (see dots in Figure 5). Using a value of 250 fg C μm^{-3} increased the N_2 fixation for *Trichodesmium* by $\sim$40% (average of 236 mmol N m^{-2} $year^{-1}$).

Cell abundance, like carbon content, was also linearly related to N_2 fixation rates, as formulated in the model. In this case, multiplication of the observed cell counts by two resulted in twice the modeled rates of N_2 fixation. It is worth noting that N_2 fixation rates are more sensitive to uncertainties in cell counts at the surface than at depth owing to the nonlinear light limitation with depth. Thus, changes to cell counts that are nonuniform with depth would lead to nonlinear changes in resulting fixation.

Growth. Modeled N_2 fixation for each organism was linearly dependent on maximum growth rate, μ_{max}. Reducing μ_{max} to the minimum value tested (0.1 day^{-1}) reduced Group A N_2 fixation by a factor of 8 and *Trichodesmium* and Group B by a factor of 5. Imposing the largest value of μ_{max} (0.9^{-1}) nearly doubled the fixation for Group B and *Trichodesmium* from their baseline values and had a small effect on Group A. The range in annual rates of N_2 fixation calculated across tested ranges of β and μ_{max} for Group A (1–14 mmol N m^{-2} $year^{-1}$) overlapped that for Group B (2–26 mmol N m^{-2} $year^{-1}$) but was lower than that of *Trichodesmium* (15–164 mmol N m^{-2} $year^{-1}$). Therefore a large discrepancy in these parameters was necessary for N_2 fixation rates of either unicellular diazotroph to compare to that for *Trichodesmium* in these statistically calculated rates.

Across the ranges of photoinhibition (β) tested, modeled annual rates of N_2 fixation varied by 19% for all diazotrophs. Imposing a β of 0.0004 for *Trichodesmium* resulted in an annual rate of N_2 fixation that remained to be twice that of each unicellular diazotroph (where $\beta = 0$). Although μ_{max} of the *Trichodesmium* strain BGRTRLI101 in the experiment of Bell and Fu (2005) was observed to decrease by $\sim$30% at the highest light level tested (150 μmol quanta m^{-2} s^{-1}), there was no evidence of β for *T. erythraeum* IMS101 (or *Crocosphaera*) at irradiance levels of 600 μE m^{-2} s^{-1} in the experiments of Goebel *et al*. (in revision). This was not surprising for *Trichodesmium*, which has high growth rates at high irradiances (Carpenter *et al*., 1993).

C:N ratio. Sensitivity of rates of N_2 fixation to variations in elemental C:N was linearly proportional to differences in biomass and growth among diazotrophs. At a constant biomass or growth rate, annual rates of N_2 fixation varied nearly twofold

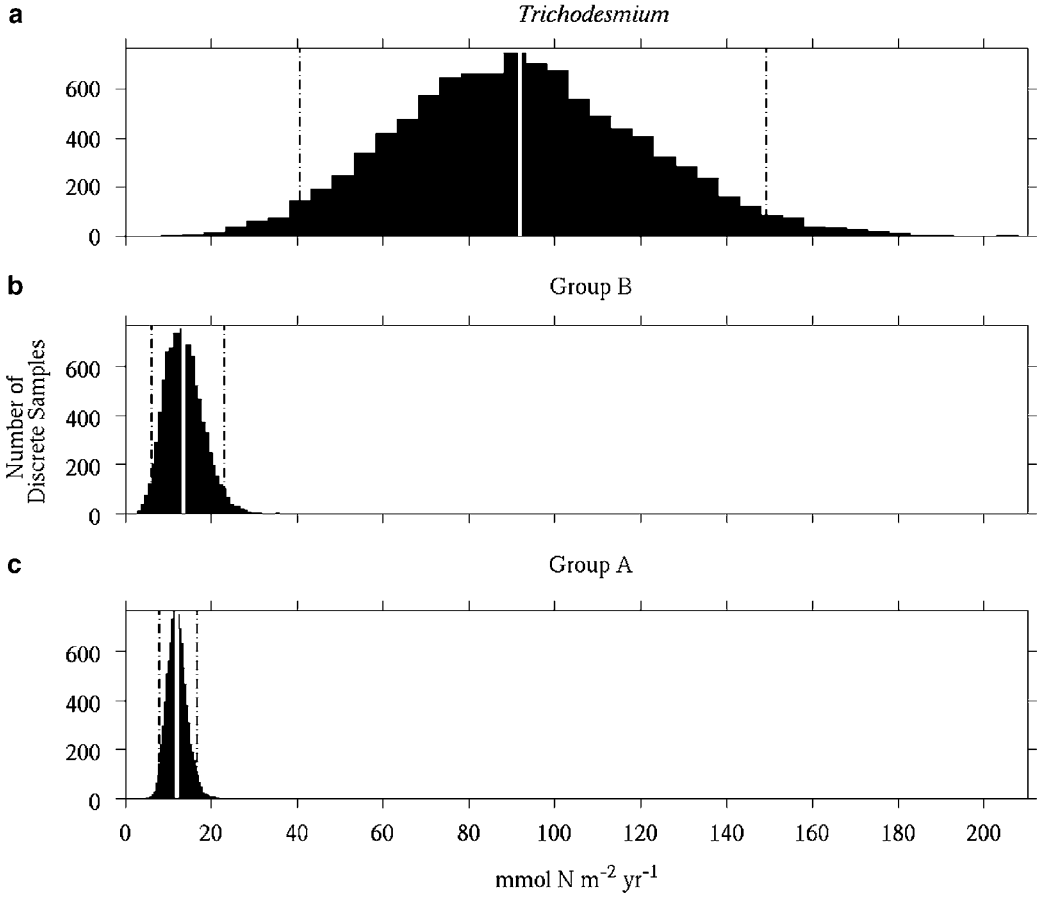

Figure 4 Mean (solid line) and 95% CI (dashed line) of modeled annual rates of N_2 fixation for (**a**) *Trichodesmium*, (**b**) Group B and (**c**) Group A cyanobacteria, modeled using a bootstrap analysis of the original, nontransformed data set.

over an equivalent range of C:N values tested for each organism. For Group B, N_2 fixation rates were almost 30% less (10 mmol N m^{-2} $year^{-1}$) using a C:N of 8.7 than that assuming the baseline value of 6.3. Within these reported ranges for C:N mass ratios, N_2 fixation of *Trichodesmium* would always be at least six times that of Group A and Group B as calculated in the base model.

Discussion

High abundances and estimated rates of N_2 fixation of unicellular cyanobacterial N_2 fixers in the North Pacific (Zehr *et al.*, 2001; Montoya *et al.*, 2004) suggest that diazotrophs other than *Trichodesmium* potentially play a significant role in the nitrogen cycle at the North Pacific Station ALOHA, and in tropical and subtropical waters worldwide. This paper describes a diagnostic model to potentially translate abundance measurements into estimates of N_2 fixation for both *Trichodesmium* and unicellular organisms for comparison of rates among different diazotrophs, and includes sensitivity analyses to parameter choices. Based on this model the dominance of N_2 fixation by *Trichodesmium* or unicellular Groups A and B was shown to depend directly on their biomass, modeled here from measured cell abundances, which varied from month to month, seasonally and between years of data collection. The average annual rate of N_2 fixation modeled for *Trichodesmium* exceeded that of unicellular Groups A and B by more than a factor of seven. However, this average annual estimate for *Trichodesmium* is dominated by two summertime cruises during year 1. As a result, the fractional contribution to instantaneous N_2 fixation by each diazotroph fluctuated substantially on interannual, seasonal and shorter time scales. For example, the average rates of modeled N_2 fixation (mmol N m^{-2} $year^{-1}$) for *Trichodesmium* were 162 during the first study year and 12 in the second. In contrast, those for Groups B and A were 17 and 8 during year 1 and 12 and 9 during year 2, respectively. Based on these numbers, unicellular organisms accounted for 13% of the total fixation by the three diazotrophs during year 1 and 63% during year 2. When including uncertainties in our estimates using the bootstrap approach, the range of unicellular N_2 fixation extended from 10% to 68% of the total annual rate of N_2 fixation for the three diazotrophs. Furthermore, on a seasonal basis, the model demonstrated that unicellular diazotrophs fixed the majority (51%–97%) of N_2 during winter and spring,

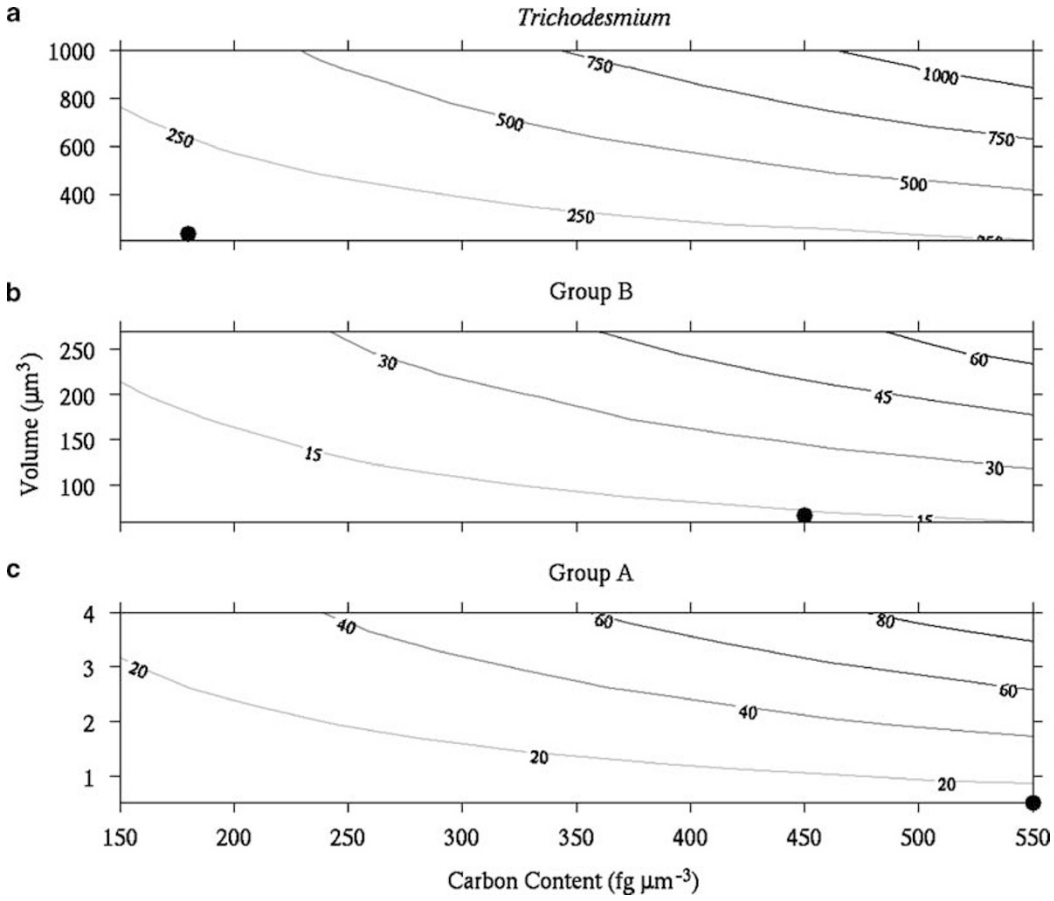

Figure 5 Modeled ranges in annual rates of N_2 fixation as calculated in a sensitivity analysis across diazotroph-specific ranges in volume and carbon content for (**a**) *Trichodesmium*, (**b**) Group B and (**c**) Group A. The data point on each plot (●) represents the modeled annual rate of N_2 fixation calculated using the parameters in Table 1. The interval between contour lines (mmol $N\,m^{-2}\,year^{-1}$) is 250 for *Trichodesmium*, 15 for Group B and 20 for Group A.

whereas *Trichodesmium* dominated total N_2 fixation by the three diazotrophs during summer and autumn (60%–96%).

Despite higher cell abundance, modeled growth rates and carbon content for Group A, the average modeled rate of N_2 fixation did not exceed that of *Trichodesmium*. While these estimates clearly varied with ranges in abundances, they also depended on chosen model parameters. Our analysis showed that this result was most sensitive to cell size owing to its square (*Trichodesmium*) or cubic (unicellular) influence on cell volume and the linear relationship between cell volume and biomass. For example, an increase in cell size of 50% for Group A to 1.5 μm diameter or 1.8 μm^3, could boost the average annual N_2 fixation rate to within the confidence limits of the *Trichodesmium* rate estimate. A viable cell size of 8 μm for Group B could potentially increase the average annual N_2 fixation rate similarly. This nonlinear sensitivity highlights the importance of establishing Group A cell dimensions from field observations as a next step.

While the set of monthly observations of cell abundance used in the present study may be the most unique and complete time series across three different types of cyanobacteria to date, it still may not represent the full range and temporal variability in abundances likely to be observed in the relatively sparsely sampled North Pacific, nor may it agree with observations that other studies base their estimates of N_2 fixation upon (see below). This may be particularly true for Group B, which has been observed at maximum volumetric and depth-integrated concentrations of at least an order of magnitude higher (Zehr *et al.*, 2001; Montoya *et al.*, 2004; Church *et al.*, 2005a) than the observations used in this study. Low cell abundances for Group B suggest that the modeled rates of N_2 fixation may have been underestimated for this diazotroph. Volumetric abundances for *Trichodesmium* and Group A used in our model were consistent with the ranges observed by Church *et al.* (2005a), also determined from *nifH* gene abundances. However, abundances of *Trichodesmium* either exceeded or were consistent with microscopic counts reported by Karl *et al.* (1992) and Letelier and Karl (1996), respectively. *Trichodesmium* abundance was highly variable. Our estimates of uncertainty attempted to take this high variability in temporal and spatial undersampling of cyanobacteria in the North Pacific

into account. However the nonparametric bootstrap method was limited by the range in available observations, therefore we could not determine any effect of sampling bias on our model results with this data set. Regardless, the relatively low sensitivity of modeled rates of N_2 fixation to cell abundance demonstrated that variations in cell concentrations were unlikely to account for large differences in modeled N_2 fixation rates among the three diazotrophs tested.

The cell abundances used in this study may have been further underestimated depending upon the number of *nifH* gene copies contained in each cell. Although it has been demonstrated that each genome of *T. erythraeum* strain IMS101 and *C. watsonii* strain WH8501 contains one gene, evidence for the presence of multiple genome copies per cell in other cyanobacteria (Binder and Chisholm, 1990 and references therein) suggests that this could also be the case for the diazotrophs investigated in the present study. If more than one genome copy existed per cell, the method used to obtain abundances from the present data set would have overestimated rates of N_2 fixation. The high, modeled rates of N_2 fixation for *Trichodesmium* compared to rates reported in the literature could be explained by the presence of multiple genome copies.

The average annual rate of N_2 fixation modeled for *Trichodesmium* (85 mmol m^{-2} $year^{-1}$) at Station ALOHA during 2004–2006 exceeded that calculated by Karl *et al*. (1997) for data collected during 1988–1995 (30–50 mmol N m^{-2} $year^{-1}$) and that modeled numerically by Fennel *et al*. (2002) (29–53 mmol N m^{-2} $year^{-1}$). In addition, the average modeled rate for *Trichodesmium* fell just above the upper limit of the diazotroph nitrogen flux estimated by Dore *et al*. (2002) for 1990–2000 (31–84 mmol N m^{-2} $year^{-1}$), also at Station ALOHA. Interannual differences in modeled rates of N_2 fixation for *Trichodesmium* of this study, however, bracketed these modeled and estimated rates reported in the literature. Furthermore, applying our model to data that excluded the high summer abundances of year 1 yielded an annual rate of N_2 fixation of 45 mmol N m^{-2} $year^{-1}$, which compared well with annual rates reported by Karl *et al*. (1997) and Dore *et al*. (2002).

In their comprehensive study, Dore *et al*. (2002) applied multiple isotopic approaches to estimate N_2 fixation and nitrogen flux in the North Pacific. Methods used in their study may provide an explanation for the comparably high rates of N_2 fixation for *Trichodesmium* modeled during year 1 in this study. Isotopic measurements by $^{15}N_2$ uptake in bottle incubations (30–110 μmol N m^{-2} day^{-1}) were likely to underestimate daily *in situ* rates of N_2 fixation by *Trichodesmium* due to the undersampling of larger diazotrophs by small-volume sampling bottles. This suggests that these rates better represent small unicellular populations, and their numbers are in good agreement with our modeled rates for Groups A and B together. Dore *et al*. (2002) also analyzed temporal changes in the stable isotopes of time-integrated, exported particulate matter. This alternate approach minimized possible biases in temporal resolution and undersampling of large diazotrophs and therefore better represented N_2 fixed by *Trichodesmium*. Their sediment trap-derived, N_2-supported rates of particulate nitrogen export were considerably larger than that measured with bottle incubations, reaching a maximum of >400 μmol N m^{-2} day^{-1}. This measurement agreed more closely with our average modeled depth-integrated rate for *Trichodesmium* (212 μmol N m^{-2} day^{-1}) but was still well below the maximum rate (2110 μmol N m^{-2} day^{-1}) calculated during the summer peak in abundance in year 1 of the present study. Sediment trap-derived estimates could also underestimate the sinking particle flux (Benitez-Nelson *et al*., 2001) and thus the derived N_2 fixation. In summary, the high variability in abundances and methodological considerations used to estimate N_2 fixation rates, as well as dependence upon seasonal and interannual variations in physical and chemical properties of the water column (for example, ENSO events; Dore *et al*., 2002), was likely to account for much of the variability in measured and modeled N_2 fixation rates.

Depth-integrated daily rates of N_2 fixation modeled in this study were consistent with those reported in the literature for unicellular diazotrophs. Modeled daily depth integrals of N_2 fixation for Groups A and B had means of 27 and 29 μmol N m^{-2} day^{-1} and maximums of 118 and 189 μmol N m^{-2} day^{-1}, respectively. These values were comparable to size-fractionated estimates of unicellular N_2 fixation of 92, 11–103 and 30–110 μmol N m^{-2} day^{-1} measured in the North Pacific by Zehr *et al*. (2001), Montoya *et al*. (2004) and Dore *et al*. (2002), respectively. Estimates by Falcon *et al*. (2004) for the North Atlantic (37 and 47 μmol N m^{-2} day^{-1}) were in reasonable agreement with our own modeled rates, but their estimates in the North Pacific (2.2 μmol N m^{-2} day^{-1}) were considerably smaller.

A final but important consideration was the physiological effect of temperature on the parameter μ_{max}. The μ_{max} measured by Goebel *et al*. (in revision) and used to parameterize growth rates of *Trichodesmium* in this study was more than double the rates reported in the literature review of LaRoche and Breitbarth (2005) and that recorded by Breitbarth *et al*. (2007). However, the Goebel *et al*. (in revision) rates for both *Trichodesmium* and *Crocosphaera* agreed with those of Tuit *et al*. (2004). The rates of Goebel *et al*. (in revision) and Tuit *et al*. (2004) were measured at a temperature of 27 °C, consistent with the temperature at which maximum growth was determined by Breitbarth *et al*. (2007). This temperature value is representative of surface waters at Station ALOHA for approximately half of the year. Since this was the period during which

highest abundances in *Trichodesmium* were observed, variation in growth rate with temperature would not have a large effect on the modeled annual rate of N_2 fixation for *Trichodesmium*. For *Crocosphaera*, Falcon *et al*. (2005) demonstrated a ~30% decrease in the growth rate of 3 μm diameter cells across a range in temperature from ~27 to 25 °C, inferring a high Q_{10} factor. Such a decrease in growth with temperature was not represented in our annual model calculations, and thus our model could overestimate rates of N_2 fixation during winter and in deeper portions of the water column. If this temperature response is representative of Group A and Group B diazotrophs, it would have a non-negligible effect on the quantitative estimates of N_2 fixation for these organisms.

With the objective to assess the role of unicellulars as potentially important N_2 fixers, we have found that during periods of moderate to high *Trichodesmium* abundance at Station ALOHA (summer and fall), modeled rates of N_2 fixation were dominated by this organism's contribution. However, during other periods (for example, winter and spring), unicellular diazotrophs were at their highest abundance and were responsible for the majority of N_2 fixation. This pattern of alternation in the dominance of N_2 fixation, as was also noted in the spatial distributions of Group B and *Trichodesmium* in the Amazon plume waters (Foster *et al*., 2007), implies that unicellular diazotrophs could have a potentially significant impact on N_2 fixation and nitrogen fluxes in mid to higher latitude regions where *Trichodesmium* has not been observed. Our next objective, to include modeled N_2 fixation rates of diatom–diazotroph associations (for example, *Rhizosolenia–Richelia*) that are known to contribute substantially to N_2 fixation in the North Pacific (Venrick, 1974; Karl *et al*., 1992; Capone, 2001), will allow for a more complete assessment of the importance of unicellular diazotrophs to N_2 fixation at Station ALOHA.

Summary
Observed cell abundances for three diazotrophs over a 2-year period (October 2004–October 2006) at Station ALOHA in the tropical North Pacific were used to model and compare rates of N_2 fixation. Although *Trichodesmium* dominated modeled N_2 fixation on an average annual basis, unicellular diazotrophs contributed the majority during periods in which *Trichodesmium* abundances were smaller (14 out of 23 cruises). Groups A and B accounted for 51%–97% of depth-integrated daily modeled rates of N_2 fixation throughout winter and spring, and could potentially account up for up to almost two-thirds of statistically modeled annual rates of N_2 fixation. Of several parameters that influenced N_2 fixation in the model, cell size was found to be the most important to constrain future measurements, particularly for Group A. While our modeled rates of daily, depth-integrated rates of N_2 fixation were consistent with other studies for unicellulars, our average and year-1 modeled rates of N_2 fixation for *Trichodesmium* were higher than annual rates reported in the literature. Our high rates resulted from two cruises in the summer of year 1 which recorded particularly high *Trichodesmium* abundances. In addition, there is some sensitivity to our choice of growth rate, and our assumption of one genome copy per cell. More generally, temporal and spatial sampling resolution as well as other model assumptions and parameter choices also affected the accuracy of the modeled results. The relatively well-characterized *Trichodesmium* is well established as an important contributor to N_2 fixation in subtropical and tropical ocean waters (Capone *et al*., 1997, 2005; Letelier and Karl, 1998; Capone and Carpenter, 1999). This study attempts to quantify the impact of relatively uncharacterized, smaller diazotrophs to this process. While this study represents a first attempt to provide estimates of ranges and relative contributions of three diazotrophs to N_2 fixation that can be used as point of comparison for future studies and by prognostic numerical models that aim to represent complex community structure that influences N_2 fixation, it also demonstrates a unique approach that indicates the potential importance of unicellular diazotrophs to N_2 fixation.

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