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Diversity trumps acidification: Lack of evidence for carbon dioxide enhancement of *Trichodesmium* community nitrogen or carbon fixation at Station ALOHA

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Abstract

We conducted 11 independent short-term carbon dioxide (CO_2) manipulation experiments using colonies of the filamentous cyanobacteria *Trichodesmium* isolated on three cruises in the North Pacific Subtropical Gyre (NPSG). Dinitrogen (N_2) and carbon (C) fixation rates of these colonies were compared over CO_2 conditions ranging from ~ 18 Pa (equivalent to last glacial maximum atmospheric P_{CO_2}) to ~ 160 Pa (predicted for ~ year 2200). Our results indicate that elevated P_{CO_2} has no consistent significant effect on rates of N_2 or C fixation by *Trichodesmium* colonies in the NPSG under present environmental conditions. Differences between P_{CO_2} treatments were not modulated by phosphorus amendments, iron amendments, or light level. Sequencing the *hetR, nifH,* 16S, and internal transcribed spacer genes of *Trichodesmium* colonies revealed a highly diverse community of *Trichodesmium* and other N_2-fixing colony-associated organisms. The species composition of *Trichodesmium* demonstrated spatiotemporal variability, but over half of total sequences were phylogenetically closely related (> 99% *hetR* sequence similarity) to isolate H9-4 of *T. erythraeum,* which showed no response to elevated P_{CO_2} in previous laboratory experiments. Our handpicked *Trichodesmium* colonies included a substantial number of organisms other than *Trichodesmium* with the metabolic capacity for N_2 and C fixation. We suggest that the diverse assemblage of *Trichodesmium* species and coexisting microorganisms within the colonies can explain the lack of an observed CO_2 enhancement of N_2 or C fixation rates, because different species are known to have different specific affinities for CO_2.

Burning fossil fuels and other human actions are adding carbon (C) to the atmosphere at unprecedented rates (IPCC 2013). This increase in atmospheric carbon dioxide (CO_2) propagates into the ocean, increasing the partial pressure of CO_2 (P_{CO_2}) of seawater and causing a suite of changes to ocean chemistry, collectively termed ocean acidification (OA; Doney et al. 2009). Among the predicted effects of OA is a possible upregulation of cellular processes by C-limited phytoplankton. Because almost all phytoplankton invest energy in carbon-concentrating mechanisms (CCMs) to help saturate their low CO_2-affinity carboxylating enzyme, ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO; Badger et al. 1998), it has been proposed that future OA may allow certain phytoplankton species to increase their C fixation rates and reallocate energy from CCMs to other metabolic processes (Hutchins et al. 2007).

Cyanobacteria, which evolved under the high CO_2 conditions of the Archaean Eon, can display an especially low CO_2-affinity RuBisCO compared with other phytoplankton, and may thus benefit from future elevated P_{CO_2} (Rost et al. 2008). There is a particular interest in the effect of OA on a class of cyanobacteria with the capacity to fix dinitrogen (N_2), termed diazotrophs. The bioavailable nitrogen (N) provided by marine diazotrophs helps fuel new production, especially in oligotrophic open-ocean environments, where N_2 fixation can account for up to half of the new N supporting export from the euphotic zone (Karl et al. 1997). A large fraction of this fixed N is provided by *Trichodesmium,* a group of filamentous, non-heterocystous cyanobacteria (Karl et al. 1997; Capone et al. 2005).

Recently, several laboratory studies on two isolates of *Trichodesmium erythraeum* have shown enhanced N_2 and, on some occasions, C fixation, in response to increases in P_{CO_2} (Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al. 2007). In addition, an isolate of *Crocosphaera watsonii,* a naturally abundant unicellular cyanobacterium (Moisander et al. 2010), also increases N_2 and C fixation rates when exposed to elevated P_{CO_2} (Fu et al. 2008; M. Gradoville unpubl.). In these studies, a doubling of present-day P_{CO_2} increased *Trichodesmium* N_2 fixation rates between 35% (Kranz et al. 2009) and 138% (Levitan et al. 2007). An enhancement of global marine N_2 fixation in this range could have substantial consequences for the coupling of elemental cycles, ocean primary productivity, carbon sequestration, and the biological pump.

Still, it is unclear whether the early evidence for a P_{CO_2} enhancement of N_2 fixation by *Trichodesmium* and *Crocosphaera* isolates manipulated in the laboratory can be used to predict the response of diazotrophs under natural environmental conditions. In most laboratory studies, *Trichodesmium* and *Crocosphaera* cultures are grown in an artificial medium with excess nutrients uncharacteristic of open-ocean habitats (Chen et al. 1996). These growth conditions may influence the effect of OA on N_2 fixation. For example, under iron- (Fe-) limiting conditions, OA has a negative or neutral effect on N_2 fixation by *Trichodesmium* and *Crocosphaera* isolates (Fu et al. 2008; Shi et al. 2012). To date, the only study of *Trichodesmium* strain IMS101 grown in nutrient-amended seawater rather than

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YBCII media (Chen et al. 1996) found that OA reduced N_2 fixation rates even in Fe-replete treatments, suggesting that C may not be the primary limiting nutrient for this strain when grown in seawater (Shi et al. 2012). Furthermore, recent evidence suggests that the N_2 fixation response to CO_2 manipulations can vary widely even within the genus *Trichodesmium* (e.g., certain laboratory strains do not exhibit the same positive response to CO_2 observed in the initial experiments that used IMS101 and GBRTRLI101 strains [Hutchins et al. 2007, 2013]).

In addition, experiments investigating the responses of naturally occurring *Trichodesmium* colonies and whole diazotrophic communities to OA have yielded conflicting results. Although elevated P_{CO_2} has stimulated N_2 fixation by *Trichodesmium* colonies isolated from the Subtropical Atlantic and the Gulf of Mexico (Hutchins et al. 2009; Lomas et al. 2012), the specific methodological approaches and the application of phosphorus- (P-) and Fe-replete conditions in these experiments (Lomas et al. 2012) make ecologically relevant interpretations difficult. In contrast, unamended whole-community experiments in the North and South Pacific gyres do not display a significant relationship between P_{CO_2} and N_2 fixation rates (Law et al. 2012; D. Böttjer pers. comm.). These conflicting results paint an unclear picture of the response of natural diazotrophic communities to OA.

Accurate forecasts of future marine nutrient cycling and associated atmospheric feedbacks require an understanding of how OA will affect biological rate processes in complex, naturally occurring microbial assemblages. A key step in this direction is determining whether the contrasting responses exhibited by *Trichodesmium* colonies and whole-water communities in previous CO_2 manipulation experiments are due to differences in community composition, physiology, environmental conditions, and/or experimental methodology. To this end, the aim of the present study is to characterize the effect of elevated P_{CO_2} on naturally occurring *Trichodesmium* assemblages in the oligotrophic North Pacific Subtropical Gyre (NPSG) under a range of nutrient and light conditions. Here, we present results of N_2 and C fixation-rate measurements from P_{CO_2} manipulation experiments, together with genetic characterization of the *Trichodesmium* assemblage and colony-associated microorganisms utilized in our incubations.

Methods

Experimental design—Experiments were carried out on three cruises aboard the R/V *Kilo Moana* at and near Sta. ALOHA (A Long-term Oligotrophic Habitat Assessment), the long-term field site of the Hawaii Ocean Time-series (HOT) program ~ 100 km north of the Hawaiian island of Oahu (Table 1). In all experiments, picked *Trichodesmium* colonies were incubated in surface-seawater cooled deck-board incubators under varying CO_2, nutrient, and light regimes. Carbon and N_2 fixation rates were measured, and samples of *Trichodesmium* colonies were saved for subsequent deoxyribonucleic acid (DNA) extraction (Table 1).

Trichodesmium colonies were collected at night using a 202 μm plankton net that was hand-towed at < 2 km h^{-1}

Table 1. Locations and ambient environmental conditions of seawater from which *Trichodesmium* colonies were collected. Sea surface temperature (SST) and chloropigment (Chl) fluorescence at 25 m depth were measured using a conductivity–temperature–depth sensor. Soluble reactive phosphorous (SRP) values are from discrete samples at 25 m taken within 1 d of the net tow. Experiments in which nutrient conditions were amended are indicated with +P or +Fe; 'ambient' indicates no added nutrients. nd, no data available.

Date	Location	SST (°C)	Chl (μg L^{-1})	SRP (μmol L^{-1} PO_4)	Morphology of colonies used	Rate and DNA measurements*	Nutrient and light treatments
22 Aug 10	22°44.96′N, 157°59.87′W	25.75	0.09	0.051	colonial	AR	ambient
24 Aug 10	24°60.00′N, 157°58.88′W	25.85	0.09	0.094	colonial	AR	ambient
26 Aug 10	24°40.28′N, 160°51.61′W	26.19	0.28	0.018	colonial	AR	ambient
14 Mar 11	22°46.40′N, 158°1.50′W	24.55	0.14	0.096	puffs and tufts	AR	+P
15 Mar 11	22°45.00′N, 158°0.01′W	24.57	0.11	0.102	puffs and tufts as separate treatments	AR	ambient
16 Mar 11	22°45.00′N, 157°57.89′W	24.42	0.14	nd	Tufts only	AR	+P, ambient
20 Mar 11	22°45.04′N, 158°0.01′W	24.23	0.16	0.072	Tufts only	AR	+P, ambient
08 Aug 12	22°48.85′N, 158°1.93′W	25.46	0.15	nd	mostly free filaments	^{13}C,^{15}N	+Fe, ambient
09 Aug 12	22°48.93′N, 158°1.96′W	25.47	0.14	0.10	mostly small puffs	^{13}C,^{15}N, DNA	+Fe, shading
10 Aug 12	22°49.00′N, 158°1.70W	25.57	0.15	nd	small puffs, large puffs, loose tufts	^{13}C,^{15}N, DNA	+Fe, ambient
11 Aug 12	22°49.00′N, 158°2.50′W	25.68	0.13	nd	small puffs, large puffs, loose tufts	^{13}C,^{15}N	+Fe, shading
12 Aug 12	22°49.01′N, 158°2.51′W	25.64	0.15	0.11	small puffs, large puffs, loose tufts	DNA only	

* AR, acetylene (C_2H_2) reduction technique.

Table 2. *Trichodesmium* colony morphology and cruise-averaged $^{15}N_2$ assimilation and C_2H_4 production (from acetylene reduction technique [AR]) rates, normalized to C and Chl *a*, respectively, with associated coefficients of variation. Mean rates and coefficients of variation are derived from treatments with ambient P_{CO_2} and nutrient conditions from each day an experiment was conducted. Standard deviations are given in parentheses. Dashes indicate experiments that were not performed on a particular cruise.

Cruise	*Trichodesmium* morphology	N_2 fixation (nmol N μmol C^{-1} d^{-1})	C_2H_4 production (nmol C_2H_4 μg Chl a^{-1} h^{-1})	Daily coefficient of variation (%)
Aug 2010	colonial	—	42.5 (52.8)	17
Mar 2011*	tufts only	—	30.2 (1.0)	2
Aug 2012	small puffs, large puffs, loose tufts	1.5 (1.0)	—	76

* Rates and coefficients of variation are from 16 March and 20 March only.

through the near-surface ocean (< 10 m depth) for 15–20 min. Colony morphology was noted (Table 2), and an inoculating loop was used to gently pick colonies from the net tow material and place them into 0.2 μm filtered surface seawater. Colonies were kept in minimal light until the initiation of the experiment (within 5 h of collection). To prepare 'media' for *Trichodesmium* incubations, surface seawater was collected using a Teflon pump or sampling bottles attached to a CTD (conductivity, temperature, depth) rosette. The seawater was filtered through a 0.2 μm in-line polycarbonate membrane filter and dispensed into acid-washed polycarbonate carboys, where the carbonate system was manipulated (*see* P_{CO_2} *manipulations and DIC measurements*) and 20 μmol L^{-1} ethylenediaminetetraacetic acid (EDTA) was added to prolong *Trichodesmium* activity (Burns et al. 2006). For P- or Fe-replete treatments, media was enriched to final concentrations of 5 μmol L^{-1} KH_2PO_4 or 100 nmol L^{-1} $FeCl_3$ EDTA. Immediately preceding inoculation, media was dispensed into acid-washed 37 mL glass serum bottles and 8–12 *Trichodesmium* colonies were added per bottle. Experiments started at sunrise and ended at sunset. Samples were incubated in flow-through deck-board incubators under blue acrylic shielding shaded to ~ 60% of sea-surface irradiance (measured using a Biospherical light meter). Black netting was used to shade bottles for experiments requiring lower light levels (~ 30%, ~ 15%, and ~ 8% incoming irradiance). Daily integrated photosynthetically active radiation (PAR) for each treatment was calculated by trapezoidal integration of measured sea-surface PAR values collected at 10 min intervals with an on-deck LI-COR® Siltronic 2π sensor and correcting for percent light levels of treatment incubations. Though surface seawater was continuously pumped through the incubator, HOBO® Pendant® temperature and light data loggers (Onset) measured a daily temperature range of 23.5–29°C in August 2012.

C and N_2 fixation rates—In August 2010 and March 2011, rates of N_2 fixation were estimated using the acetylene (C_2H_2) reduction technique (AR; Capone 1993). For these measurements, 25 mL of incubation media with the proper P_{CO_2} were dispensed into serum bottles, leaving 12 mL of headspace. Eight to twelve handpicked *Trichodesmium* colonies were added to each bottle, and the headspace was flushed with the appropriate P_{CO_2} (flushed during March 2011 cruise only), fitted with Viton septa, and sealed by crimping with aluminum caps. Acetylene was produced from calcium carbide (Aldrich) and injected into each bottle to produce a volumetric concentration of 10% C_2H_2 in the headspace. Once every 3 h through the incubation period, 100 μL of headspace was subsampled and ethylene (C_2H_4) was detected by flame ionization gas chromatography using a Shimadzu GC-8A. Samples were incubated ~ 12 h, from dawn until dusk. After the final time point, samples were filtered onto 25 mm glass-fiber filters (GF/F; Whatman), frozen at −80°C, and shipped in liquid nitrogen to Oregon State University, where they were stored at −20°C. Chlorophyll *a* (Chl *a*) from these filters was extracted in 90% acetone at −20°C for 48 h and analyzed using a Turner Model 10AU™ fluorometer via the acidification method of Strickland and Parsons (1972). Acetylene reduction rates were calculated as a linear regression of C_2H_4 produced $time^{-1}$ and normalized to the Chl *a* in each bottle. Our use of the traditional gas bubble injection AR method could increase variability in measured rates due to incomplete equilibration of C_2H_4 or C_2H_2 in liquid phase with the gas headspace (Wilson et al. 2012).

Carbon and N_2 fixation rates were measured in August 2012 using the ^{13}C method of Legendre and Gosselin (1997) and a modification of the original $^{15}N_2$ uptake method (Montoya et al. 1996). $^{15}N_2$ (99%) was added as enriched seawater (2% by volume) instead of direct gas injection to avoid problems with bubble dissolution (Mohr et al. 2010; Wilson et al. 2012). Mixtures of incubation media, $^{15}N_2$-enriched seawater, and 0.5 mL of 48 mmol L^{-1} ^{13}C bicarbonate stock were dispensed into 37 mL serum bottles using a large glass syringe to avoid air exposure. *Trichodesmium* colonies were added as described previously, and the bottles were fitted with Viton septa and aluminum caps and crimp-sealed, leaving no headspace. Incubations were terminated by vortexing and gently filtering colonies onto precombusted 25 mm GF/F filters. These filters were stored at −80°C and shipped in liquid nitrogen to Oregon State University, where they were dried at 60°C overnight and packaged tightly into tin capsules. Particulate carbon (PC) and nitrogen (PN) masses and isotopic composition were measured using an Elementar vario MICRO cube (Elementar Analysensysteme) elemental analyzer coupled to a PDZ Europa 20–20 isotope ratio mass spectrometer (SerCon) at the University of California Davis stable isotope facility. Fixation rates were calculated according to Montoya et al. (1996) and normalized to measured PC concentrations.

P_{CO_2} manipulations and DIC measurements—Two methods were used to manipulate P_{CO_2} for these experiments:

Table 3. Average measured P_{CO_2} (Pa) for all treatments on each cruise. Values are averaged from the days on which experiments were run; standard deviations between days are given in parentheses. Dashes indicate P_{CO_2} treatments that were not included on a particular cruise.

		Desired P_{CO_2} treatment (Pa)					
Cruise	Method	15	28	40	75	100	160
Aug 2010	carbonic acid addition	—	—	42	74	101	169
Mar 2011*	carbonic acid addition	—	—	42 (5)	74 (2)	108 (10)	148 (17)
Aug 2012	gas bubbling	18 (0.7)	28	37 (0.4)	65	82 (0.1)	—

carbonic acid additions and gas bubbling. Both methods manipulate P_{CO_2} by increasing DIC without altering alkalinity, similar to anthropogenic OA. In laboratory experiments aimed at comparing methods, we found that the method chosen had no significant effect on the response of *Trichodesmium* IMS101 to P_{CO_2} (M. Gradoville unpubl.).

Carbonic acid additions were used on the August 2010 and March 2011 cruises. Equal molar concentrations of HCl and $NaHCO_3$ were added to 0.2 μm filtered seawater to give P_{CO_2} values of $\sim$ 40 Pa ($\sim$ ambient P_{CO_2}), 75 Pa, 100 Pa, and 160 Pa. Gas bubbling was used in the August 2012 cruise; in order to achieve the required P_{CO_2} equilibrium in these experiments, 0.2 μm filtered seawater was bubbled for $>$ 24 h with commercially manufactured (Airgas) artificial mixtures of CO_2 and air, resulting in P_{CO_2} conditions of $\sim$ 15, 29, 42, 76, and 101 Pa.

The carbonate chemistry of incubation media was monitored on all cruises. Incubation media was dispensed into 250 mL borosilicate bottles with ground glass stoppers or 350 mL amber glass bottles, fixed with 100–300 μL $HgCl_3$, and sealed with no headspace. Samples were analyzed for dissolved inorganic carbon (DIC) and total alkalinity (TA) either onboard or at a shore-based laboratory. For August 2010 and March 2011 cruises, DIC was estimated coulometrically and TA was measured by acid titration. The DIC and TA calibrations were validated with certified CO_2 reference materials (Dickson et al. 2003). P_{CO_2} values were calculated from the DIC and TA data with the program CO2calc (Robbins et al. 2010) using the CO_2 constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987). For the August 2012 cruise, DIC and P_{CO_2} were measured according to the methods of Bandstra et al. (2006). Carbonate chemistry was analyzed each day in which an experiment was run in March 2011 and August 2012. Dissolved inorganic carbon and TA of incubation media were only measured once during the August 2010 cruise; however, pH was measured each day an experiment was run in August 2010, and did not change by $>$ 0.1 pH units through the cruise. The measured and calculated P_{CO_2} values sometimes deviated from desired P_{CO_2} (Table 3), but relative differences between treatments were achieved.

Fe measurements—For Fe measurements, 25 mL samples were collected into acid-leached, high-density polyethylene bottles and acidified on board with trace-metal clean HCl to pH = 2. Samples were analyzed by isotope dilution inductively coupled plasma mass spectrometry at the Massachusetts Institute of Technology according to the method of Lee et al. (2011).

DNA extraction, amplification, and sequencing—During the August 2012 cruise, plastic inoculating loops were used to pick duplicate or triplicate sets of *Trichodesmium* colonies into 0.2 μm filtered surface seawater. The samples were vortexed and gently filtered onto Supor membranes, which were stored at -80°C until analysis. DNA was extracted using the DNeasy Plant MiniKit (Qiagen). The protocol was modified from manufacturer's suggestions to include thawing samples, adding buffer, and bead-beading with 0.1 mm and 0.5 mm glass beads (Biospec products) for additional cell disruption prior to the extraction. DNA concentrations were determined by PicoGreen DNA quantification (Molecular Probes) using a Perkin Elmer 2030 plate reading fluorometer. The extracted DNA was stored at -80°C.

To analyze the diversity among *Trichodesmium* colonies, we amplified regions of the 16S, internal transcribed spacer (ITS), and *hetR* genes as described by Hynes et al. (2012) with minor modifications and the *nifH* gene as described in Zehr and Turner (2001) (Table 4). Polymerase chain reactions (PCR) were performed using PTC-200 Thermo Cyclers (Bio-Rad) with 50 μL reaction volumes. The 16S ribosomal ribonucleic acid (rRNA) gene was amplified in a mixture containing 1$\times$ PCR buffer, 200 μmol L^{-1} deoxynucleotide triphosphates (dNTPs), 3% bovine serum albunin (BSA), 1.5 mmol L^{-1} Mg^{2+}, 1U High Fidelity *Taq* polymerase (Invitrogen), 4 ng DNA, and 0.5 μmol L^{-1} CYA-106F and CYA-781R primers (Nübel et al. 1997). Thermocycling conditions were as follows: initial denaturing step of 95°C for 4 min, with 30 cycles of 93°C for 1 min, 57°C for 1 min, 72°C for 1 min; followed by a final extension of 72°C for 7 min. Amplification of *hetR* used the same reaction mixture as 16S but with the PH1 and PH2 primers (Lundgren et al. 2005). The temperature profile for amplification of *hetR* was 94°C for 6 min; followed by 30 cycles of 93°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 10 min. Amplification of the ITS region used a mixture containing 1$\times$ PCR buffer, 200 μmol L^{-1} dNTPs, 3% BSA, 3.5 mmol L^{-1} Mg^{2+}, 1U High Fidelity *Taq* polymerase (Invitrogen), 4 ng DNA, and 0.5 μmol L^{-1} tri16S-1247F (Orcutt et al. 2002) and tri-23SR (Hynes et al. 2012) primers. Cycling conditions for ITS were 95°C for 2 min, then 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C; and a final extension of 10 min at 72°C. A nested PCR amplification procedure was used to amplify *nifH* genes. The first round of PCR contained 1$\times$ PCR buffer, 200 μmol L^{-1} dNTPs, 3% BSA, 4 mmol L^{-1} Mg^{2+}, 1U High Fidelity *Taq* polymerase (Invitrogen), 4 ng DNA, and 1 μmol L^{-1} nif3

and nif4 primers (Zani et al. 2000). This reaction was cycled at 94°C for 7 min; then 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min; followed by 72°C for 7 min. The second *nifH* PCR reaction used the same temperature profile and mixture ingredients but used the nif1 and nif2 primers (Zehr and McReynolds 1989) and 2 μL PCR product from the first reaction.

PCR products were visualized and separated on a 1% agarose gel stained with SYBR Safe DNA gel stain. Amplified products were purified using the QIAquick® MiniElute® kit and the purified PCR products were quantified as described above. Amplified gene fragments were cloned using the TOPO® TA Cloning Kit for Sequencing (Invitrogen) according to manufacturer's instructions. Samples were sequenced on an ABI 3100 Gene analyzer (Applied Biosystems) at the Advanced Studies in Genomics, Proteomics, and Bioinformatics (ASGPB, University of Hawaii at Manoa).

Sequences were edited and trimmed using the software Geneious. Alignments were made using ClustalW (http://clustal.org) and included sequences obtained from GenBank (accession numbers from these sequences are AF490696.1, AF490680.1, AF013033.1, AF013031.1, HM486688.1, HM486704.1, HM486706.1, and EU427544.1). Sequences from our study can be found in GenBank; accession numbers are KF960112–KF960668. Phylogenetic trees were created in Geneious, using the Jukes–Cantor model and Neighbor–Joining method; genes from *O. sancta* or *A. plantensis* were used as reference outgroups. Environmental sequences were compared with sequences from the four major *Trichodesmium* clades previously identified (Lundgren et al. 2005).

Quantitative PCR—Quantitative PCR (qPCR) was used to determine the relative abundance of nitrogenase (*nifH*) genes from different groups of diazotrophs within our samples. Primers were chosen to target specific *nifH* groups identified in clone library sequencing: *Trichodesmium,* unicellular cyanobacteria groups A (UCYN-A) and *Crocosphaera*, heterocystous cyanobacteria (Het1, Het2, and Het3), and the Cluster III phylotype. Descriptions of primers, probes, PCR reactions, and thermocycling conditions can be found in previous studies by Church et al. (2005, 2008). Abundances of *nifH* gene copies were normalized to the total DNA concentrations in the extracts.

Statistical analysis—The effects of P_{CO_2} and nutrient amendments on N_2 and C fixation rates were determined using the one-way ANOVA. In some cases, the two-way ANOVA was used to test the effects of two independent variables—P_{CO_2} and the day the experiment was conducted or P_{CO_2} and light level—on the dependent variables. No post hoc tests were used. All treatments were carried out in triplicate bottles, but occasionally issues were encountered with individual samples, resulting in duplicate samples. Only measurements with replicates are reported in this study, with the exception of the 110 Pa puffs treatment on 15 March 2011; this treatment had no replication because all replicates were lost during shipment. All statistics were determined using the software R (http://www.r-project.org/).

Results

Carbon and nitrogen fixation rates—Rates of N_2 fixation by *Trichodesmium* colonies varied widely between cruises, days, and bottle replicates (Fig. 1). In general, Chl *a*–normalized AR rates were higher in August 2010, ranging from 2.9 nmol C_2H_4 μg Chl a^{-1} h^{-1} to 49.3 nmol C_2H_4 μg Chl a^{-1} h^{-1}, than on the March 2011 cruise when they ranged from 1.6 nmol C_2H_4 μg Chl a^{-1} h^{-1} to 11.7 nmol C_2H_4 μg Chl a^{-1} h^{-1} (Fig. 1). These ranges are similar to previously reported rates of AR by *Trichodesmium* colonies at Sta. ALOHA (Letelier and Karl 1998). Particulate C–normalized $^{15}N_2$ fixation rates in August 2012 ranged from 0.46 nmol N μmol C^{-1} d^{-1} to 5.1 nmol N μmol C^{-1} d^{-1}, while ^{13}C assimilation rates in August 2012 ranged between 42 nmol C μmol C^{-1} d^{-1} and 128 nmol C μmol C^{-1} d^{-1} (Fig. 2). We chose not to normalize rates to colony number because of the known variability in size and morphology of *Trichodesmium* colonies (Letelier and Karl 1996). However, our range of $\sim$ 8–12 colonies $bottle^{-1}$ gives a broad C fixation range of 0.5–7.1 nmol C $colony^{-1}$ h^{-1}, which is similar to previously reported *Trichodesmium* C fixation rates (Lomas et al. 2012). On all cruises, the variability of C and N_2 fixation rates (normalized to PC and Chl *a*) between days was greater than the variability resulting from the differences in treatment (Figs. 1, 2). On average, the coefficient of variation for N_2 fixation rates in control P_{CO_2}, ambient nutrient condition treatments was 17% in August 2010, 2% in March 2011, and 76% in August 2012 (Table 2).

Under ambient nutrient conditions, increasing P_{CO_2} did not significantly enhance N_2 fixation rates in any experiments assayed via AR or via $^{15}N_2$ assimilation (Figs. 1, 2; separate one-way ANOVAs, $p > 0.05$). Treatments in which we lowered the P_{CO_2} below present-day levels displayed a slight decrease in $^{15}N_2$ assimilation; however, this trend was not statistically significant (Fig. 3; one-way ANOVA, $p > 0.05$). Likewise, variations in P_{CO_2} did not significantly affect C fixation rates in August 2012 (Fig. 2).

Additional experiments were performed to test the possibility that nutrient limitation precluded a positive response to P_{CO_2}. We found no evidence that *Trichodesmium* N_2 fixation was limited by P availability during these cruises. At ambient P_{CO_2}, N_2 fixation rates were not enhanced by amendment with 5 μmol L^{-1} PO_4 in March 2011 (Fig. 1) or by 5 μmol L^{-1} PO_4, methylphosphonate, or amino-tris-methylene-phosphonic acid in August 2010 (data not shown). Furthermore, there was no significant difference in N_2 fixation rates between ambient and elevated P_{CO_2} treatments amended with 5 μmol L^{-1} PO_4 in March 2011 (Fig. 1). In the summer of 2012, ambient soluble reactive P (SRP) levels were consistently at the high end (125–170 nmol L^{-1}, K. Björkman pers. comm.) of the climatological range from Sta. ALOHA (Karl 2014). Hence, these experiments were all considered to be P-replete and seawater was not amended with KH_2PO_4.

Similar to P, Fe additions conducted in 2012 did not significantly affect rates of *Trichodesmium* C or N_2 fixation. At ambient P_{CO_2}, neither N_2 fixation nor C fixation were significantly different in treatments with and without added

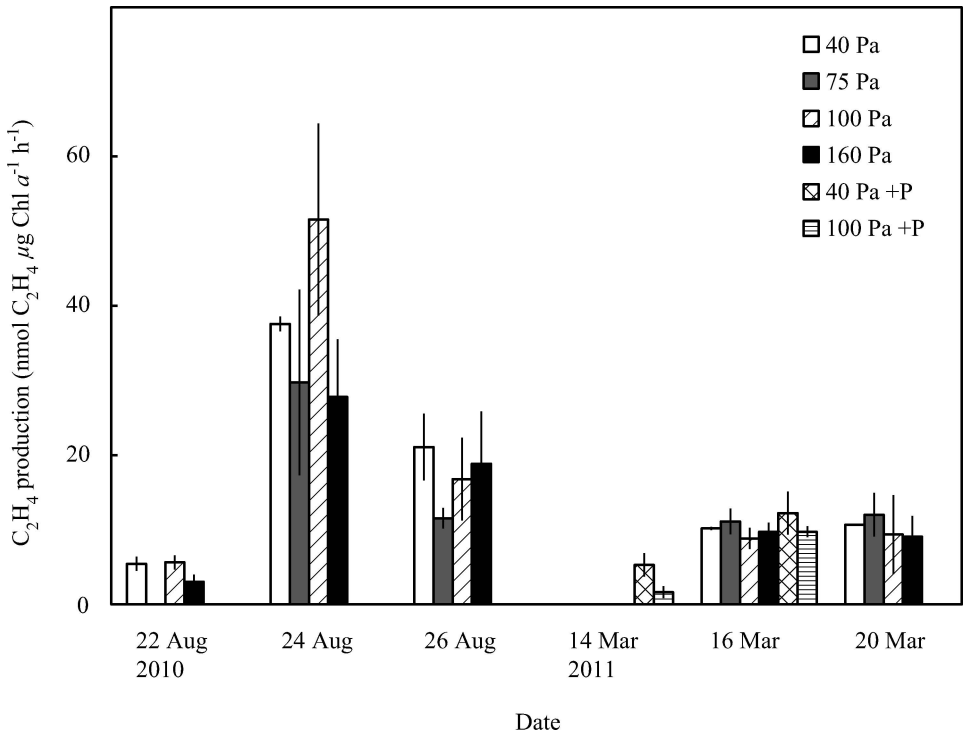

Fig. 1. Measurements of C_2H_4 production (from AR) as a proxy for N_2 fixation by *Trichodesmium* colonies in six independent incubation experiments. Treatments span a P_{CO_2} range of ~ 40–160 Pa under ambient nutrient conditions and with PO_4 amendments (+P). Rates were measured in August 2010 and March 2011 and are normalized to the Chl *a* concentration within each bottle. Error bars represent standard deviations of duplicate or triplicate bottles.

Fe (Fig. 2). Likewise, Fe amendments did not affect the N_2 fixation rates or C fixation rates in elevated P_{CO_2} treatments (Figs. 2, 3). Because all treatments were amended with EDTA, which interferes with the dissolved Fe measurements, the Fe concentrations in control treatments could not be determined. However, in two separate experiments conducted on the same cruise using whole-water samples and no EDTA additions, control treatments had 0.54 ± 0.1 and 10.8 ± 3.9 nmol Fe L^{-1}. These concentrations are both substantially lower than the Fe-replete treatment (100 nmol Fe L^{-1}), and the experiment with 10.86 nmol Fe L^{-1} did not have higher N_2 fixation rates than the experiment with 0.54 nmol Fe L^{-1} (data not shown).

Differences in the daily flux of light significantly affected *Trichodesmium* N_2 fixation rates in August 2012. Rates were higher at 60% incoming light (27.5–28.5 mol quanta m^{-2} d^{-1}) than at 8% incoming light (3.7–3.8 mol quanta m^{-2} d^{-1}) in both experiments (Fig. 4); however, light level only had a significant effect on N_2 fixation rates on 09 August (two-way ANOVA, $p < 0.05$). All light experiments were Fe-replete by addition of 100 nmol L^{-1} $FeCl_3$ EDTA and assumed to be P-replete at ambient SRP concentrations. The flux of light did not significantly change the effect of P_{CO_2} on N_2 fixation rates. In one case (11 August), rates were consistently higher at elevated P_{CO_2} regardless of light level (two-way ANOVA, $p < 0.05$); however, on 09 August there was no significant difference in N_2 fixation rates between the P_{CO_2} treatments (two-way ANOVA, $p > 0.05$).

Diversity within Trichodesmium *colonies*—Morphological diversity of *Trichodesmium* colonies varied between cruises (Table 2). In March 2011, the *Trichodesmium* community isolated from net tows included both puff and tuft morphologies. On 14 March 2011, a mixture of morphologies was used for incubations. The 15 March 2011 experiment compared the CO_2 response of the two morphologies with separate tuft, puff, and P_{CO_2} treatments (Fig. 5). For 16 March and 20 March 2011 experiments, only colonies with tuft morphologies were selected in an attempt to reduce the variability associated with puff colonies (*see* Discussion). In August 2012, isolated *Trichodesmium* consisted of mostly small puff colonies or small, loose tufts; compact tufts were generally absent. Because of the low number of *Trichodesmium* colonies collected during net tows on this last cruise, incubations contained all morphologies present: small puffs, large puffs, and loose tufts. No data are available for the morphologies of colonies used in experiments during August 2010.

DNA samples were only collected during the August 2012 cruise. Sequencing of PCR-amplified *hetR* genes revealed the majority of sequences (184 of 249) belonging to the genus *Trichodesmium.* These sequences grouped with three (of four total) major *Trichodesmium* clades (Lundgren et al. 2005; Fig. 6). Sequences grouping within each clade

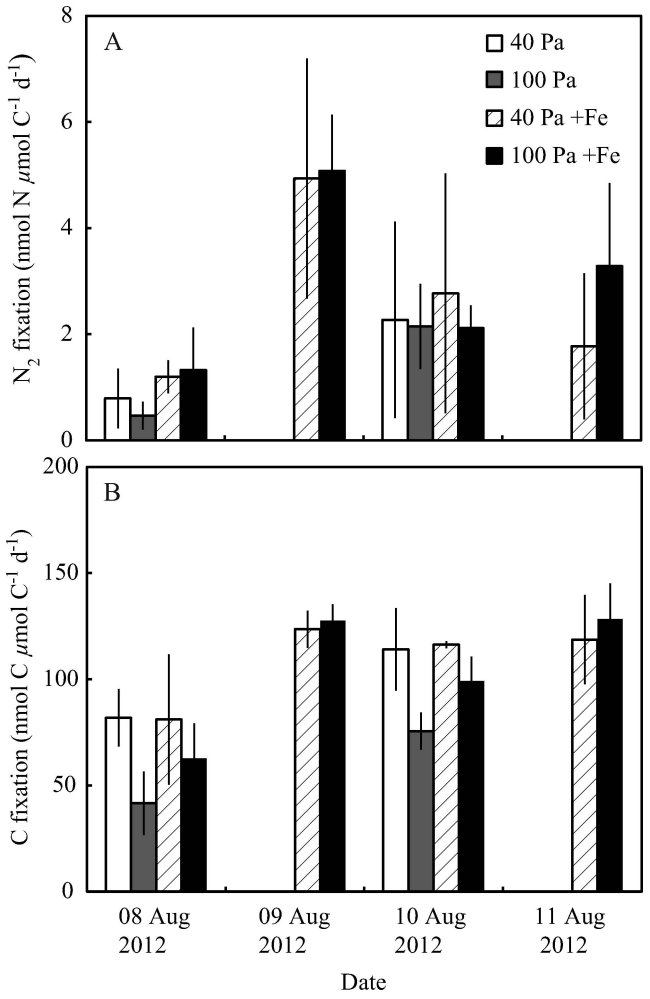

Fig. 2. Rates of (A) N_2 and (B) C fixation by *Trichodesmium* colonies under ambient (37 Pa) and elevated (82 Pa) P_{CO_2} conditions with and without Fe amendments. Rates are from $^{15}N_2$ and ^{13}C assimilation on the August 2012 cruise and are normalized to total PC content within each bottle. Error bars represent standard deviations of triplicate incubation bottles.

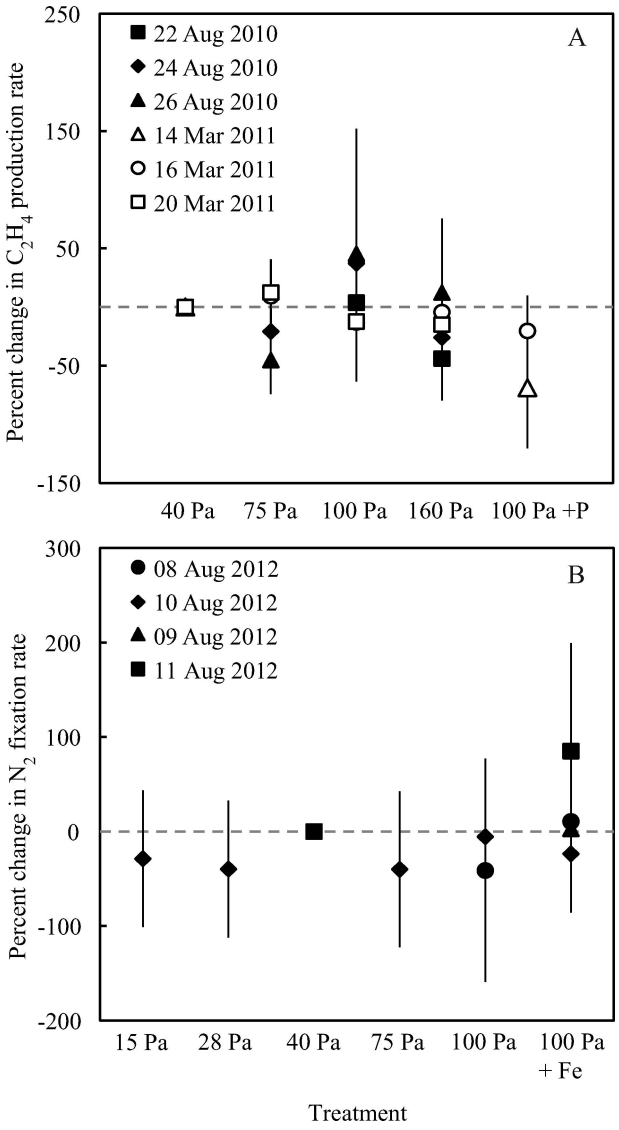

Fig. 3. Effect of P_{CO_2} and nutrient manipulations on *Trichodesmium* (A) AR and (B) $^{15}N_2$ assimilation. Rates are expressed as percent change relative to the control treatments (37 or 42 Pa). The elevated P_{CO_2}, nutrient-replete (+Fe or +P) treatments are relative to nutrient-replete (+Fe or +P) control (37 or 42 Pa) treatments. Error bars represent propagated standard deviations of duplicate or triplicate incubation bottles.

shared > 96% *hetR* gene sequence identity. Approximately 72% of the 184 sequences grouped with *Trichodesmium* clade I, which contains strains of *T. thiebautii, T. tenue* Z-1, *K. spiralis, K. pelagica,* and *T. hildebrandtii* (Hynes et al. 2012). The majority of these clade I DNA sequences (54% of the *hetR Trichodesmium* sequences) were > 99% similar to *T. thiebautii* strain H9-4 (Fig. 6). Approximately 27% of the *hetR Trichodesmium* gene sequences grouped with clade III, which contains *T. erythraeum* and some *T. contrortum* strains (Hynes et al. 2012). Over 98% of these clade III *hetR* genes were > 99% similar to *T. erythraeum* strains IMS101 and GBRTRLI101. Very few (< 2%) *hetR Trichodesmium* gene sequences grouped among the clade IV (Fig. 6). The phylogenetic representation among the 16S and ITS gene sequences showed similar patterns in *Trichodesmium* diversity as those obtained based on the *hetR* gene (Table 5). We focused our phylogenetic analyses on *hetR* sequences because this gene has been previously utilized to distinguish among the different groups of *Trichodesmium* (Lundgren et al. 2005; Hynes et al. 2012) and because we have the largest number of *hetR* sequences (Table 5).

The community composition of *Trichodesmium* in our samples varied between days (Fig. 7). Members of clade IV *Trichodesmium* were only detected on 09 August 2012. 09 August and 10 August contained nearly equal fractions of clade I and clade III *Trichodesmium.* On 12 August, *Trichodesmium* were dominated by clade I (53 out of 58 sequenced clones). However, the high variability between duplicate filters resulted in there being no significant differences among the *Trichodesmium* clades collected on different days (two-way ANOVA, $p > 0.05$).

Each set of primers we used targeted a slightly different group of organisms (Table 4), and thus showed different

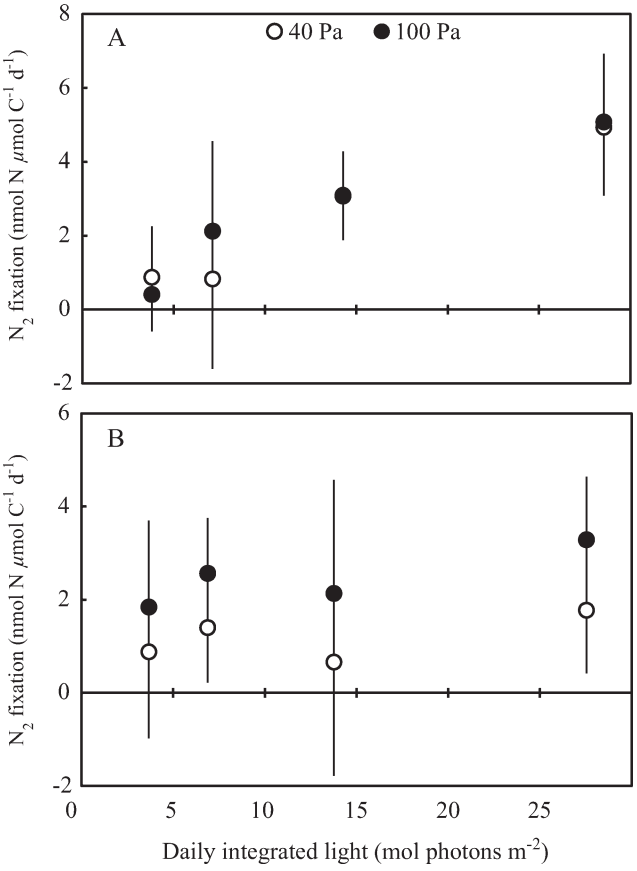

Fig. 4. *Trichodesmium* N_2 fixation rates at ambient (37 Pa, white symbols) and elevated (82 Pa, black symbols) P_{CO_2} across a range of daily light flux. All treatments were P- and Fe-replete. (A) and (B) show two independent trials on 09 and 11 August 2012, respectively. Error bars represent standard deviations of triplicate incubation bottles.

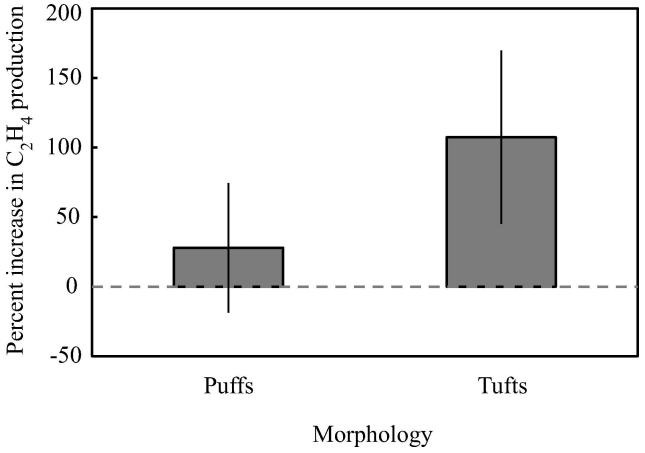

Fig. 5. Effect of elevated P_{CO_2} (108 Pa) on AR by *Trichodesmium* colonies with puff and tuft morphologies. Rates are shown as percentage change compared with ambient P_{CO_2} (42 Pa). Duplicate incubation bottles were used for all except the 108 Pa puffs treatment, which had a single bottle (*see* Methods). Error bars represent propagated error from the linear regression fit for each treatment.

patterns of *Trichodesmium*-associated organisms. Overall, we observed diverse assemblages of organisms associated with our samples. While most of the sequences from *hetR* and ITS corresponded to *Trichodesmium,* over half of the sequences from the 16S rRNA gene, which we amplified using a cyanobacterial-specific set of oligonucleotide primers, were phylogenetically related to cyanobacteria other than *Trichodesmium* (Table 5). Furthermore, among the 92 *nifH* gene sequences obtained from these colonies, only ~ 20% appeared derived from *Trichodesmium.*

To quantify the contributions of several groups of *nifH*-containing microorganisms within the *Trichodesmium* colonies, we utilized qPCR with primers targeting six different groups of N_2-fixing cyanobacteria and one Cluster III *nifH* gene sequence belonging to an organism previously described at Sta. ALOHA (Church et al. 2005) and prevalent in our *nifH* gene clone library. The resulting qPCR measurements, when normalized to the total DNA concentration per filter, show that the ratio of *Trichodesmium* to total *nifH*-containing organisms examined varied between days: on 09 August, 60% of the *nifH* genes targeted belonged to *Trichodesmium;* on 10 August, 73% were *Trichodesmium;* and on 12 August, 98% were *Trichodesmium* (Fig. 8). Next to *Trichodesmium,* the majority of *nifH*-containing organisms examined within our samples were derived from UCYN-B (*Crocosphaera* spp.) and Het1 (*Richelia* spp.). We were surprised to note the small number of Cluster III *nifH* gene copies, because sequences closely related to Cluster III represented 35% of *nifH* clone library sequences (data not shown). The different trends shown for Cluster III by clone libraries and qPCR likely reflect amplification biases associated with these *nifH* sequence-types and the primers utilized in the nested PCR reactions.

Discussion

During the period in which our experiments took place, the *Trichodesmium* assemblage did not exhibit a consistent response in N_2 or C fixation to elevated CO_2 conditions (Figs. 1–3). These results are in contrast with several laboratory studies, which report a CO_2 enhancement of N_2 and C fixation by *Trichodesmium* strains IMS101 and GBRTRLI101 grown in YBCII media (Barcelos e Ramos et al. 2007; Hutchins et al. 2007; Levitan et al. 2007). However, previous natural community experiments have shown mixed effects of CO_2 on diazotrophs: while some studies targeting *Trichodesmium* colonies have reported an enhancement of N_2 fixation due to increasing P_{CO_2} (Hutchins et al. 2009; Lomas et al. 2012), no relationship between elevated P_{CO_2} and N_2 fixation rates was observed in other studies conducted with naturally occurring diazotroph assemblages (Law et al. 2012; Böttjer et al. pers. comm.). Reconciling these conflicting results is crucial in order to determine whether future OA will increase N_2 fixation, a key process in N-limited open-ocean regions, sufficiently to produce ecological or biogeochemical implications.

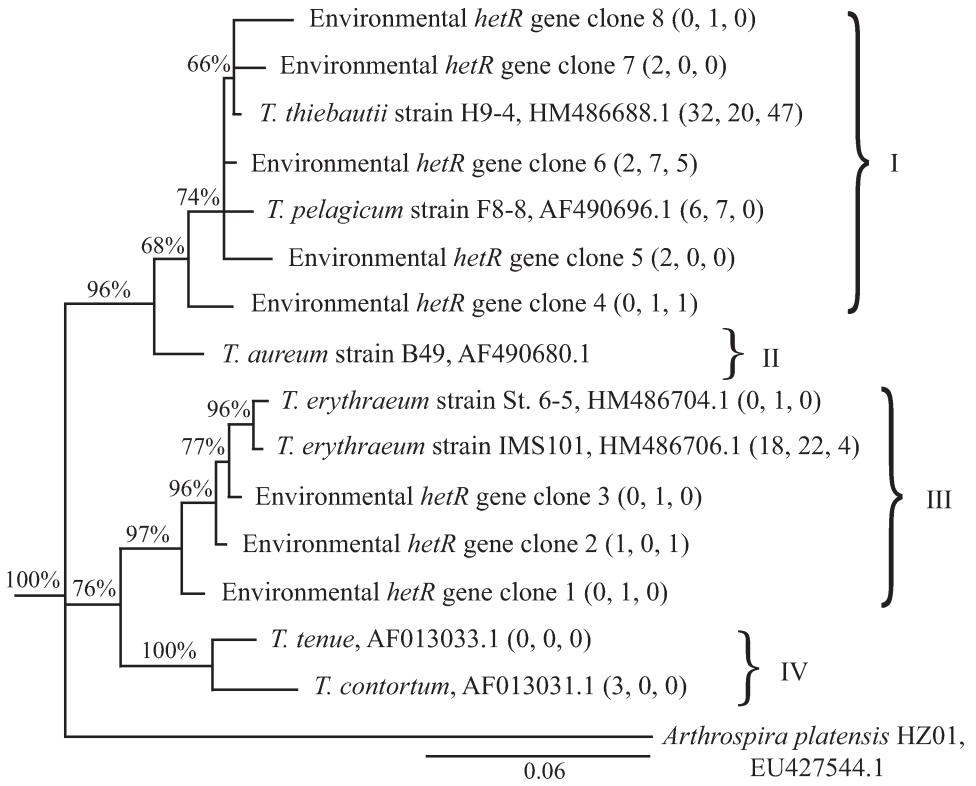

Fig. 6. Neighbor-joining phylogenetic tree depicting the relationships between *Trichodesmium hetR* gene sequences obtained from the current study together with representative sequences from cultivated strains (accession numbers given). Sequences with > 99% identical DNA were grouped together. Bootstrap support (values > 50%) for nodes are shown. Numbers in parentheses indicate the number of clones from 09, 10, and 12 August 2012, respectively. The four major clades of *Trichodesmium* phylotypes identified by Lundgren et al. (2005) are shown in roman numerals.

After our initial experiments in August 2010 found that CO_2 enhancement did not affect N_2 fixation by *Trichodesmium* at Sta. ALOHA, our efforts were aimed at testing whether the apparent discrepancy between laboratory and field studies was due to environmental conditions such as nutrients and light, experimental methodologies, or community composition. Ultimately, the results from these experiments suggest that the diversity within the community of *Trichodesmium* and colony-associated organisms at Sta. ALOHA may be the underlying reason for the lack a consistent, detectable CO_2 enhancement of N_2 fixation in the field. In this context, a recent study by Hutchins et al. (2013) has shown a six-fold range in the carbon affinity of diverse *Trichodesmium* strains. The diversity and relative abundance of DNA sequences in *Trichodesmium* colonies from our August 2012 cruise may explain the lack of CO_2 stimulation and the overall biological variability seen in our results, assuming the sampled population represented species with a range of CO_2 affinities.

Table 4. Summary of primers used for PCR amplification of 16S rRNA, ITS, *hetR,* and *nifH* genes. for, forward primer; rev, reverse primer.

Genetic marker	Sequence	Primers	Primer targets	Sequence (5′-3′)
16S	for	CYA-106F	cyanobacteria	CGGACGGGTGAGTAACGCGTGA
16S	rev	CYA-781R	cyanobacteria	GACTACTGGGGTATCTAATCCCATT
ITS	for	tri16S-1247F	*Trichodesmium*	CGTACTACAATGGTTGGG
ITS	rev	tri-23SR	*Trichodesmium*	TTCGCTCACCGCTACA
hetR	for	PH1	heterocyst differentiation gene	TGYGCKATTTAYATGACCTA
hetR	rev	PH2	heterocyst differentiation gene	ATGAANGGTATKCCCCAAGGA
nifH (outer)	for	nifH3	nitrogenase reductase gene	ATRTTRTTNGCNGCRTA
nifH (outer)	rev	nifH4	nitrogenase reductase gene	TTYTAYGGNAARGGNGG
nifH (inner)	for	nifH1	nitrogenase reductase gene	TGYGAYCCNAARGCNGA
nifH (inner)	rev	nifH2	nitrogenase reductase gene	ADNGCCATCATYTCNCC

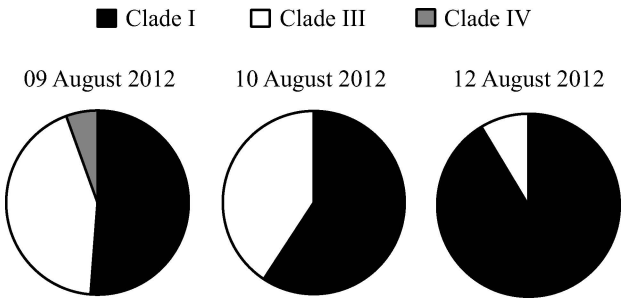

Fig. 7. Major clades of *Trichodesmium* based on *hetR* gene sequences from the field colonies over a 4 d period. Clade designations are based on *hetR* gene phylogeny compared with sequences from GenBank. $n = 184$ total *hetR Trichodesmium* sequences.

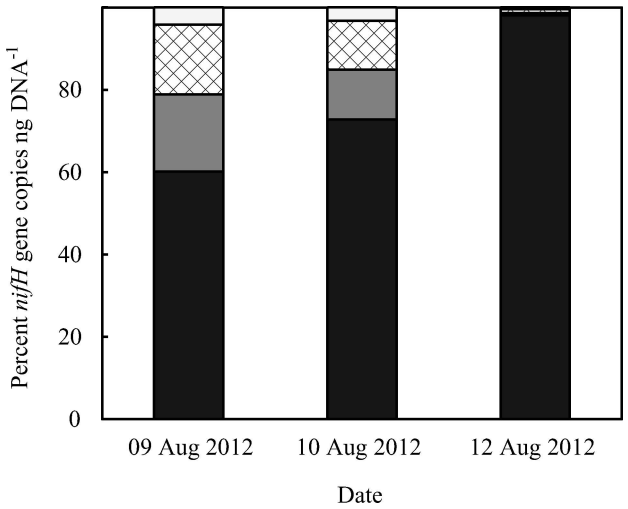

Fig. 8. *nifH* gene copies from *Trichodesmium* (black), *Crocosphaera* (grey), *Richelia* (hatched), and the sum contribution of UCYN-A, Het2, Het3, and Cluster III phylotypes (white). Values are normalized to DNA extract concentrations and presented as percentages of the total quantified phylotypes.

Mechanistically, enhanced P_{CO_2} has been linked to changes in the CCM of *Trichodesmium* IMS101 (Kranz et al. 2009, 2010); reallocation of ATP and reductants from the CCM is thought to provide the extra energy needed for increased rates of N_2 and C fixation (Kranz et al. 2011). CO_2 also changes the ratios of certain protein pools in *Trichodesmium;* for example, higher P_{CO_2} has been shown to increase the photosystem I : photosystem II ratio, possibly implying increased cyclic electron transport (Levitan et al. 2010). However, elevated P_{CO_2} does not appear to increase the intracellular nitrogenase protein pool, suggesting that the specific activity of the nitrogenase protein increases with P_{CO_2} (Levitan et al. 2010). Certain environmental and experimental conditions may regulate these physiological responses to CO_2 perturbations. For example, because light provides the ultimate source of energy for C fixation, N_2 fixation, and CCM operation, light levels would be expected to moderate the effect of P_{CO_2} on C and N_2 fixation. Indeed, light has been shown to affect the response of *Trichodesmium* to P_{CO_2}, with the largest enhancements observed under low light intensities (Kranz et al. 2010; Garcia et al. 2011). Ample nutrient conditions may also be a prerequisite for CO_2 stimulation of C and N_2 fixation; *Trichodesmium* N_2 fixation can be limited by the availability of P and Fe (Sañudo-Wilhelmy et al. 2001; Chappell et al. 2012). Finally, methodological choices may affect the CO_2 response; for example, elevated CO_2 has been shown to change the ratio of $^{15}N_2$ fixation to nitrogenase activity assayed via AR (Garcia et al. 2011), so the choice of measurements used in the field to estimate N_2 fixation rates could affect the interpretation of results. In light of these possibilities, we explored whether differences in environmental conditions, methodological approaches, or colony diversity might explain why we did not observe a CO_2 enhancement of N_2 or C fixation by the community of *Trichodesmium* at Sta. ALOHA.

Environmental conditions—Nutrient limitation appears to be an important control on rates of N_2 fixation by *Trichodesmium* and there is evidence that diazotrophs at Sta. ALOHA can be limited by the availability of P and Fe (Grabowski et al. 2008; Watkins-Brandt et al. 2011; Chappell et al. 2012). Hence, under P and/or Fe limitation, *Trichodesmium* N_2 and C fixation could be insensitive to a P_{CO_2} enhancement as a result of primary limitation by the availability of these nutrients. However, we found that the response of *Trichodesmium* assemblages to CO_2 was not significantly affected when amending treatments with these nutrients (Fig. 3). Because P has been shown to limit N_2 fixation at Sta. ALOHA when in situ SRP concentrations are below $\sim$ 40 nmol L^{-1} (Grabowski et al. 2008), the relatively high background P observed during our cruises (Table 1; *see* Results) may explain the absence of an observed P stimulation of N_2 fixation.

In contrast to P, concentrations of Fe during the August 2012 cruise appear typical for Sta. ALOHA (*see* results; Boyle et al. 2005). Recent studies have suggested that the effect of P_{CO_2} on diazotrophs is particularly sensitive to Fe availability. Reducing seawater pH changes Fe speciation, resulting in decreased phytoplankton Fe uptake, and N_2

Table 5. Summary of results from sequencing PCR-amplified *hetR,* 16S, ITS, and *nifH* genes. Total numbers of sequences for each gene are given by *n;* these sequences are further segregated into the four *Trichodesmium* clades (*see* Fig. 6) and other organisms associated with *Trichodesmium* colonies.

Gene	*n*	Total *Trichodesmium*	*Trichodesmium* clade I	*Trichodesmium* clade III	*Trichodesmium* clade IV	Associated organisms
hetR	249	184	132	49	3	65
16S	93	42	27	15	0	51
ITS	123	120	99	21	0	3
nifH	92	16	16	0	0	76

fixation is reduced under low-Fe conditions (Fu et al. 2008; Shi et al. 2012). However, in our study elevated P_{CO_2} did not statistically increase N_2 or C fixation rates in the Fe-amended treatments (Fig. 2).

A fundamental difference between our observations and results from previous laboratory studies is our use of natural seawater amended with only one nutrient at a time; most laboratory experiments use growth media that has been amended with a full suite of inorganic nutrients and trace metals. Thus, our observed lack of physiological enhancement under P- or Fe-enriched conditions could be explained by limitation of some other micronutrient. *Trichodesmium* isolates grown in amended seawater under more realistic nutrient concentrations have shown a negative response to P_{CO_2} (Shi et al. 2012), suggesting that the chemistry of the media used plays a key role when studying the response of *Trichodesmium* to OA.

As with P and Fe manipulations, changes in the daily light flux did not affect the response of *Trichodesmium* N_2 fixation to elevated P_{CO_2} (Fig. 4). These results contrast with laboratory experiments showing that CO_2 has a stronger effect on *Trichodesmium* N_2 fixation under low-light conditions (Kranz et al. 2010; Garcia et al. 2011). However, direct comparisons between our study and these previous studies are difficult due to differences in the quantity and quality of light provided. In the laboratory, cultures were grown under low light on a 12 h : 12 h light : dark cycle using white light; light fluxes for these experiments were 38 (Garcia et al. 2011) and 50 μmol photons m^{-2} s^{-1} (Kranz et al. 2010). In contrast, our incubations took place under natural sunlight (sinusoidal cycle with clouds) with blue screening and neutral density filters for bottles being shaded. The mean daily surface irradiance (photon flux) was 975 μmol photons m^{-2} s^{-1}, making the average light level in our lowest light treatment (8% of the surface irradiance) $\sim$ 78 μmol photons m^{-2} s^{-1}. Though this intensity is higher than the low-light conditions used by Kranz et al. (2010) and Garcia et al. (2011), it is still well below the light half-saturation constant reported for natural *Trichodesmium* colonies of $\sim$ 300 μmol photons m^{-2} s^{-1} (Kana 1992). However, because we utilized a mixture of blue and neutral density screens while Kranz et al. (2010) and Garcia et al. (2011) used white light, the photosynthetically usable radiation in those earlier studies may be substantially lower than in ours. At present we cannot discard the possibility that the light levels we used were insufficiently low to affect the response to P_{CO_2}.

Methodological approaches—Our results contrast not only with previous laboratory studies but also with *Trichodesmium* incubation field experiments in the Subtropical Atlantic (Lomas et al. 2012) and the Gulf of Mexico (Hutchins et al. 2009). In both of these studies, N_2 fixation was enhanced by elevated P_{CO_2}. However, key differences in the experimental design, methodologies, and environmental conditions in these studies may help explain the apparent discrepancy with our field observations. For example, Hutchins et al. (2009) found that elevating P_{CO_2} increased $^{15}N_2$ assimilation rates by 6–41%, but did not affect AR rates (Garcia et al. 2011). However, in our field experiments we observed no CO_2 enhancement of N_2 fixation rates based on AR or $^{15}N_2$ assimilation methods (Fig. 3).

Differences in replication and the manner in which N_2 fixation rates are normalized can also affect the comparison of results between research groups. While Hutchins et al. (2009) normalized rates of $^{15}N_2$ assimilation by *Trichodesmium* colonies to the volume of seawater sampled, Lomas et al. (2012) normalized rates per unit colony or colony C and found that elevated CO_2 enhanced N_2 fixation colony^{-1} in 9 out of 9 experiments but enhanced N_2 fixation colony C^{-1} in 7 out of 9 experiments. Furthermore, a lack of sample replication in the Lomas et al. experiments precludes an assessment of the statistical significance of the reported trends. Colony size in our experiments was extremely variable and incubations included *Trichodesmium* ranging from small, loose tufts to large puffs. For this reason, rates in our study are normalized to PC (for $^{15}N_2$ and C uptake experiments) or Chl *a* concentration (for AR experiments).

Finally, the environmental nutrient conditions in experimental treatments and these three distinct study regions may play a role in the observed field result discrepancies. While Lomas et al. (2012) amended all treatments from the Subtropical Atlantic with both P and Fe, Hutchins et al. (2009) did not include nutrient-amended P_{CO_2} treatments from the studies in the Gulf of Mexico and ambient nutrient concentrations in his study were not reported. In our study, P and Fe amendments did not significantly affect the response of *Trichodesmium* N_2 or C fixation rates to elevated P_{CO_2}. In addition, we did not see evidence that the *Trichodesmium* assemblage was P- or Fe-limited at ambient P_{CO_2}, a condition that is not always the case in the NPSG (Watkins-Brandt et al. 2011; Chappell et al. 2012). In summary, although differences in experimental design complicate the comparison of field experiments, at present none of these differences appears to be sufficient to explain the discrepancies between our results and those of Lomas et al. (2012) and Hutchins et al. (2009).

Diversity—We investigated whether the lack of a P_{CO_2} response could be explained by the diversity of N_2-fixing microorganisms within our samples, both in terms of nucleic acid sequences (analyzed in 2012) and colony morphology (recorded in 2011 and 2012). Though morphology may not be an accurate taxonomic predictor, *Trichodesmium* species have been historically associated with particular morphotypes (Hynes et al. 2012). Additionally, colony morphology may have a direct effect on CO_2 acquisition by controlling the surface area : volume ratio of colonies as well as the cohesiveness between filaments, thus affecting the rate of gas diffusion toward the center of colonies (Paerl and Bebout 1992). Indeed, on 15 March 2011 when the effect of elevated P_{CO_2} was tested on puff and tuft colonies separately, the tuft colonies showed a stronger response to elevated P_{CO_2} (Fig. 5). However, in subsequent experiments carried out on 16 and 20 March 2011 using only tuft colonies, P_{CO_2} did not affect AR (Fig. 1).

The relatively high coefficient of variation in rates of N_2 fixation among replicate treatments seemed to correlate

with the morphological diversity of *Trichodesmium* colonies utilized in incubations (Table 2). The lowest coefficient of variation for N_2 fixation rates (2%) was observed during the 16 and 20 March 2011 experiments when only tuft colonies were selected. In August 2010, when no preference was given to a particular morphology, the coefficient of variation was greater (17%). Finally, the highest variation was observed in August 2012 (76%), when, due to the low *Trichodesmium* abundance observed in the field, colonies of all sizes and forms, including loose tufts, were used for experiments. It is conceivable that the high variability in rates observed in August 2012 reflects variability in the diversity of *Trichodesmium* within incubation bottles.

In order to further explore the role that *Trichodesmium* diversity played in our observed N_2 and C fixation response to elevated P_{CO_2}, we analyzed nucleic acid sequences from colonies isolated on the August 2012 cruise. These analyses provide evidence for genetic variability and its potential role in the variability in C and N_2 fixation rates observed on these cruises. The observed variability in community composition between days (Figs. 7, 8) likely reflects small-scale spatial heterogeneity of *Trichodesmium* and associated microorganisms. Combining data from all sampling days shows *hetR Trichodesmium* gene sequences clustering with three out of the four major *Trichodesmium* clades (Fig. 6). Interestingly, a majority ($\sim$ 79% of total) of these sequences were $>$ 99% similar to isolates of two different *Trichodesmium* strains that have been used in laboratory CO_2 manipulation experiments. Approximately 24% of *hetR Trichodesmium* gene sequences grouped with *T. erythraeum* IMS101 and GBRTRLI101, strains that have both shown enhanced N_2 fixation rates at elevated P_{CO_2}. However, the majority of these sequences (54% of total clones sequenced) grouped with the strain *T. thiebautii* H9-4 (Fig. 6). A recent laboratory study comparing the effect of CO_2 on N_2 fixation by several previously untested *Trichodesmium* isolates found that the response to CO_2 varies between isolates: CO_2 half saturation constants ($K_{1/2}$) ranged from 6 Pa to 41 Pa, with the strain H9-4 having the lowest $K_{1/2}$ value of any strain tested. In contrast, strain IMS101 had one of the highest values measured in *Trichodesmium* (Hutchins et al. 2013).

The dominance of *hetR* gene sequences clustering close to *T. thiebautii* H9-4, as well as the diversity of total *Trichodesmium* sequences, may help explain our experimental results. If the distribution of *hetR* gene sequences reflects the variability of *Trichodesmium* CO_2 $K_{1/2}$, the dominance of *hetR* gene sequences similar to H9-4 suggests low CO_2 $K_{1/2}$ in our samples. If our interpretation is correct, samples collected during August 2012 were dominated by a strain of *Trichodesmium* that is fully saturated at present CO_2 conditions; even the last glacial maximum CO_2 treatments (17 Pa) would have been significantly above the CO_2 $K_{1/2}$ reported by Hutchins et al. (2013) for H9-4.

Furthermore, the community did not consist of only H9-4, but included *Trichodesmium* resembling strain IMS101 and other phylotypes within clades I, III, and IV of the major *Trichodesmium hetR* gene clades (Fig. 6). Unequal distribution of species into incubation bottles would contribute to the high variability we observed between replicates. Additionally, the differences in N_2 fixation rates observed between days probably result from changes in the community composition of *Trichodesmium* over space and time (Fig. 7). Nevertheless, a change in the dominant community can lead to a change in the mean $K_{1/2}$ observed in our samples and cause changes in N_2 fixation rates and response to long-term P_{CO_2} perturbations.

It is relevant to note the diversity and metabolic capacities of organisms other than *Trichodesmium* found within colonies. Quantitative PCR showed that on 09 August 2012, *Trichodesmium* accounted for only 60% of *nifH* genes from organisms targeted in this study; this fraction increased to 73% on 10 August and 98% on 12 August (Fig. 8). The other major constituents of the *Trichodesmium* colonies quantified included *Crocosphaera* spp. and *Richelia* spp. *Crocosphaera* did not likely contribute much to N_2 fixation rates, because the incubations were only during the light period and *Crocosphaera* fixes N_2 in the dark. We do not have data to show whether *Richelia* or the other *nifH* organisms were actively fixing N_2 during the incubations. However, our data suggest that on 09 August, organisms other than *Trichodesmium* may have contributed substantially to the N_2 fixation rates. It is also likely that our samples contained substantial fractions of other diazotrophs that we did not target with qPCR primers; thus, the percentages of *nifH* genes belonging to *Trichodesmium* and other groups presented in Fig. 8 represent upper limits. Our incubations also contained organisms other than *Trichodesmium* with the ability to fix C; for instance, both *Crocosphaera* and *Richelia* are N_2-fixing cyanobacteria. Additionally, based on 16S rRNA gene sequences, which specifically targeted cyanobacteria, only 45% of clones were derived from *Trichodesmium* (Table 5). Though clone library data may not be quantitatively reliable, these results suggest that organisms other than *Trichodesmium* contributed to C fixation during our experiments. In summary, it is important to consider that while our study targeted *Trichodesmium,* we measured community rates normalized to community PC or Chl *a.* Elevated P_{CO_2} did not enhance C or N_2 fixation rates by this community.

Ecological significance and future directions—Our overall results show no evidence that *Trichodesmium* community N_2 or C fixation responds rapidly to abrupt shifts in P_{CO_2}. These results are in agreement with two independent studies of whole diazotrophic communities in the North and South Pacific Gyres, which have observed no increase in N_2 fixation rates with elevated P_{CO_2} (Law et al. 2012; Böttjer et al. pers. comm.), and with conceptual suggestions that microbial communities may be relatively resilient to OA (Joint et al. 2011). Our results may be explained by both the dominance of *T. thiebautii* in the NPSG and the diversity of diazotrophs associated with *Trichodesmium* colonies. Globally, *T. thiebautii* is often more abundant than *T. erythraeum* (Marumo and Nagasawa 1976; Carpenter and Price 1977); thus, the lack of P_{CO_2}

enhancement observed in our study may represent the response of diazotrophic communities in many ocean regions. However, one important question that our experimental approach was not able to address is how OA will affect diazotrophic communities on longer timescales. For CO_2 perturbations on the timescale of days, certain species may acclimate through physiological changes that allow them to benefit from elevated P_{CO_2}. Over longer, multiyear timescales, it is possible that elevated P_{CO_2} conditions will favor the growth of certain species, such as *T. erythraeum,* which are able to increase growth rates with elevated P_{CO_2}. For this reason, further experiments addressing the species-specific responses to P_{CO_2}, the environmental regulation of these responses, and the possibility for natural selection over long timescales are needed in order to improve our capabilities to model and predict how microbial assemblages and processes will change in response to long-term changes in OA.

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