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Key Points:
- Short-term responses of diazotrophs to increases in seawater pCO_2 are described
- N_2 fixation rates, *nifH* gene abundances and transcripts were largely unaffected
- Diazotrophs in the North Pacific Subtropical Gyre appear relatively resilient

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Experimental assessment of diazotroph responses to elevated seawater pCO_2 in the North Pacific Subtropical Gyre

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Abstract We examined short-term (24–72 h) responses of naturally occurring marine N_2 fixing microorganisms (termed diazotrophs) to abrupt increases in the partial pressure of carbon dioxide (pCO_2) in seawater during nine incubation experiments conducted between May 2010 and September 2012 at Station ALOHA (A Long-term Oligotrophic Habitat Assessment) (22°45′N, 158°W) in the North Pacific Subtropical Gyre (NPSG). Rates of N_2 fixation, nitrogenase (*nifH*) gene abundances and transcripts of six major groups of cyanobacterial diazotrophs (including both unicellular and filamentous phylotypes), and rates of primary productivity (as measured by ^{14}C-bicarbonate assimilation into plankton biomass) were determined under contemporary (~390 ppm) and elevated pCO_2 conditions (~1100 ppm). Quantitative polymerase chain reaction (QPCR) amplification of planktonic *nifH* genes revealed that unicellular cyanobacteria phylotypes dominated gene abundances during these experiments. In the majority of experiments (seven out of nine), elevated pCO_2 did not significantly influence rates of dinitrogen (N_2) fixation or primary productivity (two-way analysis of variance (ANOVA), $P > 0.05$). During two experiments, rates of N_2 fixation and primary productivity were significantly lower (by 79 to 82% and 52 to 72%, respectively) in the elevated pCO_2 treatments relative to the ambient controls (two-way ANOVA, $P < 0.05$). QPCR amplification of *nifH* genes and gene transcripts revealed that diazotroph abundances and *nifH* gene expression were largely unchanged by the perturbation of the seawater pCO_2. Our results suggest that naturally occurring N_2 fixing plankton assemblages in the NPSG are relatively resilient to large, short-term increases in pCO_2.

1. Introduction

Anthropogenic fossil fuel combustion and deforestation continue to alter both atmospheric and oceanic carbon dioxide (CO_2) inventories [*Meehl et al.*, 2007]. In large regions of the world's oceans, the partial pressure of carbon dioxide (pCO_2) in seawater is increasing at a rate similar to atmospheric pCO_2 [*Keeling et al.*, 2004; *Santana-Casiano et al.*, 2007; *Dore et al.*, 2009; *Bates et al.*, 2012]. Changes in the ocean carbonate system have significantly decreased upper ocean pH [*Doney et al.*, 2008; *Dore et al.*, 2009; *Feely et al.*, 2009]. By the year 2150, atmospheric pCO_2 is predicted to have tripled relative to contemporary conditions (increasing from ~390 ppm to >1100 ppm), with a concomitant decrease in surface ocean pH of ~0.5 units [*Caldeira and Wickett*, 2003]. In addition to long-term (decadal-scale) anthropogenic changes in seawater CO_2, various naturally occurring physical and biological processes also impart higher frequency variability on the seawater carbonate system. For example, diurnal to seasonal-scale heating and cooling of the upper ocean, or vertical entrainment of dissolved inorganic carbon (DIC)-enriched deep waters through processes such as upwelling or convective mixing can all influence seawater pCO_2 and pH [*Feely et al.*, 1999; *Dore et al.*, 2003; *Bates*, 2007]. Biological processes such as photosynthesis, respiration, and calcification also alter the seawater carbonate system. However, despite considerable research, there remains uncertainty regarding how progressive or abrupt changes in the ocean carbonate system influence marine ecosystems and biogeochemical cycles [*Doney et al.*, 2008; *Joint et al.*, 2011].

Nitrogen (N) is an essential element for biological production in both terrestrial and marine environments [*Vitousek and Howarth*, 1991]. The most abundant form of N in the biosphere is dinitrogen gas (N_2). However, the biological capacity to reduce N_2 to ammonia (NH_3), a process referred to as N_2 fixation, is governed by a restricted group of prokaryotes (termed diazotrophs). In the marine environment, diazotrophs are genetically, physiologically, and ecologically diverse, including free-living and/or symbiotic, filamentous, and unicellular microorganisms [*Carpenter and Capone*, 2008; *Sohm et al.*, 2011]. Biological N_2 fixation represents

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an important supply of new N to the oligotrophic regions of the open ocean, thereby partly regulating net community production and carbon export [*Capone et al.*, 2005]. In the North Pacific Subtropical Gyre, supply of fixed N by N_2 fixation is estimated to be comparable to the delivery of N via mixing, upwelling, and/or advection [*Karl et al.*, 1997; *Dore et al.*, 2002; *Johnson et al.*, 2010]. As a result, oceanic diazotrophs play functionally important roles in marine biogeochemistry.

Most of our understanding of how diazotrophs might respond to future changes in the ocean's carbonate system stems from laboratory studies using cultivated model microorganisms. Such studies have demonstrated that rates of N_2 fixation by cyanobacteria belonging to the genera *Trichodesmium* and *Crocosphaera*, are sensitive to increases in pCO_2, with rates of N_2 fixation increasing between 30% to nearly 200% with increases in pCO_2 over the range of 750 to 1000 ppm [*Barcelos e Ramos et al.*, 2007; *Hutchins et al.*, 2007; *Levitan et al.*, 2007; *Fu et al.*, 2008]. Similarly, field-based manipulation experiments conducted in both the subtropical Atlantic and the Gulf of Mexico using isolated ("hand-picked") *Trichodesmium* colonies from net tows observed 6 to 156% enhancement in rates of N_2 fixation during short-term ($<$24 h) pCO_2 enrichment (750 and 800 ppm) experiments [*Hutchins et al.*, 2009; *Lomas et al.*, 2012].

In contrast to these studies demonstrating increases in N_2 fixation, others have reported that diazotroph growth and rates of N_2 fixation were either unchanged or inhibited under conditions of elevated pCO_2. A laboratory study using the heterocyst-forming diazotroph *Nodularia* (a bloom-forming diazotroph dwelling in brackish, relatively eutrophic inland aquatic ecosystems) revealed that rates of N_2 fixation decreased by approximately 40% with increases in pCO_2 (750 ppm [*Czerny et al.*, 2009]). In a series of experiments conducted using whole seawater collected in the subtropical South Pacific Ocean, where the diazotroph assemblages were dominated by a group of uncultivated unicellular cyanobacteria referred to as UCYN-A, rates of N_2 fixation were largely unaffected by increased pCO_2 (750 ppm [*Law et al.*, 2012]). Similarly, a recent study by *Gradoville et al.* [2014] demonstrated no enhancement of N_2 fixation by *Trichodesmium* colonies isolated from the North Pacific Subtropical Gyre (NPSG) in response to increased seawater pCO_2 (up to ~2000 ppm).

In this study, we sought to quantify the short-term (24 to 72 h) physiological responses of diverse, naturally occurring N_2 fixing marine cyanobacteria to abrupt increases in seawater pCO_2. We conducted a series of shipboard CO_2 enrichment experiments at Station ALOHA (A Long-term Oligotrophic Habitat Assessment), the field site for the Hawaii Ocean Time-series (HOT) program in the NPSG. We experimentally examined responses in rates of N_2 fixation and primary productivity, together with changes in nitrogenase gene (*nifH*) abundances and transcription, to pCO_2 enrichments (targeting ~1100 ppm). Overall, we observed no consistent response in diazotroph activities in the pCO_2-enriched treatments. Our results provide new information on possible implications of a changing ocean carbon cycle on the dynamics of a functionally important group of marine microorganisms.

2. Methods

2.1. Sample Location, Collection, and Experimental Setup

Carbonate system perturbation experiments were conducted aboard the R/V *Ka'imikai-O-Kanaloa* and R/V *Kilo Moana* during several cruises to the field site Station ALOHA between May 2010 and September 2012. Near-surface seawater (~250 L from 5 m depth) was collected prior to midnight using polyvinyl chloride sampling bottles affixed to a conductivity-temperature-depth rosette. Seawater was subsampled under subdued light into acid-washed 20 L polycarbonate carboys fitted with sterile, gas-vented caps. After filling, carboys were placed in outdoor, shaded (~50 % surface irradiance), and surface seawater-cooled incubators. Care was used in setting up and sampling the experiments to minimize trace nutrient contamination; however, none of the experiments were conducted under rigorous trace metal clean conditions. For most experiments, target pCO_2 levels (~390 or ~1100 ppm) were achieved by gently bubbling the seawater in the carboys with either air (controls, 390 ppm) or a mixture of air and CO_2 (treatments, 1100 ppm) for approximately 6–8 h at 3 L min^{-1} until the desired, elevated pCO_2 was attained. Seawater pH was measured at regular intervals over this 6–8 h period and used to compute the approximate seawater pCO_2 using CO2SYS software [*Lewis and Wallace*, 1998]. When the targeted pCO_2 was reached (predawn), the flow rate of the air-CO_2 mix was adjusted to maintain near-constant seawater pCO_2 for the duration of the experiments ($<$1.5 L min^{-1}). Subsequently, samples for determinations of total alkalinity (TA), dissolved inorganic carbon

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(DIC), ^{14}C-based primary productivity, chlorophyll *a* (Chl *a*) concentrations, rates of N_2 fixation, and diazotroph *nifH* gene abundances and transcripts were collected from each carboy just before dawn (representing the first sampling time point, T_0) as described in the following section 2.2. Typically, two additional time points were sampled during each experiment (hence, the resulting sampling points are reported as T_0, T_{24}, T_{48}, or T_{72}). Subsamples were withdrawn from the carboys by blocking the vent on the carboy caps and utilizing the resulting positive pressure to draw seawater through silicone tubing.

We also conducted a single experiment (September 2012) to test whether rates of N_2 fixation and C fixation were sensitive to "bubbling" as a means of modifying the carbonate system. For this experiment, bubbled treatments consisted of whole seawater (triplicate 20 L carboys) bubbled with air or mixtures of air and CO_2 (as described above) to achieve the targeted pCO_2 of ~390 and 1100 ppm. Nonbubbled treatments consisted of whole seawater (triplicate 20 L carboys) where trace metal grade hydrochloric acid (together with sodium bicarbonate) was added to increase pCO_2 to ~ 1100 ppm while minimizing changes in seawater TA. Controls (~390 ppm) for the nonbubbled treatments consisted of unperturbed whole seawater in triplicate carboys held in the same seawater incubators. DIC and TA were measured at each time point to examine possible daily scale variations in pCO_2.

2.2. Total Alkalinity, Dissolved Inorganic Carbon, and pH

Seawater samples for determinations of TA, DIC, and pH were collected and analyzed following HOT program procedures and methodologies [*Winn et al.*, 1998; *Dore et al.*, 2009]. DIC and TA samples were collected into 300 mL, precombusted borosilicate bottles and immediately preserved with 100 μL of saturated mercuric chloride ($HgCl_2$) for subsequent analysis in the laboratory. TA was determined using an automated, closed-cell potentiometric titration and DIC was measured coulometrically using a Versatile INstrument for the Determination of Total inorganic carbon and titration Alkalinity 3S system [*Dickson et al.*, 2007]. Accuracy and precision of the DIC and TA measurements were validated through analysis of certified seawater CO_2 reference material [*Dickson*, 2001], with a resulting accuracy of approximately 1 μmol L^{-1} and 3 μmol L^{-1}, respectively. Salinity-normalized values for DIC and TA were calculated as follows: nDIC = (35 × DIC)/(measured salinity) and nTA = (35 × TA)/(measured salinity). Seawater pH (measured at 25°C) was analyzed by the spectrophotometric detection of *m*-cresol purple with a precision of 0.001 [*Clayton and Byrne*, 1993]. To examine seasonal variability in the carbonate system at Station ALOHA, long-term (1988–2011) trends in seawater pCO_2, pH, nDIC, and nTA (determined by least squares linear regression analyses) were subtracted from the time series data.

2.3. ^{14}C-based Primary Productivity (C Fixation) and Concentrations of Chlorophyll *a*

We used the ^{14}C-radiotracer method to measure the assimilation of inorganic carbon by phytoplankton as an estimate of the rate of primary productivity [*Steeman-Nielsen*, 1952], following the procedures described by *Letelier et al.* [1996]. For every time point of our experiments, seawater was subsampled prior to dawn from each experimental carboy into clean, acid-washed 500 mL polycarbonate bottles. Each sample bottle received 250 μL of a ^{14}C-sodium carbonate stock solution to yield final activity of ~ 0.05–0.1 μCi mL^{-1}. Sample bottles were incubated in outdoor, light-shaded (~50% surface irradiance), seawater-cooled incubators for the entire photoperiod (generally ~12 h). After dusk, 250 μL of the incubated seawater was removed from each bottle and placed into 20 mL glass scintillation vials containing 500 μL β-phenethylamine for subsequent determination of the total radioactivity added to each sample. The remaining seawater was filtered through a 25 mm diameter glass microfiber (GF/F) filter and the filters were placed in scintillation vials and stored at −20°C. At the shore-based laboratory, filters were acidified by the addition of 1 mL of 2 M hydrochloric acid and allowed to passively vent in a fume hood for 24 h. To determine the ^{14}C activity, scintillation vials received 10 mL of liquid scintillation cocktail (Ultima Gold©) and were counted in a liquid scintillation counter (Packard model 4640). Estimates of carbon fixation rates based on ^{14}C activity were computed based on measured DIC concentrations for all treatments, accounting for changes in seawater carbonate chemistry resulting from pCO_2 manipulations. Seawater samples for subsequent determinations of Chl *a* concentrations (150 mL) were collected at all time points from each carboy and filtered onto 25 mm diameter GF/F filters. Following filtration, filters were transferred into 10 mL glass screw cap tubes containing 5 mL of cold (−20°C) 100% acetone. Tubes were wrapped in aluminum foil, stored at −20°C for approximately 7 days to passively extract pigments [*Letelier et al.*, 1996]. Back in the shore-based laboratory, the samples were analyzed by fluorometry [*Holm-Hansen et al.*, 1965].

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2.4. Inorganic Nutrients

Samples for analyses of inorganic nutrient concentrations were subsampled from each carboy into clean, acid-washed 500 mL polyethylene bottles and immediately frozen upright at −20°C until analyses. At the shore-based laboratory, samples were thawed and split for measurements of nitrate plus nitrite ($NO_3^- + NO_2^-$), phosphate (PO_4^{3-}), and silicic acid ($Si(OH)_4$). High-sensitivity analytical techniques were chosen for [PO_4^{3-}] and [$NO_3^- + NO_2^-$] because concentrations of these nutrients at Station ALOHA are at or below the detection limits of standard autoanalyzer methods [*Karl et al.*, 2001]. Samples for the determination of [$NO_3^- + NO_2^-$] were analyzed using the chemiluminiscent method described by *Garside* [1982] as modified by *Dore and Karl* [1996]. [PO_4^{3-}] was determined using the MAGnesium Induced Coprecipitation assay [*Karl and Tien*, 1992]. [$Si(OH)_4$] was quantified following the procedures described by *Hansen and Koroleff* [1999].

2.5. N_2 Fixation Rates, Gene Abundances, and Transcripts of Diazotrophs

Rates of N_2 fixation were assessed by the $^{15}N_2$ isotopic tracer technique [*Montoya et al.*, 1996]. For experiments conducted between May 2010 and April 2011, whole seawater samples were subsampled from each carboy into clean, acid-washed 4.5 L polycarbonate bottles without introducing air bubbles. Bottles were sealed with caps fitted with silicone septa. After capping, 3 mL of $^{15}N_2$ gas (98%; Isotech Laboratories®) was injected into each polycarbonate bottle through the silicone septum using a gas-tight syringe and a stainless steel needle. However, a previous study [*Mohr et al.*, 2010] reported underestimation of rates of N_2 fixation by these standard procedures, a finding these authors attributed to the time required for the $^{15}N_2$ gas bubble to equilibrate with the seawater. Hence, for the experiment carried out in September 2012, the $^{15}N_2$ gas was first dissolved into seawater (prepared as described in *Wilson et al.* [2012]) and the resulting $^{15}N_2$-enriched water was added to 4.5 L polycarbonate sampling bottles. Regardless of the method used to estimate rates of N_2 fixation (adding $^{15}N_2$ gas in gaseous or dissolved form) in the present study, rates of N_2 fixation in the pCO_2 perturbed treatments were compared to controls, which were measured by identical procedures during each individual experiment. All $^{15}N_2$-enriched bottles were incubated for 24 h in the same surface seawater shaded incubator previously described. After the incubation period, the entire volume was filtered onto a precombusted 25 mm diameter, GF/F filter and subsequently stored frozen at −20°C. In the shore-based laboratory, these filters were dried for 24 h at 60°C and pelleted, and ^{15}N assimilation on each filter was analyzed with an elemental analyzer-isotope ratio mass spectrometer (Carlo-Ebra EA NC2500®). Rates of N_2 fixation were calculated as described in *Capone and Montoya* [2001].

In addition, whole seawater samples (4 L) were collected from each carboy into clean, acid-washed 4 L polycarbonate bottles for subsequent determination of cyanobacterial *nifH* genes and transcripts. Approximately 2 L each was filtered through 25 mm diameter 0.22 μm Durapore filters (Millipore®) to concentrate cells for subsequent extraction of DNA and RNA, respectively; nucleic acid filtrations were typically completed within 45 min. Filters for subsequent RNA analysis were stored in microcentrifuge tubes containing 500 μL of a buffer containing 10 μL ß-mercaptoethanol to 1 mL Buffer RLT (Qiagen®). Filters for subsequent DNA analysis were kept in 500 μL buffer containing 20 m*M* Tris-HCl, pH 8.0; 2 m*M* EDTA, pH 8.0; 1.2% Triton X and 20 mg mL^{-1} lysozyme. Microcentrifuge tubes containing the filters and buffers were flash frozen in liquid nitrogen and kept at −80°C until extraction. In the shore-based laboratory, DNA and RNA were extracted from filters using the DNeasy kit (Qiagen®) or RNeasy Mini Kit (Qiagen®), respectively, following the manufacturer's protocols.

Determinations of diazotroph *nifH* gene abundances (gene copies L^{-1}) and *nifH* gene transcripts (gene transcripts L^{-1}) were assessed by quantitative polymerase chain reaction (QPCR). Six different *nifH* phylotypes were targeted by QPCR, including UCYN-A, *Crocosphaera* spp., *Trichodesmium* spp., and three types of heterocyst-forming cyanobacteria (termed Het1, Het2, and Het3) identified as deriving from *Richelia* spp. (Het1 and Het2) and *Calothrix* spp. (Het3 [*Foster and Zehr*, 2006]). These phylotypes are commonly retrieved from *nifH* clone libraries at Station ALOHA [e.g., *Church et al.*, 2009] and were amplified using group specific primers and probes as described in *Church et al.* [2005a, 2005b]. Both QPCR (for gene abundances) and reverse transcriptase (RT)-QPCR (for gene transcripts) reactions were performed in duplicate. For RT-QPCR, extracted mRNA was reverse transcribed (SuperScript III RT cDNA synthesis Kit, Invitrogen®) as described in *Church et al.* [2005a, 2005b], and the resulting cDNA was amplified in the QPCR reactions.

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Table 1. Near-Surface (5 m) Seawater Characteristics and Sampling Time Points for Each of the CO_2 Perturbation Experiments Between May 2010 and April 2011[a]

Date	Time points (hours)	T (°C)	PO_4^{3-} (nmol P L^{-1})	$NO^{3-}+NO^{2-}$ (nmol N L^{-1})	Chl *a* (μg L^{-1})	^{14}C-PP (μmol C L^{-1} d^{-1})	nDIC (μmol C L^{-1})	nTA (μmol L^{-1})	pH	pCO_2 (μatm)
May 2010	0, 24	24.3	75	1.5	0.08	0.52	2006	2327	8.081	356
June 2010	0, 24	24.7	61	2.3	0.07	0.56	2014	2335	8.076	391
July 2010	0, 24	25.8	64	2.0	0.09	0.70	1992	2324	8.072	379
August 2010	0, 24, 48	25.5	103	2.8	0.07	0.33	1996	2324	8.055	369
September 2010	0, 24, 48	25.6	84	4.0	0.07	0.48	1998	2321	8.063	404
October 2010	0, 48, 72	26.0	64	3.1	0.10	0.70	1997	2323	8.059	385
March 2011	0, 48	24.4	68	3.1	0.08	0.53	1996	2321	8.078	357
April 2011	0, 24, 72	24.0	103	5.1	0.11	0.42	1993	2311	8.068	339
September 2012	0, 24, 72	25.7	70	3.2	0.05	0.68	1990	2313	ND	ND

[a]Temperature (T), phosphate (PO_4^{3-}), nitrate + nitrite ($NO_3^- + NO_2^-$), chlorophyll *a* (Chl *a*), C fixation (^{14}C-PP), salinity-normalized dissolved inorganic carbon (nDIC), salinity-normalized total alkalinity (nTA), and partial pressure of carbon dioxide (pCO_2). ND = not determined.

2.6. Statistical Analysis

Two-way analysis of variance (two-way ANOVA) was utilized to identify statistical patterns, using treatments (ambient versus CO_2-elevated) and incubation time as factors, checking data for normality and heterogeneity of variance [*Zar*, 1998]. Standard deviations of the replicate treatments ($n = 3$) are reported whenever applicable.

3. Results

3.1. Habitat Characteristics and Diazotroph Community Structure at Station ALOHA

Short-term CO_2 perturbation experiments were conducted on nine cruises to Station ALOHA between May 2010 and September 2012 with the majority of experiments occurring during the spring and summer months (Table 1). Near-monthly HOT program sampling of this site since October 1988 provides robust temporal context in which to place our carbonate system perturbation experiments. The CO_2 perturbation experiments conducted for this study were initiated under conditions spanning the full annual range of surface ocean carbonate system properties observed at Station ALOHA (Figure 1). During our study period, seawater pCO_2 in the near-surface ocean (5 m) fluctuated between 339 and 404 μatm (Figure 1a). Detrended seawater pCO_2 revealed a seasonal pattern of elevated pCO_2 (>365 μatm) during the late summer and fall (August– October) when seawater temperatures peak (>25°C; Table 1), with lower pCO_2 during the winter months. Near-surface ocean pH varied by ~0.03 units during our sampling (ranging from 8.055 to 8.081), typical for the region. Removal of the long-term downward trend in seawater pH revealed a seasonal pattern in pH out of phase with pCO_2, with lowest pH observed in the summer when pCO_2 was maximal (Figure 1b). Detrended nDIC fluctuated ~10 μmol C L^{-1} over the study, varying between 1964 and 1974 μmol C L^{-1}, with elevated concentrations in the late winter and spring (Figure 1c). Consistent with the long-term HOT measurements, nTA varied little during our sampling, ranging between 2302 and 2315 μmol L^{-1} with no apparent seasonality (Figure 1d). In addition, the near-surface waters at Station ALOHA were persistently oligotrophic throughout our study, as reflected by low concentrations of inorganic nutrients (i.e., N + N was consistently <5 nmol N L^{-1}, PO_4^{3-} was <75 nmol P L^{-1}), and chlorophyll *a* (<0.15 μg L^{-1}; Figures 2a–2c and Table 1). Rates of ^{14}C-based carbon fixation in the near-surface ocean were typical for the site, ranging between 0.3 and 0.7 μmol C L^{-1} d^{-1} (Figure 2d, Table 1).

3.2. Stability and Variability of pCO_2 During Experiments

We measured DIC and TA at all sampling time points during the CO_2 perturbation experiments and used those two variables (as recommended by *Dickson* [2011]) to compute seawater pCO_2 in the control and CO_2 perturbed treatments (Table 2) using CO2SYS software. The pCO_2 in the control carboys varied between 336 ± 4 and 415 ± 7 μatm, but was relatively constant (varying <5%) over the course of any one experiment (Table 2). The pCO_2 attained in the perturbed treatments ranged from 920 ± 17 to 1144 ± 30 μatm, and temporal variations within each experiment were comparable to the control carboys. During the experiment conducted in September 2012, we perturbed the CO_2 system by adding hydrochloric acid (HCl) and sodium bicarbonate ($NaHCO_3$). However, the pCO_2 was not maintained throughout the remaining duration of the experiment (as compared to the experiments which were continuously aerated with mixtures of CO_2 and air),

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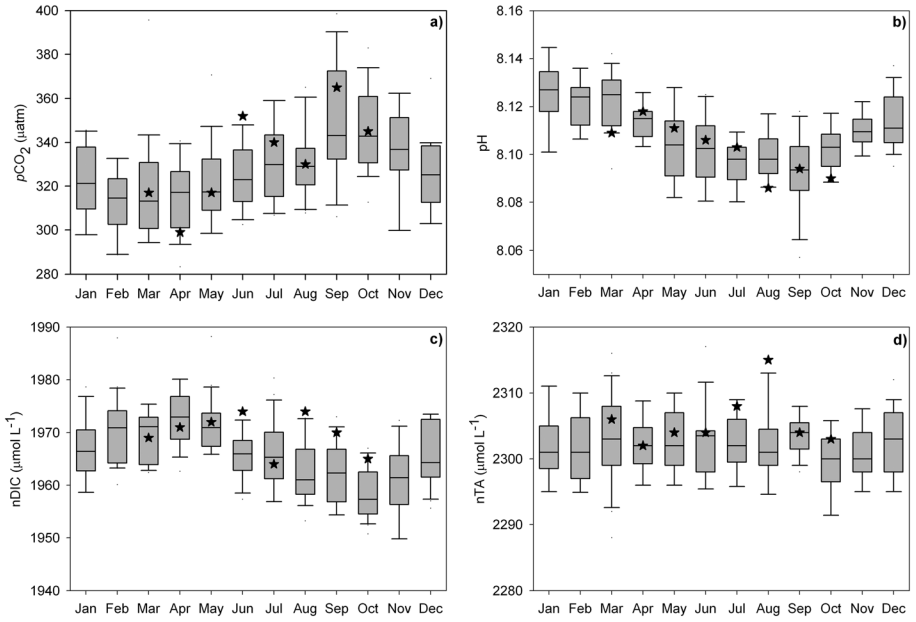


Figure 1. Climatology of monthly (a) seawater pCO_2, (b) pH (on the total scale), (c) normalized dissolved inorganic carbon (nDIC), and (d) normalized total alkalinity (nTA) in the near-surface ocean (5 m) at Station ALOHA between 1989 and 2011. Box plots of detrended (removal of 1988–2011 long-term trends) monthly mean values with box boundaries representing the 25th and 75th percentiles, the line inside each box shows the median, and box whisker caps define the 10th and 90th percentiles of the full time series data set for each parameter shown. nDIC and nTA were normalized to salinity of 35; pH and pCO_2 were calculated from nDIC and nTA. The star indicates measurements made when a CO_2 perturbation experiment was conducted.

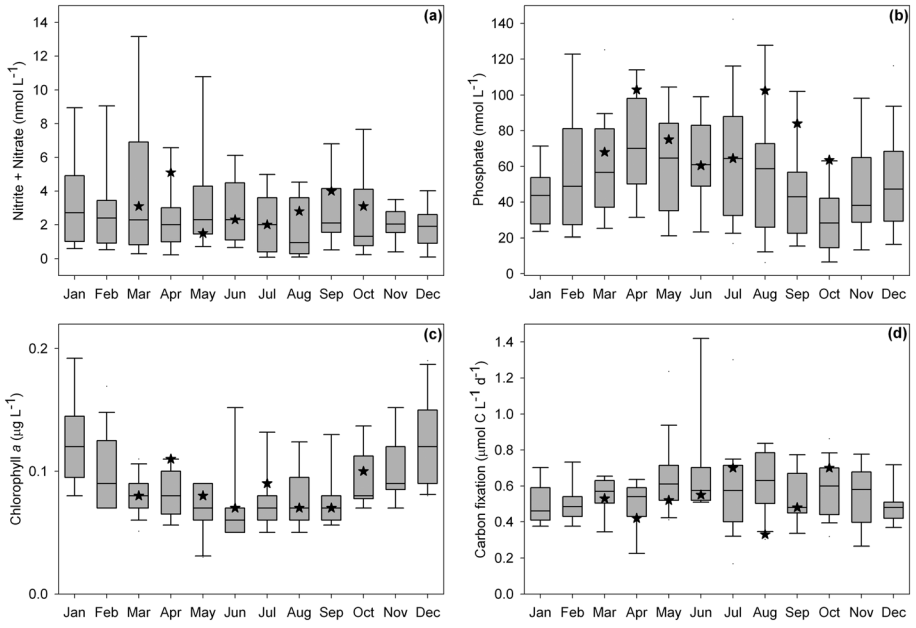


Figure 2. Mean monthly concentrations of (a) nitrate + nitrite, (b) phosphate, (c) chlorophyll *a*, and (d) rates of primary productivity in the near-surface ocean (5 m) at Station ALOHA between 1989 and 2011 depicted as box plots. The box boundaries present the 25th and 75th percentiles, the line inside each box the median, and box whisker caps define the 10th and 90th percentiles of the full time series data set for each parameter shown. The star indicates measurements made when a CO_2 perturbation experiment was conducted.

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Table 2. Mean (±Standard Deviation of Triplicate Carboys When Available) Partial Pressure of Carbon Dioxide (pCO_2) in the Control and CO_2 Perturbed Treatments at the Various Time Points of Each Experiment

		Control pCO_2 (μatm)			Elevated pCO_2 (μatm)		
Experiment	Time Points (h)	First Time Point	Second Time Point	Third Time Point	First Time Point	Second Time Point	Third Time Point
May 2010[a]	0, 24	341 ± 6	336 ± 4		920 ± 17	926 ± 30	
Jun 2010[a]	0, 24	371 ± 8	346 ± 3		1084 ± 42	1006 ± 36	
Jul 2010[a]	0, 24	359 ± 5	341 ± 5		1030 ± 17	1076 ± 40	
Aug 2010[a]	0, 24	379 ± 1	379 ± 1	373 ± 9	958 ± 14	970 ± 29	954 ± 36
Sep 2010[a]	0, 24, 48	405	362 ± 6	361 ± 1	1029	1068 ± 61	1084 ± 8
Oct 2010[a]	0, 48, 72	393	354 ± 2	337 ± 3	989	967 ± 10	1004 ± 16
Mar 2011[a]	0, 48	380	363 ± 2		1079	1031 ± 43	
Apr 2011[a]	0, 48, 72	352	350 ± 4	340 ± 2	938	1003 ± 19	973 ± 0.2
Sep 2012[a]	0, 24, 72	415 ± 7	387 ± 4	389 ± 2	1093 ± 11	1048 ± 12	1060 ± 28
Sep 2012[b]	0, 24, 72	395 ± 2	381 ± 20	390 ± 3	1144 ± 30	1114 ± 44	898 ± 13

[a] CO_2 system was modified through bubbling mixtures of CO_2 and air.
[b] CO_2 system was modified through the addition of hydrochloric acid (HCl) and sodium bicarbonate ($NaHCO_3$).

resulting in a decline (by ~19%) in pCO_2 toward the end of the experiment (72 h) in the pCO_2-elevated treatment (Table 2).

3.3. N_2 and C Fixation Under Elevated pCO_2

Rates of N_2 fixation in experiments conducted between May 2010 and April 2011 in both the controls (0.6 ± 0.2 and 6.7 ± 3.5 nmol N L^{-1} d^{-1}) and CO_2 treatments (0.3 ± 0.1 and 6.8 nmol N L^{-1} d^{-1}; Table 3) were in the range of previously reported data for the region [*Church et al.*, 2009]. The only exception when N_2 fixation rates were substantially higher occurred during the experiment conducted during September 2012, where we utilized a revised $^{15}N_2$ tracer technique; the $^{15}N_2$ tracer was dissolved in seawater prior to adding to the experimental incubations instead of adding the $^{15}N_2$ as a gaseous bubble [*Mohr et al.*, 2010; see section 2.5]. As has been previously observed [*Wilson et al.*, 2012; *Großkopf et al.*, 2012], N_2 fixation rates in both the controls and CO_2 perturbed treatments during this experiment were substantially greater than in experiments where $^{15}N_2$ was added as a bubble with rates ranging between 19.4 ± 2.8 and 32.2 ± 2.2 in the controls and from 16.8 ± 2.5 to 31.5 ± 0.9 nmol N L^{-1} d^{-1} in the pCO_2-elevated samples (Table 3). Rates among the replicate carboys varied by 3–71% (= coefficient of variance), with variability among replicates similar in both the controls and CO_2 perturbed treatments (Table 3). In most cases (seven of the nine experiments), rates of N_2 fixation in the CO_2 perturbed treatments were not significantly different from the controls (two-way ANOVA, $P > 0.05$; Table 3). In the remaining two experiments, rates of N_2 fixation in the CO_2 treatments were significantly lower (two-way ANOVA, $P < 0.05$) than the controls (Table 3 and Figure 3a).

We also investigated the response in rates of ^{14}C-based primary productivity to CO_2 enrichment. Rates of primary productivity measured in the controls ranged from 0.3 ± 0.03 to 1.2 ± 0.3 μmol C L^{-1} d^{-1}, while rates in the CO_2 treatments ranged between 0.2 ± 0.03 and 0.8 ± 0.2 μmol C L^{-1} d^{-1}. As observed with N_2 fixation, rates of productivity did not demonstrate consistent changes in response to CO_2 enrichment (Table 4). In seven of the nine experiments, primary productivity in the CO_2 treatments was not significantly different from the controls (two-way ANOVA, $P > 0.05$), and in the remaining two experiments, rates were significantly lower (by 52 to 72%) than the controls (two-way ANOVA, $P < 0.05$; Figure 3b).

During one of our field-based experiments (September 2012), we simultaneously estimated rates of N_2 fixation and primary productivity in treatments that were aerated at the targeted pCO_2 ("bubbled") or altered by the addition of HCl and $NaHCO_3$ ("nonbubbled"; Figure 4). Comparison of the resulting rates of N_2 fixation and ^{14}C-bicarbonate fixation revealed generally no significant differences among these methods of perturbing the seawater carbonate system (one-way ANOVA, $P > 0.05$; Figure 4).

3.4. Response of *nifH* Gene Abundance and Transcripts to Increasing Seawater pCO_2

We examined diazotroph population dynamics in our experiments based on QPCR and RT-QPCR amplification of *nifH* genes and gene transcripts. In five of eight experiments where *nifH* genes were quantified, the UCYN-A

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Table 3. Mean Rates (±Standard Deviation of Triplicate Carboys) of N_2 Fixation and Primary Productivity in Control and CO_2-Elevated Treatments at Different Time Points of the Experiments[a]

		N_2 Fixation				Primary Productivity			
Date	Sampling Time (h)	Control (nmol N L^{-1} d^{-1})	CO_2 Treatment (nmol N L^{-1} d^{-1})	P Level (Treatment)	P Level (Time)	Control (μmol C L^{-1} d^{-1})	CO_2 Treatment (μmol C L^{-1} d^{-1})	P Level (Treatment)	P Level (Time)
May 2010[b,d]				ns	**				
	0	0.6 ± 0.2	1.0 ± 0.4			—	—		
	24	1.4 ± 0.4	1.4 ± 0.2			—	—		
Jun 2010[b,d]				ns	ns			ns	ns
	0	1.8 ± 0.5	1.5 ± 0.3			0.5 ± 0.3	0.6 ± 0.2		
	24	2.5 ± 0.1	1.8			0.7 ± 0.1	0.5 ± 0.2		
Jul 2010[b,d]				ns	ns			**	**
	0	1.5 ± 0.9	1.07 ± 0.7			0.3 ± 0.1	0.2 ± 0.1		
	24	1.3 ± 0.9	1.5 ± 0.2			0.6 ± 0.2	0.3 ± 0.1		
Aug 2010[b,d]				ns	ns			ns	ns
	0	0.6 ± 0.2	0.5			0.2 ± 0.002	0.2 ± 0.02		
	24	1.2 ± 0.4	1.1 ± 0.3			0.4 ± 0.3	0.2 ± 0.03		
	48	1.1 ± 0.4	1.1 ± 0.4			0.3 ± 0.03	0.4 ± 0.2		
Sep 2010[b,d]				*	ns			ns	ns
	0	1.4	1.4			0.2	0.3		
	24	4.5 ± 1.2	1.1 ± 0.5			0.3 ± 0.03	0.3 ± 0.1		
	48	3.5 ± 0.1	0.7 ± 0.5			0.6 ± 0.1	0.5 ± 0.2		
Oct 2010[b,d]				ns	ns			ns	ns
	0	5.0	6.8			0.5	0.5		
	48	6.7 ± 3.5	3.7 ± 2.4			0.4 ± 0.1	0.5 ± 0.1		
	72	—	0.7 ± 0.5			0.5 ± 0.1	0.4 ± 0.002		
Mar 2011[b,d]				ns	ns			ns	ns
	0	0.6	0.4			0.4	0.3		
	48	0.7 ± 0.1	0.5 ± 0.1			0.3 ± 0.1	0.3 ± 0.1		
Apr 2011[b,d]				**	*			*	ns
	0	6.6	0.3			0.7	0.7		
	24	3.9 ± 1.3	0.3 ± 0.1			1.0 ± 0.2	0.3 ± 0.03		
	72	1.7 ± 0.3	0.3 ± 0.1			1.2 ± 0.3	0.5 ± 0.1		
Sep 2012[b,e]				ns	**			ns	**
	0	19.4 ± 2.8	19.6 ± 3.2			1.0 ± 0.2	0.7 ± 0.1		
	24	32.3 ± 2.2	22.3 ± 3.8			0.4 ± 0.2	0.7 ± 0.03		
	72	31.7 ± 9.3	31.5 ± 0.9			0.5 ± 0.2	0.6 ± 0.1		
Sep 2012[c,e]				ns	***			ns	***
	0	19.9 ± 3.1	16.8 ± 2.5			0.9 ± 0.1	0.9 ± 0.03		
	24	31.8 ± 5.8	29.7 ± 3.0			0.6 ± 0.1	0.5 ± 0.1		
	72	24.8 ± 2.6	18.3 ± 4.5			0.9 ± 0.04	0.8 ± 0.2		

[a]Also depicted are statistical results from two-way analyses of variance. Significance level (p): ns = $p > 0.05$,
* = $p < 0.05$,
** = $p < 0.01$, and
*** = $p < 0.001$.
[b]CO_2 system was modified through bubbling mixtures of CO_2 and air.
[c]CO_2 system was manipulated by the addition of hydrochloric acid (HCl) and sodium bicarbonate ($NaHCO_3$).
[d]N_2 fixation rates were estimated by injecting $^{15}N_2$ in gaseous form.
[e]N_2 fixation rates were assessed by adding $^{15}N_2$ dissolved in seawater.

phylotype accounted for between 40 and 98% of the gene abundances at the beginning of the experiments (Figure 5). In a single experiment (August 2010), gene abundances of *Crocosphaera* rivaled those of UCYN-A, and in the remaining two experiments (September and October 2010), *Crocosphaera* dominated gene abundances, contributing 80% and 95%, respectively, of the quantified *nifH* genes (Figure 5). Relative to unicellular cyanobacteria, the abundances of filamentous diazotrophs (including *Trichodesmium* and the three phylotypes of heterocystous cyanobacteria) were low in all of the experiments. The *nifH* gene abundances of the two unicellular phylotypes (UCYN-A and *Crocosphaera*) were often at least 2 orders of magnitude greater than abundances of these filamentous phylotypes (Figure 6). Overall, there were no significant differences in *nifH* gene abundances for any of the six phylotypes between the control and CO_2-enriched treatments (Figure 6). In addition, gene abundances did not vary

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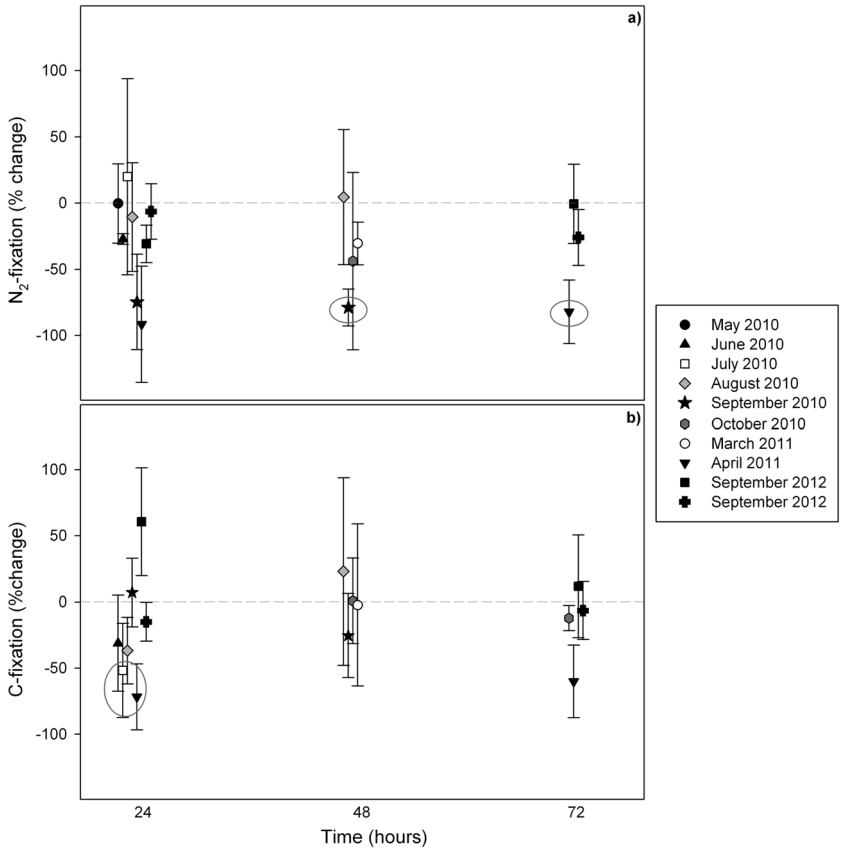


Figure 3. Percent change in rates of (a) N_2 fixation and (b) primary productivity after 24, 48, and 72 h of incubation in CO_2-enriched relative to control treatments. Error bars represent propagated standard errors of the mean rates and the circles indicate occasions when the percent change was statistically significant ($p < 0.05$; two-way ANOVA).

significantly in time over the course of the various experiments (two-way ANOVA, $p > 0.05$), suggesting the incubation conditions (confinement of the organisms) did not stimulate or inhibit the growth of these organisms.

We also quantified *nifH* gene transcription by these same six cyanobacterial phylotypes (Figure 6). In all of our experiments, gene transcripts of *Trichodesmium* spp. were 1–2 orders of magnitude greater than *nifH* gene transcripts derived from the unicellular or the heterocystous phylotypes quantified. The dominance of *Trichodesmium nifH* transcripts likely reflects the time of day the samples were collected (predawn), which is consistent with previously reported diel changes in *Trichodesmium nifH* expression at this location [*Church et al.*, 2005a]. As observed with rates of N_2 fixation and primary productivity, increasing pCO_2 had little influence on *nifH* gene transcription (Figure 6) with the exception of *Crocosphaera nifH* gene transcripts (during the experiment conducted in October 2010), which were nearly tenfold greater in the pCO_2 treatment than the control after 72 h of incubation ($1.9 \times 10^4 \pm 9.8 \times 10^3$ versus $2.1 \times 10^5 \pm 1.4 \times 10^5$ gene transcripts L^{-1}, respectively).

4. Discussion

We sought to examine whether projected future changes in the ocean carbonate system might impact diazotroph activities and abundances in the NPSG, one of the largest marine ecosystems on the planet. Although our goal was to gain insight into potential responses by contemporary marine diazotroph assemblages to shifts in the seawater carbonate system projected for the latter part of this century, we acknowledge that results from short-term incubation experiments do not provide information on shifts in the microbial species or gene evolution resulting from selective pressure due to long-term environmental changes. In this context, our experiments were designed to examine the physiological responses of naturally occurring contemporary diazotroph assemblages to short-term (48 to 72 h) abrupt perturbations in a single global change variable, namely seawater pCO_2.

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Table 4. Summary of the Effects of Ocean Acidification on Processes Associated With Marine Diazotrophs[a]

Approach	Targeted Organism	pCO_2 Conditions	Measured Property	Effect	Reference
(a) Culture-based	*Trichodesmium erythraeum* (IMS 101)	Between 140 and 850 µatm	N_2 fixation	+	*Barcelos e Ramos et al.* [2007]
	T. erythraeum (IMS 101)	385 and 750 ppm (P-replete/P-deplete)	N_2 fixation	+	*Hutchins et al.* [2007]
	T. erythraeum (GBRTRLI101)	385 and 750 ppm (P-replete/P-deplete)	N_2 fixation	+	*Hutchins et al.* [2007]
	T. erythraeum (IMS 101)	250, 400, and 900 ppm	N_2 fixation	+	*Levitan et al.* [2007]
	T. erythraeum (IMS 101)	150 and 900 µatm (50 µmol photons m^{-2} s^{-1})	Fe protein subunit of *nifH*	o	*Levitan et al.* [2010]
	T. erythraeum (IMS 101)	150 and 900 µatm (200 µmol photons m^{-2} s^{-1})	Fe protein subunit of *nifH*	-	*Levitan et al.* [2010]
	T. erythraeum (IMS 101)	365 and 750 ppm (Fe-deplete)	N_2 fixation	-	*Shi et al.* [2012]
	T. erythraeum (KO4-20, GBR), *T. contortum* (2174), *T. thiebautii* (H9-4)	100, 190, 280, 750, 1500, and 2000 ppm	N_2 fixation	o/+	*Hutchins et al.* [2013]
	Nodularia spuminga (IOW-2000/1)	Between 153 and 731 ppm	N_2 fixation	-	*Czerny et al.* [2009]
	Crocosphaera watsonii (WH8501)	190, 355, and 750 ppm (Fe-deplete)	N_2 fixation	o	*Fu et al.* [2008]
	C. watsonii (WH8501)	190, 355, and 750 ppm (Fe-replete)	N_2 fixation	+	*Fu et al.* [2008]
	C. watsonii (WH0401, WH0003, WH8501)	100, 190, 280, 750, 1500, and 2000 ppm	N_2 fixation	+	*Hutchins et al.* [2013]
(b) Field-based	*Trichodesmium* colonies (GOM)	380 and 750 ppm	N_2 fixation	+	*Hutchins et al.* [2009]
	Trichodesmium colonies (SNA)	150, 390, and 800 ppm	N_2 fixation	+	*Lomas et al.* [2012]
	Trichodesmium colonies (NPSG)	Between 280 and 1500 ppm	N_2 fixation	o	*Gradoville et al.* [2014]
	Mixed assemblages (SPSG)	390 and 750 ppm	N_2 fixation	o	*Law et al.* [2012]
	Mixed assemblages (NPSG)	390 and 1100 ppm	N_2 fixation	o	This study
	Mixed assemblages (NPSG)	390 and 1100 ppm	*nifH* gene abundances	o	This study
	Mixed assemblages (NPSG)	390 and 1100 ppm	*nifH* gene expression	o	This study

[a]Laboratory experiments targeted monospecific cultures, whereas field-based experiments were conducted with isolated field populations or whole, mixed assemblages. + = increase in measured property under elevated relative to contemporary pCO_2 conditions, − = decrease in measured property under elevated pCO_2 conditions, and o = no difference between elevated and contemporary pCO_2 conditions. Gulf of Mexico (GOM), Subtropical North Atlantic (SNA), North Pacific Subtropical Gyre (NPSG), South Pacific Subtropical Gyre (SPSG).

In the majority of our experiments, we observed no statistically significant responses (positive or negative relative to the controls) in rates of N_2 fixation, *nifH* gene abundances, or *nifH* gene transcription in response to increases in seawater pCO_2. During two experiments, rates of N_2 fixation were significantly lower in the pCO_2-enriched treatments relative to the experimental controls (Table 3). Similarly, we did not observe consistent responses in rates of primary productivity (Table 3) or concentrations of Chl *a* to elevated seawater pCO_2. In most experiments, rates of primary productivity in the CO_2 perturbed treatments were statistically indistinguishable from the controls. During two experiments, rates of productivity were significantly lower in the elevated CO_2 treatments than in the controls. Such findings suggest that rates of both N_2 fixation and primary productivity in the NPSG are relatively insensitive to large, abrupt changes in seawater pCO_2.

Findings from our field-based experiments using naturally occurring N_2-fixing cyanobacteria, including those for which there are currently no cultivated representatives, contrast with results from laboratory-based studies in which significant stimulation of both C and N_2 fixation by cultivated representatives of the marine cyanobacteria *Trichodesmium* and *Crocosphaera* has been observed under enhanced pCO_2 conditions similar to those tested in the present study (Table 4). Such laboratory studies have highlighted the possibility that long-term increases in seawater pCO_2 could promote the growth and activities of marine diazotrophs. However, there

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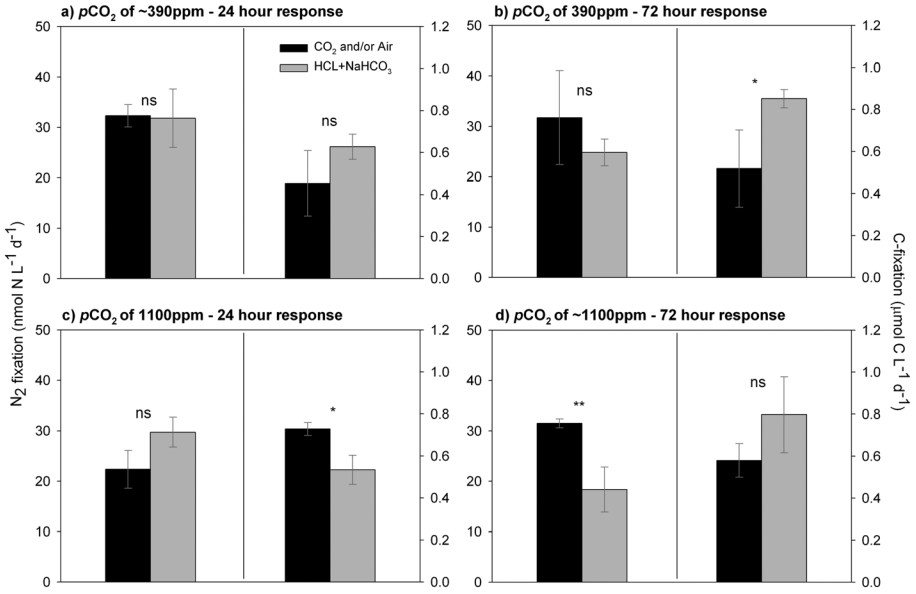


Figure 4. N_2 fixation and primary productivity at different time points during an experiment comparing two approaches for manipulating seawater pCO_2 (aerated with CO_2/air in black versus additions of acid/bicarbonate in grey). (a and c) Rates of N_2 fixation are shown and (b and d) primary productivity depicted. Subsamples were withdrawn from carboys after 24 or 72 h and subsequently incubated for an additional 12 h (primary productivity) or 24 h (N_2 fixation). Vertical bars represent mean rates (±standard deviation) and the statistical significance is based on results from an analysis of variance, where ns = $p > 0.05$, * = $p < 0.05$, and ** = $p < 0.01$.

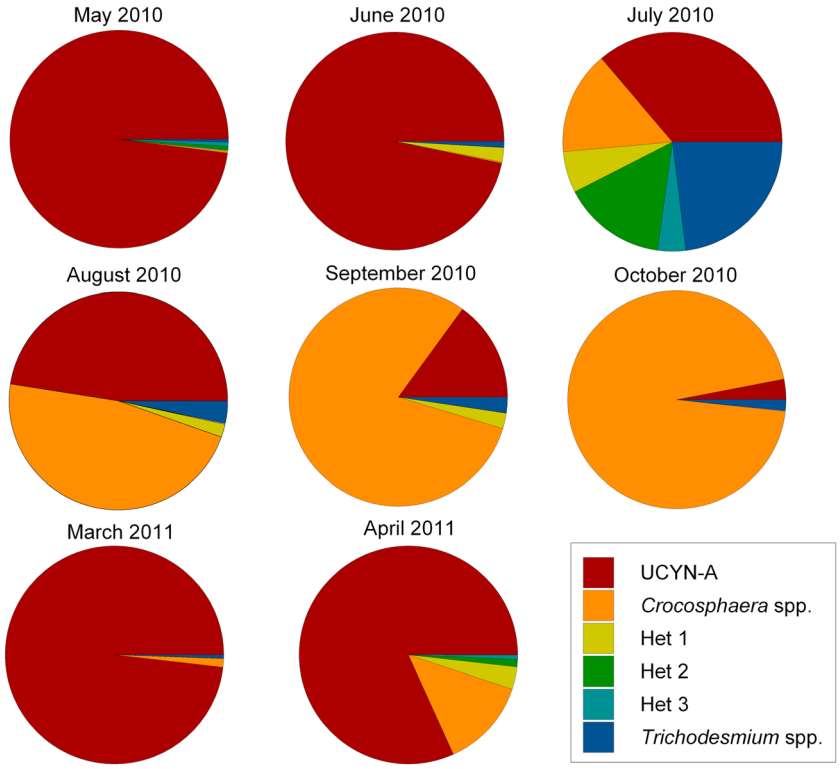


Figure 5. Relative contribution (% of total quantified *nifH* genes) of six different *nifH*-containing cyanobacteria at the beginning of each CO_2 manipulation experiment at Station ALOHA. Depicted are UCYN-A, *Crocosphaera* spp., *Trichodesmium* spp., Het-1 and Het-2 (*Richelia* spp.), and Het 3 (*Calothrix* spp.).

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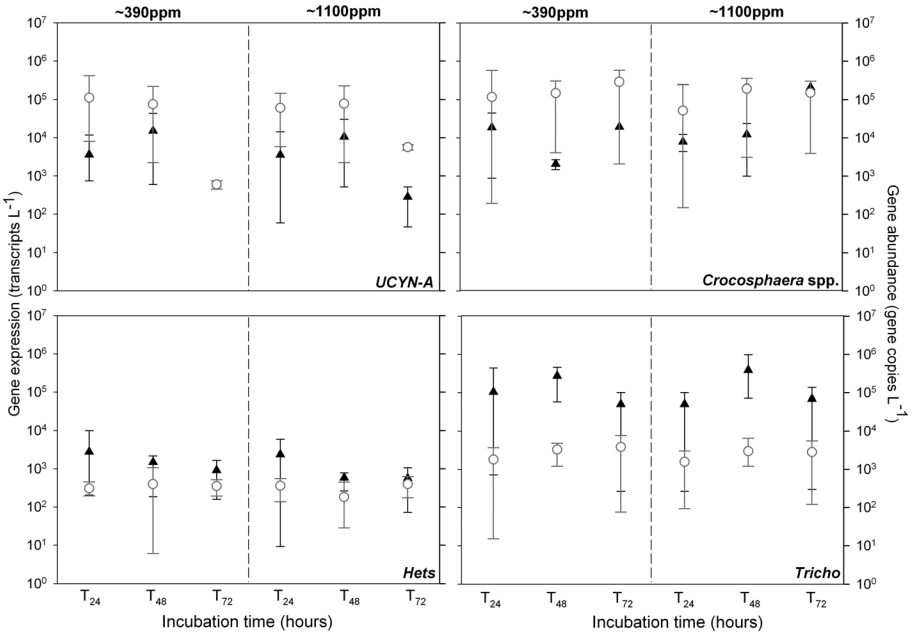


Figure 6. Comparison of *nifH* gene expression (gene transcripts L^{-1}; black triangles) and gene abundances (gene copies L^{-1}; open circles) of UCYN-A, *Crocosphaera* spp., *Trichodesmium* spp. (Tricho), and the sum of the heterocystous cyanobacteria *Richelia* spp., and *Calothrix* spp. (Hets). Controls are depicted on the left side of each panel (~390 ppm) and the CO_2 perturbed treatments on the right side of each panel (~1100 ppm). Symbols depict mean gene abundances or transcripts and error bars represent the minimum and maximum gene abundances and transcripts measured during experiments.

have been comparatively few field-based assessments on the responses of diazotrophs to changes in seawater CO_2 (Table 4). In two field experiments conducted using naturally occurring *Trichodesmium* colonies from the Gulf of Mexico and the Sargasso Sea, rates of N_2 fixation (as measured by acetylene reduction or $^{15}N_2$ assimilation) increased between 6 and 156% in pCO_2-enriched treatments (where pCO_2 was ~750 ppm) relative to unperturbed controls [*Hutchins et al.*, 2009; *Lomas et al.*, 2012]. However, a similar study conducted in the NPSG detected no enhancement of N_2 fixation by isolated *Trichodesmium* colonies to elevated pCO_2 [*Gradoville et al.*, 2014].

In the South Pacific Subtropical Gyre (SPSG), *Law et al.* [2012] conducted a series of pCO_2 perturbation experiments using whole seawater, and similar to the results of the present study, these authors found no consistent response in rates of N_2 fixation to abrupt increases (750 ppm) in seawater pCO_2. Taken together, results from the *Law et al.* [2012] and *Gradoville et al.* [2014] studies and the experiments conducted as part of the present work suggest that CO_2 is not a limiting factor controlling the growth and activities of natural assemblages of ocean diazotrophs in the large regions of the Pacific Ocean (NPSG and SPSG). Moreover, these results suggest that the activities of contemporary diazotroph assemblages in these habitats are relatively insensitive to large, abrupt changes in the seawater carbonate system. Such findings have potentially important implications for our capacity to predict future changes to plankton biogeochemistry and net ecosystem productivity in some of the largest ecosystems on the planet. Specifically, based on results from laboratory studies, marine diazotrophs are often predicted to become increasingly important to ocean carbon cycling with progressive increases to seawater pCO_2. However, our field-based experiments, together with those of *Law et al.* [2012], suggest that enrichment of the upper ocean with CO_2 may not stimulate the activities or net growth (as reflected by no changes in *nifH* abundances in our experiments) of naturally occurring N_2 fixing microorganisms in the Pacific Ocean.

There are a number of reasons diazotroph responses in our field-based experiments might differ from those observed in laboratory-based studies. The lack of response, or in some cases, detrimental impacts of increased pCO_2 on rates of N_2 fixation and primary productivity observed in the present study presumably reflect differences in the physiological responses of diverse, naturally occurring planktonic assemblages in the NPSG relative to the marine diazotroph isolates examined to date. The resulting responses to CO_2 in these field experiments reflect the aggregate behavior of a mixed assemblage of plankton, including diazotrophs, which

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possess diverse metabolic capabilities and presumably differing physiological sensitivities to variations in CO_2. Similar to the *Law et al.* [2012] study, in the majority of our experiments, the diazotroph assemblage (as assessed based on *nifH* gene quantifications) was dominated by UCYN-A (comprising between ~40 and 98% of the quantified *nifH* genes; Figure 5). Time series measurements of N_2 fixation rates at Station ALOHA suggest that small, unicellular diazotrophs (<10 μm) consistently dominate rates of N_2 fixation at this site [*Church et al.*, 2009]. Although UCYN-A remains uncultivated, gene-based surveys and isotope-labeling studies have provided insights into the ecology and metabolic potential of these microorganisms. The recent work of *Thompson et al.* [2012] for instance discovered that this organism can exist as an epiphyte associated with a unicellular picoplanktonic prymnesiophyte. Metagenomic analyses of cell sorts enriched in UCYN-A revealed the absence of suites of genes necessary for oxygenic photosynthesis (including photosystem II) and fixation of inorganic carbon (via the Calvin-Benson-Basham cycle); however, these organisms appear to have retained key genes encoding components of photosystem I [*Zehr et al.*, 2008; *Tripp et al.*, 2010]. Such results hint at a presumed photoheterotrophic metabolism for UCYN-A [*Zehr et al.*, 2008]. A number of studies have hypothesized that photoautotrophs relying on carbon-concentrating mechanisms (CCM) to concentrate CO_2 at the active site of the RuBisCO enzyme may benefit from increases in pCO_2 through reduction in energetic demands of the CCM components [*Kaplan et al.*, 1991; *Price et al.*, 2008; *Raven*, 2003]. There is some evidence supporting this hypothesis in laboratory experiments with *Trichodesmium* [*Levitan et al.*, 2007; *Kranz et al.*, 2009]; in these studies, pCO_2 was increased (up to 900 ppm) and *Trichodesmium* was observed to downregulate CCM activity and allocate greater energy reserves toward fueling N_2 fixation. The absence of RuBisCO and CCMs in the UCYN-A genome suggests that these organisms may not experience the same increase in growth efficiency under conditions of elevated pCO_2. The dominance of UCYN-A (and relatively low abundance of *Trichodesmium*) and the inability of UCYN-A to fix CO_2 may underlie the lack of response in N_2 fixation to increases in CO_2 observed in our experiments.

In an attempt to assess the potential impact of the mode of manipulating the CO_2 system in field-based experiments, we simultaneously estimated rates of N_2 fixation (and primary productivity) in gently aerated versus carboys where the carbonate system was perturbed through the addition of HCl and $NaHCO_3$ (Figure 4). We observed no significant difference in rates of N_2 fixation between the controls and CO_2 perturbed treatments in this experiment. Moreover, we also observed no significant differences in rates of N_2 fixation and ^{14}C-carbon fixation between the gently aerated and the $HCl/NaHCO_3$ perturbed carboys. These results suggest that the gentle aeration procedures recommended for pCO_2 perturbation experiments [*Riebesell et al.*, 2011] and utilized for most of our experiments were not detrimental to N_2 fixation.

On three occasions (August, September, and October 2010), we initiated experiments in which the unicellular cyanobacterium *Crocosphaera* contributed significantly to the total *nifH* gene abundances (47–95%; Figure 5). *Crocosphaera* can be a significant contributor to oceanic N_2 fixation [e.g., *Montoya et al.*, 2004; *Moisander et al.*, 2010] and cultured isolates of *Crocosphaera* (strain WH8501) have been shown to increase rates of N_2 fixation in iron (Fe)-replete conditions after brief acclimation to elevated CO_2 concentrations [*Fu et al.*, 2008]. In one of our experiments (October 2010), we did observe an increase in *Crocosphaera nifH* expression under elevated pCO_2 conditions (Figure 6), but even when *Crocosphaera* was a dominant component of diazotroph abundance (based on quantification of *nifH* genes), rates of N_2 fixation in our experiments remained either unchanged or decreased under enhanced pCO_2 conditions. To date, it remains unclear how variability in gene transcript abundances relate to rates of N_2 fixation; however, the lack of a measurable response in N_2 fixation to elevated pCO_2, despite significant increases in transcriptional activity could reflect posttranscriptional and/or posttranslational modification of *nifH* gene transcripts by *Crocosphaera*. In a study examining genetic variability among strains of *Crocosphaera* (including several from the North Pacific), *Webb et al.* [2009] found that despite low apparent genetic variability, these strains exhibited notable differences in their physiologies. Hence, there may be "ecotype"-dependent differences in the response of *Crocopshaera* to changes in seawater pCO_2; unfortunately, the QPCR *nifH* gene assays utilized in the present study do not allow us to distinguish potential ecotype-specific responses by *Crocosphaera*. In addition, a recent study by *Hutchins et al.* [2013] shows considerable taxon-specific differences in the CO_2 sensitivities of rates of N_2 fixation. For example, the CO_2 half-saturation constant for *Crocosphaera watsonii* (WH8501) was estimated at 162 ppm, suggesting N_2 fixation by this strain might be nearly saturated under present CO_2 conditions.

Our results may also differ from findings with monospecific cultures due to the types of resources limiting diazotroph growth in the ocean relative to conditions examined in the laboratory. Besides temperature and

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light, various essential nutrients have been hypothesized as possible controls on diazotroph growth in the open sea, including phosphorus, molybdenum, iron, and nickel [e.g., *Sañudo-Wilhelmy et al.*, 2001; *Mills et al.*, 2004; *Ho*, 2013]. Several laboratory studies have examined whether nutrient limitation plays an interactive role in CO_2 stimulation of diazotroph growth. In a series of laboratory-based experiments with a strain of *Crocosphaera*, *Fu et al.* [2008] measured elevated rates of N_2 fixation in response to increased CO_2 in Fe-replete cultures, but no CO_2-dependent response was observed under Fe-limited growth conditions. *Shi et al.* [2012] observed a pH-dependent decrease in rates of N_2 fixation in iron-limited cultures of *Trichodesmium*, a finding the authors attribute to changes in iron chemistry of the seawater medium rather than physiological alteration of *Trichodesmium* growth. A recent field-based study conducted by *Gradoville et al.* [2014] found that increasing pCO_2 did not significantly enhance N_2 fixation by *Trichodesmium* colonies under Fe-enriched conditions (100 nmol Fe L^{-1}). We did not employ rigorous trace metal clean sampling techniques for the experiments described in the present study and did not measure concentrations of iron in our incubations; hence, we are unable to determine the extent to which we may have inadvertently introduced trace nutrients to these incubations. Iron concentrations in the near-surface ocean at Station ALOHA have been reported to range between 0.2 and 0.7 nmol L^{-1} [*Boyle et al.*, 2005] and these concentrations have been considered nonlimiting [*Dutkiewicz et al.*, 2012]. Thus, we suspect that the elevated Fe concentrations typical of ALOHA likely resulted in Fe-replete conditions during our experiments, making it unlikely that Fe limitation resulted in the lack of response in our CO_2 perturbation experiments.

In contrast, the availability of phosphorus (P) in the upper ocean at Station ALOHA, where concentrations are generally <0.1 μmol L^{-1} [*Björkman and Karl*, 2003], has been shown to restrict the metabolic activity of natural populations of diazotrophs [e.g., *Grabowski et al.*, 2008; *Sohm et al.*, 2008; *Watkins-Brandt et al.*, 2011]. *Hutchins et al.* [2007] demonstrated that rates of N_2 fixation by *Trichodesmium* strains GBR (isolated from the Pacific) and IMS (isolated from the Atlantic) grown either under P-replete (20 μmol P L^{-1}) or P-limiting (0.2 μmol P L^{-1}) conditions were enhanced with increasing CO_2. Thus, even if the diazotrophs sampled as part of these experiments were P limited, we still should have anticipated stimulation in rates of N_2 fixation in response to increases in CO_2. The lack of response in our experiments suggests that CO_2 is not limiting diazotroph growth in the NPSG; rather, we suspect the persistently low concentrations of other important growth-requiring nutrients (e.g., vitamins and nickel) observed in the surface ocean of this ecosystem might be more likely to restrict diazotroph growth [e.g., *Barada et al.*, 2013; *Ho*, 2013; *Sañudo-Wilhelmy et al.*, 2014] and obscure potentially stimulatory influences of CO_2 on these microorganisms.

The inconsistencies between field-based CO_2 manipulation experiments conducted with diverse natural assemblages of marine diazotrophs and laboratory experiments conducted using monospecific isolates highlights the utility of both cultivation-dependent and independent approaches for informing our understanding of diazotroph physiology, ecology, and biogeochemistry. While field-based experiments such as those conducted as part of this study provide insight into plankton ecology and biogeochemistry in a more realistic setting that includes interactions among organisms, laboratory experiments provide important clues into potential physiological and genetic changes by isolated microorganisms. Moreover, the relatively slow growth rates of diazotrophs (~0.15 day^{-1} for *Trichodesmium* spp. or ~0.35 for *Crocosphaera* spp. [*LaRoche and Breitbarth*, 2005; *Webb et al.*, 2009]) make laboratory experiments well suited for examining hypotheses focused on acclimatization and adaptability by diazotrophs to slow and progressive ecosystem changes, including those accompanying future changes to seawater CO_2. Further laboratory-based and field-based experiments will hopefully provide additional information on how rising CO_2 in combination with other changes in climate-sensitive processes that were not explored in the present study (e.g., warming) may influence the physiology of marine N_2 fixing cyanobacteria, and the ocean C and N cycle.

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