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Experiments linking nitrogenase gene expression to nitrogen fixation in the North Pacific subtropical gyre

Jonathan P. Zehr[1]
Department of Ocean Sciences, University of California, Santa Cruz, California 95064

Joseph P. Montoya
School of Biology, Georgia Institute of Technology, Atlanta, Georgia 30332

Bethany D. Jenkins, Ian Hewson, Elizabeth Mondragon, and Cindy M. Short
Department of Ocean Sciences, University of California, Santa Cruz, California 95064

Matthew J. Church, Andrew Hansen, and David M. Karl
School of Ocean and Earth Science and Technology, University of Hawai'i, Honolulu, Hawai'i 96822

Abstract

We examined diazotroph activity in controlled experiments containing natural populations of nitrogen (N_2)-fixing microbes collected offshore from Kane'ohe Bay, Hawai'i, and from Station ALOHA (A Long-term Oligotrophic Habitat Assessment) in the North Pacific subtropical gyre. Quantitative polymerase chain reaction and reverse transcription-polymerase chain reaction approaches showed that the abundances of the major phylotypes did not change substantially during experimental incubations. Diel phasing of *nifH* gene expression in the experiments was similar to that documented for natural populations. Cell-specific N_2 fixation rates, calculated as $^{15}N_2$ incorporation rate divided by specific phylotype abundance (gene copy number), varied between the experiments, but generally agreed with the abundance and levels of *nifH* expression of each group of diazotrophs. Phosphorus additions had little effect on the abundance, gene expression, or N_2 fixation activity of diazotrophs across the course of the 36-h experiments, indicating that other factors may limit the growth, gene expression, and activity of diazotrophs in the North Pacific subtropical gyre.

Oceanic biological nitrogen (N_2) fixation (BNF) has received increasing attention because of its important role in controlling the availability of nitrogen (N) for primary productivity and carbon sequestration (Gruber 2005). Oceanic BNF is controlled by several groups of cyanobacteria and possibly other noncyanobacterial prokaryotes (Zehr et al. 2001; Montoya et al. 2004; Church et al. 2005*a*). However, the contribution of individual diazotroph groups and the factors that constrain the growth of N_2-fixing microorganisms and N_2 fixation rates in the oceans are not well known. Redfield's analysis of ocean elemental stoichiometry suggested that imbalances in the availability of nitrogen (N) and phosphorus (P) relative to the needs of phytoplankton would select for N_2-fixing microorganisms (Redfield 1958), which was echoed by the idea of a nitrogen homeostat ("nitrostat") in a modeling analysis of the N cycle of the global ocean (Tyrrell 1999). Conversely, Klausmeier et al. (2004) note that there was significant variability in N : P ratios that is species-specific and is affected by physiological state and growth rate. Resolving this controversy and understanding the factors that limit and select for diazotrophs is critical for understanding the dynamics of the N cycle in the global ocean.

P and/or iron (Fe) are essential nutrients, the availability of which might control the growth of diazotrophs (Sañudo-Wilhelmy et al. 2001; Karl 2002). In the North Pacific subtropical gyre, increased rates of N_2 fixation are hypothesized to drive the system toward P limitation, even though the in situ concentrations of inorganic P are higher than in some P limited systems (Karl et al. 2001). There may be colimitation by Fe and P, and the interactions and relative availability of these nutrients may be highly dynamic, with short-term and long-term changes that affect N_2-fixing microorganisms. The limitation of N_2 fixation by P may depend on the characteristics of the specific diazotroph, including uptake capabilities and cell size.

There are several groups of *nifH*-containing microorganisms, including two distinct groups of unicellular cyanobacterial *nifH* sequences (termed Groups A and B) present at Station (Sta.) ALOHA (A Long-term Oligotrophic Habitat Assessment) of the Hawai'i Ocean Time-Series (HOT) program station near Oahu (Zehr et al. 1998; Zehr

[1] Corresponding author (zehrj@pmc.ucsc.edu).
[2] Present address: Department of Cell and Molecular Biology, and Graduate School of Oceanography, University of Rhode Island, Kingston, Rhode Island 02881.

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Table 1. Summary of experiments using oceanic water from Sta. ALOHA and coastal water from outside Kane'ohe Bay. Experiments G1 and G4 were initiated with water collected from HOT cruises 117 and 129, respectively.

Exp. No.	Dates	Experiment	Source water	Analyses
G1	28 Jul 00–30 Jul 00	Phosphorus addition versus control	Sta. ALOHA	Clone libraries
G4	10 Aug 01–13 Aug 01	Phosphorus addition versus control	Sta. ALOHA	Clone libraries QPCR QRT-PCR, epifluorescence microscopy, chorophyll *a*
G5	13 Aug 01–15 Aug 01	Prefiltered with 10-μm mesh versus control	Kane'ohe Bay	QPCR, epifluorescence microscopy
G6	17 Feb 02–19 Feb 02	Phosphorus addition versus control (prefiltered with 10-μm mesh)	Kane'ohe Bay	Clone libraries QPCR QRT-PCR, epifluorescence microscopy, chorophyll *a*

et al. 2001). Quantitative polymerase chain reaction (QPCR) and reverse transcription-polymerase chain reaction (QRT-PCR) results showed that both of the unicellular groups were abundant and expressed *nifH* and that the Group A unicellular cyanobacterial phylotype was one of the most abundant diazotrophs at this location (Church et al. 2005*a*). Montoya et al. (2004) showed that diazotrophs <10 μm in diameter contributed substantially to overall N_2 fixation rates, and this size fraction included Group A and B *nifH* phylotypes. It is still not known what the contributions of individual *nifH* phylotypes are to overall N_2 fixation rates or whether the growth and activity of individual groups of diazotrophs are limited by the availability of P (and/or Fe).

In this study, we performed enclosure (microcosm) studies and $^{15}N_2$ uptake experiments to examine the relationships between variability in N_2 fixation rates and the abundance of microbes containing *nifH*. In addition, we examined whether phosphate additions influenced the abundances, nitrogenase gene expression, and $^{15}N_2$ fixation rates by major diazotroph groups.

Materials and methods

Open ocean water samples were collected from Sta. ALOHA (22°45′N, 158°00′W) in the North Pacific subtropical gyre (approximately 100 km north of Oahu), and coastal water samples were collected outside the mouth of Kane'ohe Bay, Hawai'i (approximately 21°26′N, 157°47′W) on the windward (northeastern) side of Oahu. The source water and experimental conditions for the experiments are summarized in Table 1. Water samples (120 liters) were collected from a depth of 25 m at Sta. ALOHA immediately before departing for shore-based laboratories (approximately 10-h transit) on HOT cruises 117 and 129. The water was collected using 10–12-liter polyvinylchloride (PVC) bottles mounted to a conductivity-temperature-depth (CTD) rosette, prefiltered through 110-μm pore size nylon mesh (Nitex) to remove metazoan grazers and larger colony-forming diazotrophs (*Trichodesmium* colonies and symbiotic chain forming diatoms). The water was transported to the Hawai'i Institute for Marine Biology (HIMB) facility in acid-washed (1 mol L^{-1} hydrochloric acid [HCl] wash, followed by deionized water rinses) polycarbonate carboys (20 liters) for the experimental incubations. The carboys were kept shaded throughout the trip to HIMB. All experiments began less than 24 h from the time of water collection. Experiments were initiated (T_0) near dusk after a period of acclimation (2–4 h) in the flume.

For coastal experiments, seawater was collected approximately 1 km outside the mouth of Kane'ohe Bay using a small boat and 20-liter acid-washed carboys (Table 1). The water was prefiltered with 110-μm pore size nylon mesh. In two experiments, (G5 and G6), the water was also prefiltered using 10-μm pore size nylon mesh into acid-washed (1 mol L^{-1} HCl) polycarbonate bottles (to remove *Trichodesmium* trichomes and diatom cells that might harbor endosymbiotic N_2 fixers, before the incubation). Water was transferred from the carboys into acid-washed Nalgene® 2-liter (2.3 liters total volume) and 4-liter (4.5 liters total volume) polycarbonate bottles for the experiment. The 4-liter bottles were used for the $^{15}N_2$ incubations and were closed with airtight septa caps. The 2-liter bottles were used for the nucleic acid and microscopy sampling and were closed with polycarbonate-threaded caps. All bottles were immediately placed in running seawater flumes at HIMB to maintain ambient surface-water temperature (between 20°C and 25°C, depending on the season). The flumes were shaded with neutral density screening to mimic 25% of incident surface irradiance.

The experiments (Table 1) were designed to quantify the abundance and nitrogenase gene expression of major N_2-fixing groups from the open ocean relative to $^{15}N_2$ fixation rates. In addition, the experiments were designed to determine if the addition of phosphate (PO_4^{3-}) stimulated growth (abundance), *nifH* gene expression, or N_2 fixation activity. At T_0, $^{15}N_2$ was added to the 4-liter bottles, and P (0.5 μmol L^{-1} Na_2HPO_4) was added to the 2-liter and 4-liter treatment bottles. Subsamples (500 mL) were taken from 2-liter bottles at each time point (starting with T_0) for nucleic acid sampling, and 4-liter bottles were sampled (entire 4-liter bottle filtered) for ^{15}N isotopic measurements. Samples were collected for DNA once per day, and RNA samples were collected at intervals (4–6 h) during the course of the 36-h incubation. The DNA samples were filtered through 10-μm pore size, 47-mm diameter, nylon or polyester filters and then through low-protein and nucleic-acid–binding, 0.2-μm pore size, 25-mm diameter Supor (Pall Gelman) filters. Both filter sizes for DNA extraction

were stored in 500 μL of Tris EDTA (10 mmol L^{-1} Tris-HCl at pH 7.4; 1 mmol L^{-1} ethylenediaminetetraacetic acid at pH 8.0).

Water filtered for RNA samples was not size-fractioned, because the size class of the nitrogenase phylotypes could be determined from analysis of the size-fractioned DNA samples. RNA samples were filtered through 0.2-μm pore size, 25-mm diameter Supor (Pall Gelman) or Durapore (Millipore) filters. The filters were placed in 2-mL bead beater vials (Biospec Products) containing 600-μL RLT buffer (Qiagen RNeasy kit, Qiagen), 1% β-mercaptoethanol, and 0.2 g of 0.1-mm-diameter glass beads (Biospec Products). The samples were frozen in liquid nitrogen and stored at −80°C until processed.

Samples (50 mL) were also collected for enumeration of the unicellular diazotrophic cyanobacteria by epifluorescence microscopy. Although the epifluorescence and size alone do not imply diazotrophy, *Crocosphaera*-like cyanobacteria that have been confirmed to be N_2-fixing by cultivation and molecular analysis are the only 2–5-μm diameter organisms that contain phycoerythrin currently known in oceanic waters. Samples were fixed with 2% paraformaldehyde and filtered through 1–2-μm pore size, 25-mm diameter, black polycarbonate filters (GE Osmonics). The abundance of autofluorescing phycoerythrin-containing cells (3–8-μm diameter) was determined on a Zeiss Axioplan epifluorescence microscope using green excitation (Zeiss filter set 20, excitation 546-nm bandpass, and emission 575–640-nm bandpass filters) and a 40× objective. At least 10 fields of view or 300 cells were counted. It was not possible to enumerate Group A unicellular cyanobacteria, because we are not yet certain of their pigment content or cell size (*see Discussion*), and they may be similar in size and characteristics to non–N_2-fixing *Synechococcus*.

Samples (700–1,000 mL) for chlorophyll *a* (Chl *a*) measurements were filtered through Whatman GF/F filters and extracted in acetone overnight. Chl *a* was measured on a Turner Designs TD-700 fluorometer.

DNA and RNA samples were extracted as described in Church et al. (2005*a*) and Church et al. (2005*b*), respectively. DNA was extracted using methods based on the xanthogenate method (Tillett and Neilan 2000), with the addition of 2× xanthogenate buffer to the samples stored in TE. The samples were incubated in xanthogenate buffer for 2 h at 70°C, vortexed for 30 s, placed on ice for 30 min, and treated as described in Church et al. (2005*a*). RNA was extracted using the Qiagen RNEasy kit (Qiagen). The bead beater tubes containing RLT lysis buffer and filters were agitated for 1.5 min (30-s intervals) with a Fast Prep Machine (ThermoSavant, Bio 101 FP-120, Qbiogene). The filters were removed and the tubes centrifuged briefly to pellet the beads. The supernatant was removed to a clean 2-mL microcentrifuge tube, and an equal volume of 70% ethanol added. The samples were purified according to the manufacturer's instructions with the addition of an on-column DNase incubation according to the manufacturer's protocol. The DNA and RNA was quantified with Pico (DNA) or Ribo (RNA) Green (Molecular Probes) using a plate reading spectrofluorometer (Varian).

A *nifH* gene fragment was amplified by RT-PCR using degenerate primers and a one-step RT-PCR kit (Superscript One-Step RT-PCR, Invitrogen) to determine the phylogenetic composition of the active diazotrophs in the experiments relative to previously determined *nifH* phylotypes found in the ocean environment,. The RT-PCR reactions were as described in Church et al. (2005*b*).

Two pairs of degenerate *nifH* primers were used in a nested PCR strategy as described previously (Church et al. 2005*b*). DNA (3–5 ng) was used for PCR, and RNA (3–5 ng) was used for the RT-PCR reactions. The amplification products were cloned into P-GEM T vectors, and the cloned inserts were sequenced using BigDye reagents (Applied Biosystems) on an ABI 3100 sequencer. The sequences are available from GenBank under accession numbers DQ062516-DQ062537, DQ088684-DQ088687, and DQ118185- DQ118239.

The DNA sequences obtained were imported into an ARB (Ludwig et al. 2004) *nifH* database of sequences that had previously been aligned with a *nifH* protein family (PFAM) (multiple sequence alignment of a protein family) using HMMER (profile hidden Markov model software, www.sanger.ac.uk, or hmmer.wustl.edu) (Zehr et al. 2003*b*). DNA sequences in this database were aligned according to the aligned amino acid sequences in ARB. Because all of the sequences in this study were very similar to ones previously obtained at Sta. ALOHA, the sequences reported here were aligned using the Fast Aligner function in ARB. The resulting DNA sequence alignment was used for phylogenetic analysis to identify subtle differences within the clades. Phylogenetic analysis of the DNA sequences was performed with the neighbor-joining algorithm with Jukes-Cantor correction in ARB using all nucleotide positions of the *nifH* alignment between, but not including, the primer sites for the Zehr and McReynolds (1989) primers. Sequences representing the closest cultivated representatives and environmental sequences from the Pacific Ocean were included in the analysis presented here.

QPCR was performed using Taqman probes and primers designed with the *nifH* sequences of the Group A and Group B unicellular cyanobacteria, *Trichodesmium*, a heterocystous cyanobacterium (specifically the heterocyst-1 cluster, here called het-1, (Church et al. 2005*a*) believed to be symbionts of diatoms, and a γ Proteobacterium (AO15). The primer design and QPCR and QRT-PCR methods have been described previously (Short et al. 2004; Church et al. 2005*a*; Church et al. 2005*b*). Samples were processed using an ABI GeneAmp 5700 (Applied Biosystems). The cycle threshold (Ct) for each sample was determined with replicates (either duplicate or triplicate DNA or cDNA samples), and Ct values converted to gene copy numbers using a standard curve constructed from amplification of linearized plasmids containing the target gene (Short et al. 2004). The standard deviation was calculated from the experimental replicates when present or from the analytical replicates (replicate QPCR on same sample) if there were not sufficient experimental replicates. The significance of changes in abundance attributable to treatments was tested with a paired, two-tailed Student's *t*-test using the TTEST function in Microsoft Excel.

Rate measurements

N_2 fixation rate measurements were carried out in 4-liter polycarbonate bottles equipped with silicone septum caps. A gas-tight syringe was used to add $^{15}N_2$ (99 atom%, Cambridge Isotope Laboratories). Incubations were terminated by filtration under gentle pressure through a precombusted 25-mm diameter GF/F filter after passage through a 10-μm pore size, 25-mm diameter, nylon prefilter to remove larger phytoplankton from the sample. Sample filters were dried at 60°C then stored over desiccant until analyzed by continuous-flow isotope ratio mass spectrometry using a Micromass Optima interfaced to a CE Elantech NA2500 elemental analyzer. N_2 fixation rates were calculated by isotope mass balance as described in Montoya et al. (1996).

The incubations and isotope ratio measurements provide specific rates [h^{-1}] of activity normalized to the standing stock of biomass (particulate N) because we measured the accumulation of tracer as a proportion of the final pool of particulate organic matter sampled. In the case of N_2 fixation, specific rates represent activity during the entire incubation period of each sample (all samples started at T_0 and terminated at different end time points). In all cases, biomass specific rates were multiplied by the biomass concentration to produce volumetric rates of activity [nmol N L^{-1} h^{-1}].

Note that our experimental approach differs from a classic time series experiment in that each rate measurement is the cumulative activity across increasing lengths of incubation time. Thus, our second time point represents the activity of the sample through the entire interval from the start of the time series experiment until the sample was filtered and includes the time interval sampled by our first time point. For example, identical rates measured from incubation during a dark period and a dark + light period suggest that N_2 fixation occurred at similar rates in both the light and dark period. We adopted this approach because we initially anticipated very low rates of activity based on previous work in the North Atlantic. The cumulative nature of our measurements increases the chance of resolving low rates, but can increase the difficulty of resolving temporal patterns of variation in activity.

Statistical analysis

The effect of PO_4^{3-} treatment on abundance of the diazotroph phylotypes was performed with 2-way analysis of variance (ANOVA; effect of P addition and time points) on the *nifH* gene copy number L^{-1} data using Sigma Stat (Systat Software). ANOVA was performed on the abundances of *Trichodesmium* and *Richelia* in the >10 μm and Group A and B unicellular cyanobacteria in the <0.2 μm size fractions to test for treatment effects. There is no reason to expect a time-dependent effect of the fractionation, and the small numbers associated with the diazotrophs in the "wrong" size fraction had high variances.

The rates of N_2 fixation during dark, light + dark, and dark + light + dark periods in control and PO_4^{3-}-amended treatments were analyzed by 2-way ANOVA using Sigma Stat (Systat Software). Analysis of the complete N_2 fixation time series (which included multiple time points) was analyzed by nested ANOVA using JMP software (SAS Institute).

Results

nifH Diversity

The *nifH* PCR or RT-PCR products obtained from G1, G3, G4, G6, and a vertical profile from Sta. ALOHA clustered with the same phylogenetic groups as previously reported from Sta. ALOHA (Zehr et al. 1998; Church et al. 2005*b*) and other locations (Fig. 1).

Clone libraries from freshly collected samples were similar in composition to those from the experimental manipulations. Sequences amplified from samples collected on HOT cruise 117 in July 2000 clustered in groups 1, 2, 5, 9, 11, and 12, and sequences obtained from the experiment using water collected on that cruise (G1) clustered in groups 1, 2, 5, 9, 11, 12, 13, and 15 (Table 2).

Different groups of sequence types were obtained from the various experiments (Table 2). The relative abundance of different phylogenetic groups in experiments G4 and G6 were assessed by QPCR and QRT-PCR (Table 3).

Abundance of diazotrophs

There was no detectable change in abundances of the individual diazotroph groups during the time courses of experiments G4 and G6 (Figs. 2, 3; data not shown for G6). The most abundant diazotrophic unicellular cyanobacteria group in experiment G4 (August 2001, Sta. ALOHA) was the Group B unicellular cyanobacteria phylotype (Table 3). In experiment G6 (February 2002, offshore of Kane'ohe Bay), the Group A unicellular cyanobacterial phylotype was the most abundant (Table 3).

The group B phylotype is closely related to the sequences from the cultivated unicellular cyanobacterium, *Crocosphaera watsonii* sp. WH8501. There are no sequences from cultivated organisms that cluster as closely with the Group A phylotype (Fig. 1). *Cyanothece* sp. is the closest *nifH* phylogenetic relative, but it is not as closely related to the Group A phylotype as is the *Crocosphaera nifH* phylotype to Group B sequences. *Trichodesmium* and the heterocystous phylotype (het-1) were abundant in the large size fraction of the G4 experiment (Figs. 2, 3), but were in low abundances in the G6 experiment, which had been prefiltered (Table 3). Subsequent to this study, probes for the het-2 and het-3 diatom symbionts were developed (Foster and Zehr, unpubl. data). The het-2 and het-3 symbionts were present in experiments G4 and G6 but at much lower concentrations than the het-1 phylotype (data not shown).

Microscopic counts of 3–8-μm diameter phycoerythrin-containing cells (the size range of *Crocosphaera* sp. WH8501 in culture) in the experiments agreed well with QPCR measurements of abundances of Group B, but not the Group A unicellular cyanobacterial phylotype abundances (Table 3).

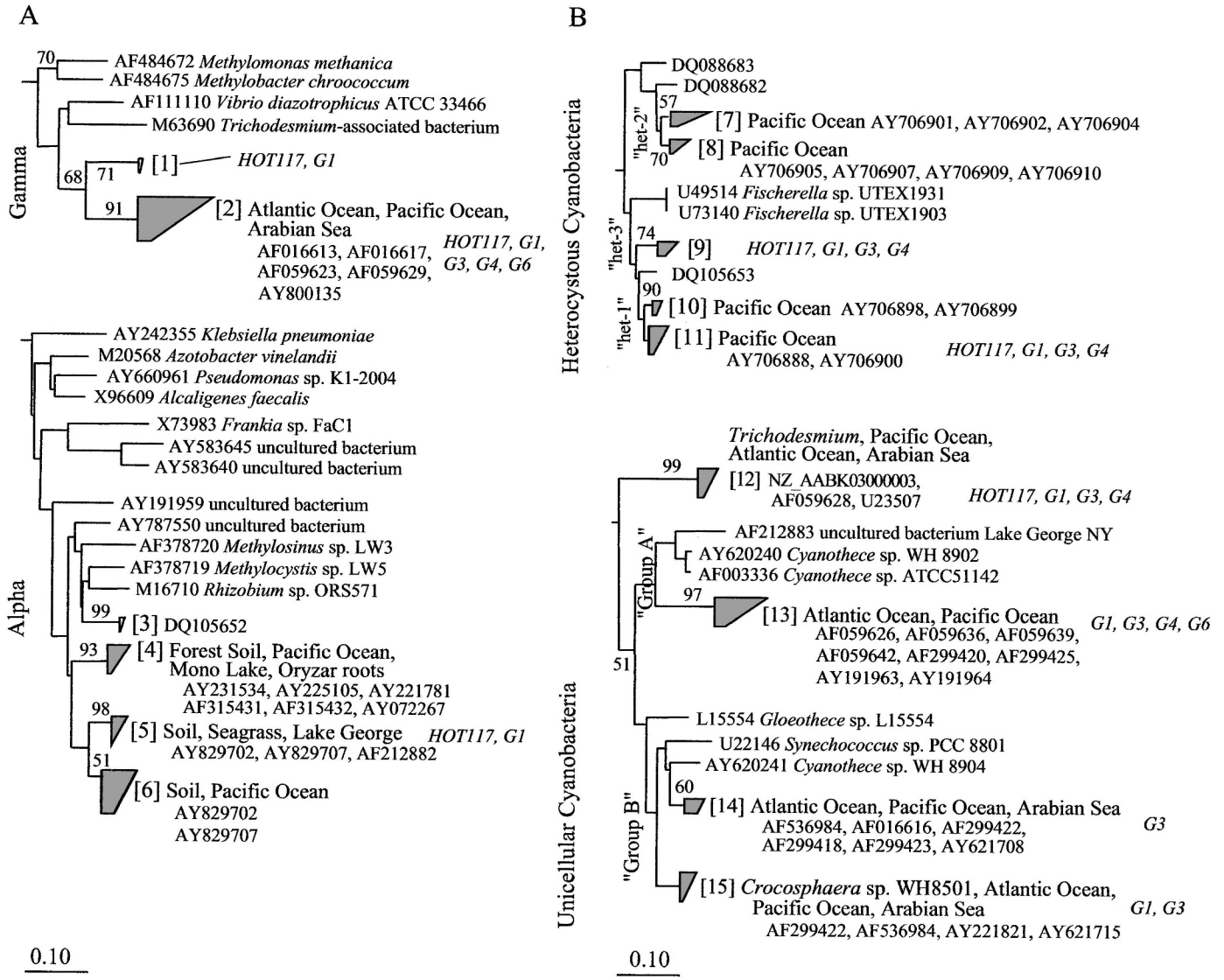


Fig. 1. Phylogenetic trees of *nifH* gene DNA sequences from Sta. ALOHA and Kane'ohe Bay. Trees were constructed by neighbor joining using the Jukes-Cantor correction in ARB (scale bar represents 0.10 fixed substitutions per site). Bootstrap values (of 100 replicates) for the major oceanic clusters are shown at nodes. Samples containing sequences in different groups and representative GenBank accession numbers from other studies are shown. GenBank accession numbers for representative sequences from this study are shown in Table 2. Group numbers (in brackets) are discussed in Results. (A) Phylogenetic tree of Proteobacterial *nifH* gene DNA sequences. (B) Phylogenetic tree of cyanobacteria *nifH* gene DNA sequences.

Size-fraction filtration partially separated the Proteobacterial and unicellular cyanobacterial *nifH* genes from the *Trichodesmium* and heterocystous phylotypes (het-1, believed to be *Richelia* or a *Richelia*-like symbiont of diatoms). Some *Trichodesmium* and het-1 sequences were also detected in the <10 μm but >0.2 μm size fraction, but generally the abundance of these diazotrophs was 10-fold greater on the 10-μm filters (Figs. 2, 3, Table 3). The unicellular cyanobacteria were primarily detected in the 0.2–10-μm size fraction (Figs. 2, 3, Table 3).

The addition of phosphorus had little influence on the abundance of diazotrophs detected in experiments G4 or G6 (Fig. 3). No significant increase in abundance was observed in any diazotroph group. The only significant differences were detected on day two, which were reductions in the control for Group A (G4) and a reduction in Group B in PO_4^{3-} treatment in G6. There was no consistent effect of PO_4^{3-} addition on Chl *a* concentrations in the experiments with open ocean (G4) or coastal water (G6) (G4 control 0.16 ± 0.02 μg Chl *a* L^{-1}, PO_4^{3-} treatment 0.19 ± 0.02 μg Chl *a* L^{-1}; G6 control 0.48 ± 12 μg Chl *a* L^{-1}, PO_4^{3-} treatment 0.5 ± 0.03 μg Chl *a* L^{-1}). No obvious effect of P was detected during the first 1.5 days of the G6 experiment.

Table 2. Summary of *nifH* sequences obtained from experiments with Sta. ALOHA and Kane'ohe Bay water. The number of sequences of each type relative to the total number of sequences found in each sample is shown. Phylogenetic group numbers are as indicated in Fig. 1.

Phylogenetic group	Sample or experiment	No. of sequence type/ total sequences/sample	Time (depth or size fraction and experimental treatment)	Accession numbers of representative sequences
1	HOT117	36/89	03:45 h (5 m, 25m, 50 m)	DQ062516, DQ062524, DQ062525, DQ062517, DQ062526, DQ062518
1	HOT117	16/89	12:00 h (5 m, 25 m)	DQ118218, DQ118223, DQ118225
1	G1	8/113	18:30 h (0.2 μm,PO_4^{3-}) 19:00 h (10 μm, Control) 19:00 h (10 μm, PO_4^{3-})	DQ062533, DQ118220, DQ118222
2	HOT117	2/89	12:00 h (25 m)	DQ118227
2	G1	11/113	18:30 h (10 μm, control; 0.2 μm, PO_4^{3-}; 0.2 μm, PO_4^{3-})	DQ062532, DQ062535, DQ062537
2	G4	2/25	17:45 h (0.2 μm, control)	DQ118230
2	G6	56/62	08:15 h (0.2 μm, control) 12:00 h (0.2 μm, control) 20:00 h (0.2 μm, control) 00:00 h (0.2 μm, control)	DQ118232, DQ118233, DQ118234, DQ118235
5	HOT117	2/89	03:45 h (100 m)	DQ088685
5	G1	10/113	19:00 h (0.2 μm, PO_4^{3-})	DQ088684
9	HOT117	5/89	12:00 h (5 m, 25 m, 100 m)	DQ118224, DQ118226, DQ118228
9	G1	11/113	09:55 h (10 μm, PO_4^{3-}) 19:00 h (10 μm, control) 19:00 h (10 μm, PO_4^{3-})	DQ062530, DQ118219, DQ118221
9	G4	1/25	08:30 h (0.2 μm, control)	DQ118231
11	HOT117	19/89	12:00 h (5 m, 25 m, 100 m)	DQ118195, DQ118198, DQ118199
11	G1	2/113	09:55 h (10 μm, control) 19:00 h (10 μm, PO_4^{3-})	DQ062528, DQ118191
11	G4	11/25	17:45 h (0.2 μm, control)	DQ118203
12	HOT117	9/89	12:00 h (5 m, 25 m)	DQ118196, DQ118197, DQ118186
12	G1	66/113	09:15 h (10 μm, control; 10 μm PO_4^{3-}; 0.2 μm PO_4^{3-}); 09:55 h (10 μm control; 10 μm PO_4^{3-}); 18:30 h (10 μm control; 0.2 μm PO_4^{3-}; 0.2 μm PO_4^{3-}) 19:00 h (10 μm control; 10 μm PO_4^{3-})	DQ118188, DQ118187, DQ062527, DQ062519, DQ062529,DQ062520, DQ062531, DQ062521, DQ062534, DQ062536, DQ118189, DQ118190, DQ118185, DQ118193
12	G4	10/25	08:30 h (0.2 μm, control) 17:45 h (0.2 μm, control)	DQ118204, DQ118202
13	G1	2/113	18:30 h (0.2 μm, PO_4^{3-})	DQ062522
13	G4	1/25	08:30 h (0.2 μm, control)	DQ118206
13	G6	6/62	08:15 h (0.2 μm, control) 12:00 h (0.2 μm control) 20:00 h (0.2 μm, control) 00:00 h (0.2 μm, control)	DQ118207, DQ118208, DQ118209, DQ118210
15	G1	3/113	18:30 (0.2 μm, PO_4^{3-};10 μm PO_4^{3-})	DQ062523, DQ118192

nifH Gene expression

Gene expression was normalized to cell abundance based on the gene copy concentration measured with QPCR. There were diel variations in *nifH* transcript abundances for all of the diazotroph phylotypes in experiments G4 and G6 (Figs. 4, 5). The Group A and B unicellular cyanobacteria phylotypes had opposite patterns of transcription, with maximum expression during the day and night, respectively (Figs. 4, 5). *Trichodesmium* and the heterocystous cyanobacteria expressed *nifH* during the day in G4 (Fig. 4). The γ Proteobacteria (probe AO15) had only a slight diel cycle, with greatest expression during the day (Fig. 4).

Maximum expression per gene copy of the Group A *nifH* phylotype in experiment G4 was <10 transcripts per gene copy (Fig. 4), with expression increasing (100 transcripts per gene copy) in experiment G6 (Fig. 5). The Group B expression per gene copy was also higher in experiment G6 than in experiment G4 (Figs. 4, 5). The heterocystous cyanobacteria phylotype (het-1) had the highest transcript abundances (between 100 and 1,000 transcripts per gene copy) (Fig. 4). *Trichodesmium* maximum transcript abundances were approximately 100 transcripts per gene copy in experiment G4 (Fig. 4).

There was no detectable effect of PO_4^{3-} addition on unicellular cyanobacteria *nifH* gene expression in either experiment G4 or G6 (Figs. 4, 6).

Table 3. Abundance of unicellular cyanobacteria diazotrophs in experiments G4, G5, and G6, determined by QPCR and epifluorescence microscopy in relation to cell-specific (assuming single gene copy per cell) N_2 fixation rates. The mean (±SE, $n = 3$) number of copies mL^{-1} of each *nifH* phylotype was calculated for two size fractions: small (<10 μm but >0.2 μm) and total of small and large (>10 μm, plus <10 μm but >0.2 μm) size fractions. The N_2 fixation rates were not significantly different between light and dark periods (Table 4) so the average N_2 fixation rates for G4 (0.095 nmol N L^{-1} h^{-1}) and G6 (0.048 nmol N L^{-1} h^{-1}) were used to calculate light and dark cell specific rates by dividing by *nifH* gene copies (in the small size fraction) of cyanobacteria that fix N_2 in the light (*Trichodesmium*, *Richelia*, and Group A unicellular cyanobacterial phylotype) or dark (Group B unicellular cyanobacteria). *Richelia* gene copy numbers were divided by the average number of heterocysts (N_2-fixing cells in heterocyst-forming cyanobacteria) in a filament, because heterocysts are the site of N_2 fixation in heterocyst-forming species. Proteobacterial N_2 fixation was not included in cell-specific rate calculations, because they are likely to be smaller than the cyanobacteria and are assumed to be unlikely to contribute significantly relative to cyanobacteria.

			nifH gene abundance (gene copies mL^{-1})†						Cyano specific N_2 fixation rate (nmol N $cell^{-1}$ h^{-1})	
			Dark	Light				Dark + light		
Exp. No. (avg. N_2 fixation rate)‡	Counts§	Size fraction	Group B	Group A	*Richelia*	*Richelia* (heterocysts)	*Trichodesmium*	Proteobacteria AO15	Dark	Light
G4 (0.095±0.047)	678	0.2 μm	428±53.3	7 ±1.5	13±4.1	2	7±2.7	92±27	2.2×10^{-7}	3.3×10^{-6}
		10 μm + 0.2 μm	44 ±53.6	8±1.5	165±84	28	83±46.5	BD	ND	ND
G5 (0.042±0.016)	70	0.2 μm	17±3.7	22±7.7	ND	ND	ND		2.2×10^{-6}	1.6×10^{-6}
G6 (0.048±0.017)	BD	0.2 μm	0.2±0.09	62±8	0.4±0.06	0.07	0.02±0.003	BD	2.4×10^{-4}	7.7×10^{-7}

BD, below detection; ND, not determined.
† Average±standard deviation of experimental replicates.
‡ nmol N L^{-1} h^{-1}.
§ 3–8-μm diameter phycoerythrin-containing (autofluorescent) cells by epifluorescence microscopy (cells mL^{-1}).

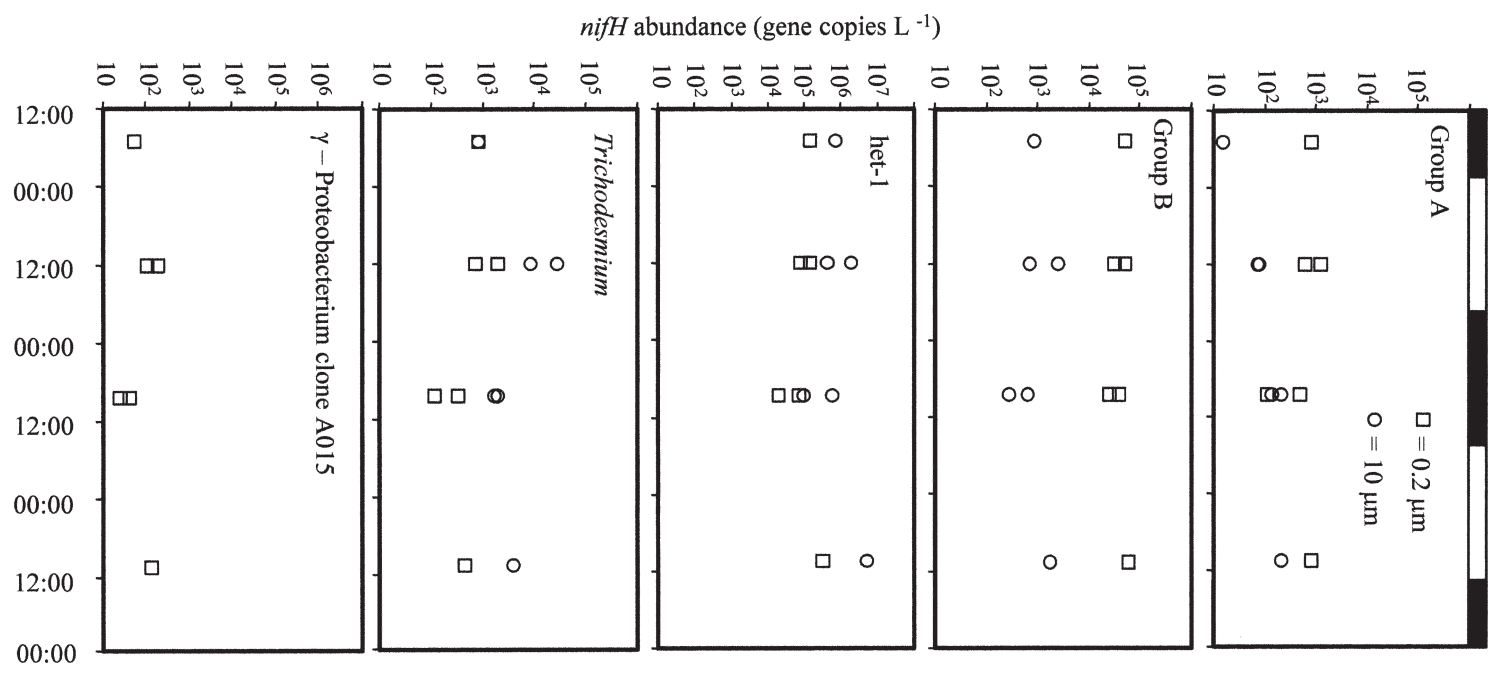


Fig. 2. Abundances of diazotrophs determined by Taqman QPCR in experiment G4 (Sta. ALOHA, 10–13 Aug 2001). Data points are from replicate experimental bottles. Time from the initiation of the incubation experiments is indicated on the X axis, and light and dark periods are indicated by the black (dark) and white (light) bar at the top of the graph.

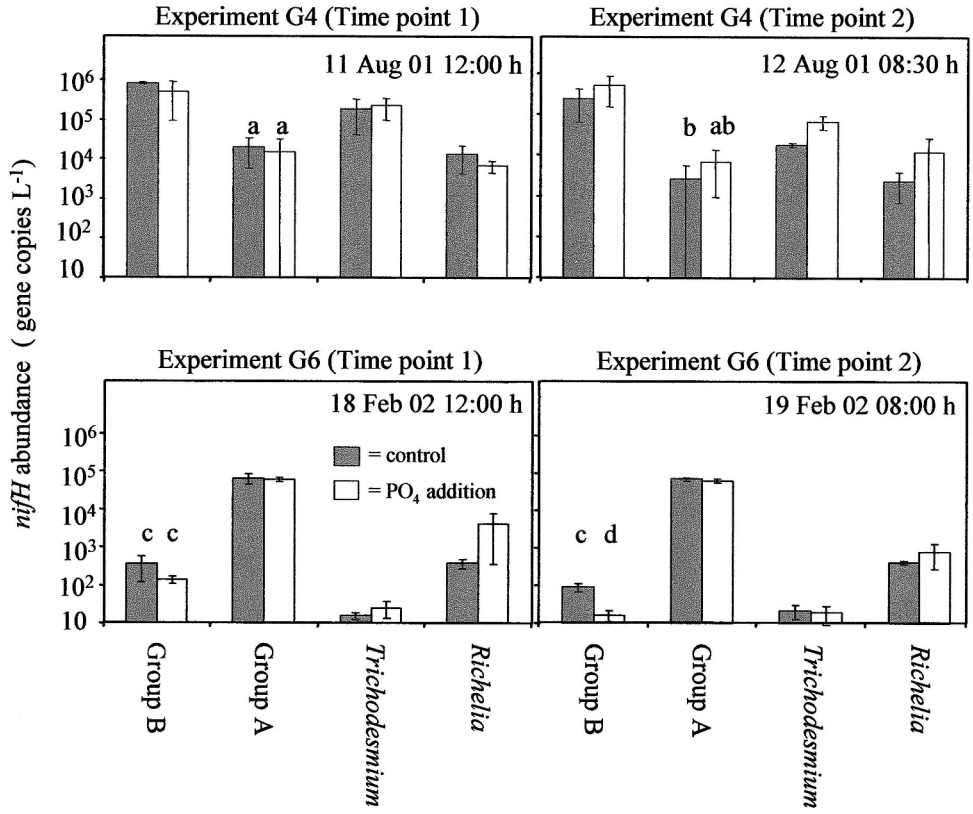


Fig. 3. Abundance of diazotrophs determined by Taqman QPCR in control and PO_4^{3-} addition treatments in experiments G4 (Sta. ALOHA, August 2001) and G6 (outside of Kane'ohe Bay, February 2002). Samples were filtered sequentially through 10-μm and 0.2-μm filters. Data are shown for *Richelia* and *Trichodesmium* in the 10-μm size class and the Groups A and B unicellular cyanobacteria in the <0.2-μm size class. Treatment effects (time point and PO_4^{3-} treatment) were tested with 2-way ANOVA. Significant differences are shown with letters a, b (G4) and c, d (G6). Bars with the same letter are not significantly different from each other.

N_2 fixation rates

N_2 fixation rates were measurable in all experiments (Table 4). Average rates of N_2 fixation in Sta. ALOHA water ranged from 0.014 (G1) to 0.53 (G5) nmol N L^{-1} h^{-1}. Rates were lowest in experiment G1 and highest in experiment G4, which was also performed with water from Sta. ALOHA (Table 4).

Two experiments with coastal water from outside Kane'ohe Bay (G2 and G6) focused on the effect of PO_4^{3-} amendment on the N_2 fixation rates. The G5 experiment was designed to test the effect of 10-μm prefiltration on measured N_2 fixation rates (Table 4). The subsequent G6 experiment was prefiltered to focus on N_2 fixation by the unicellular cyanobacteria. The mean volumetric N_2 fixation rates were similar in all three experiments, ranging between 0.029 nmol N L^{-1} h^{-1} and 0.053 nmol N L^{-1} h^{-1}.

There were some significant differences ($k < 0.05$) among rates measured during only the dark period, a full day (dark + light) or a day and a half (dark + light + dark) in experiments G4 and G6 (Table 3). However, differences in rates between the light-dark periods were relatively small, and significant N_2 fixation rates occurred during both light and dark periods suggesting that N_2 fixation occurred during the light and dark periods in all experiments.

Gene copy specific rates were calculated assuming observed N_2 fixation rates in light and dark periods were caused by the organisms expressing *nifH* during the light or dark periods. The gene copy specific rate in the dark in experiment G4 was 2.2×10^{-7} nmol N gene $copy^{-1}$ h^{-1} assuming the N_2 fixation was caused by Group B unicellular cyanobacteria. The specific rate in the dark in experiment G6 was substantially higher (2.0×10^{-4} nmol N gene $copy^{-1}$ h^{-1}).

N_2 fixation in the light was assumed to be caused by Group A unicellular cyanobacteria, *Richelia* (specifically the heterocysts, which are about one out of six cells of a *Richelia* filament), and *Trichodesmium*. The cell-specific rate in the light in experiment G6 was about 8×10^{-7} nmol N gene $copy^{-1}$ h^{-1}, which is higher than the rate in the light in experiment G4 (3.3×10^{-6} nmol N gene $copy^{-1}$ h^{-1}, Table 3). Subsequent to this study, Taqman probes for two additional heterocystous cyanobacterial sequence types previously seen in clone libraries from the Pacific Ocean and HOT (Fig. 1) (Foster and Zehr, unpubl. data) were developed. These sequences correspond

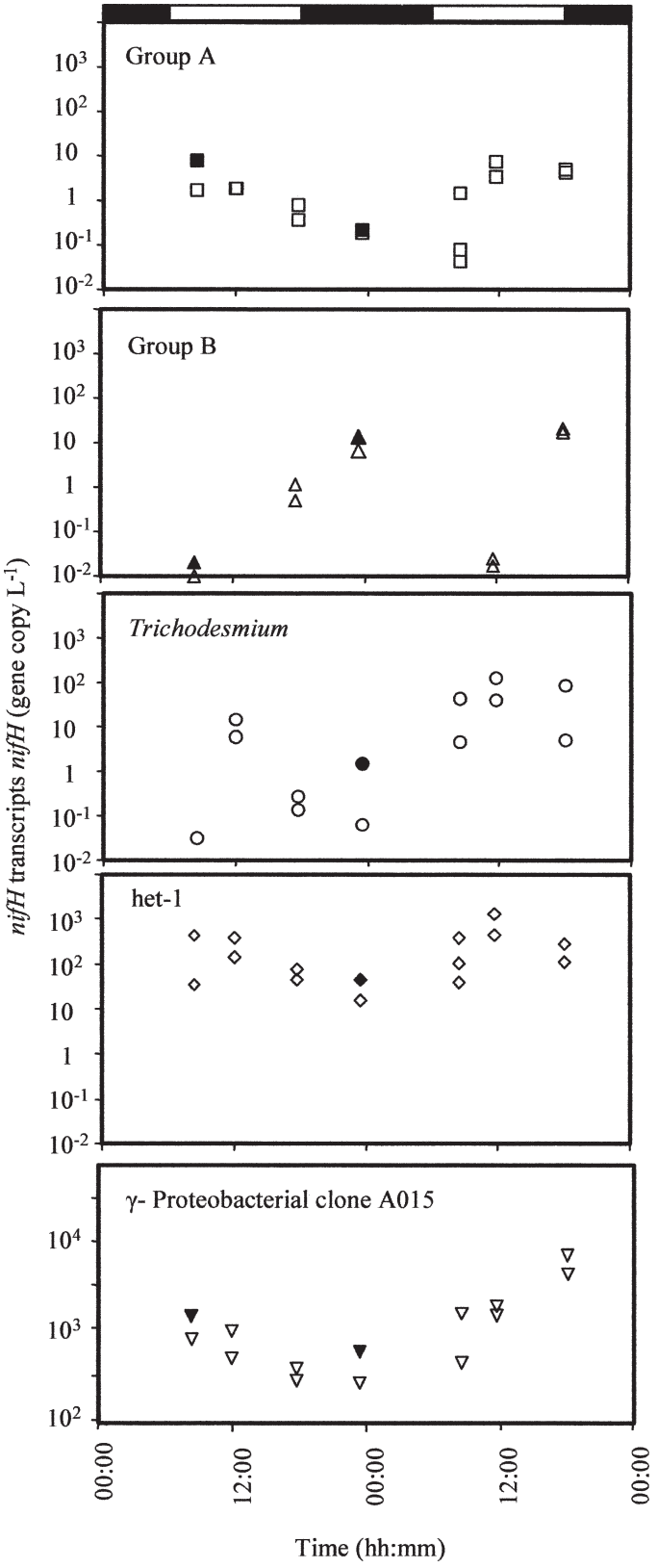


Fig. 4. Diel expression of *nifH* normalized to *nifH* gene copies during the daily cycle in experiment G4 (Sta. ALOHA, August 2001) determined by QRT-PCR using Taqman primers and probes for Group A and B unicellular cyanobacteria sequence types, *Trichodesmium*, a heterocystous cyanobacterial group (het-1) believed to be a diatom symbiont, and the γ Proteobacteria AO15. Filled symbols indicate *nifH* expression in PO_4^{3-} treatments.

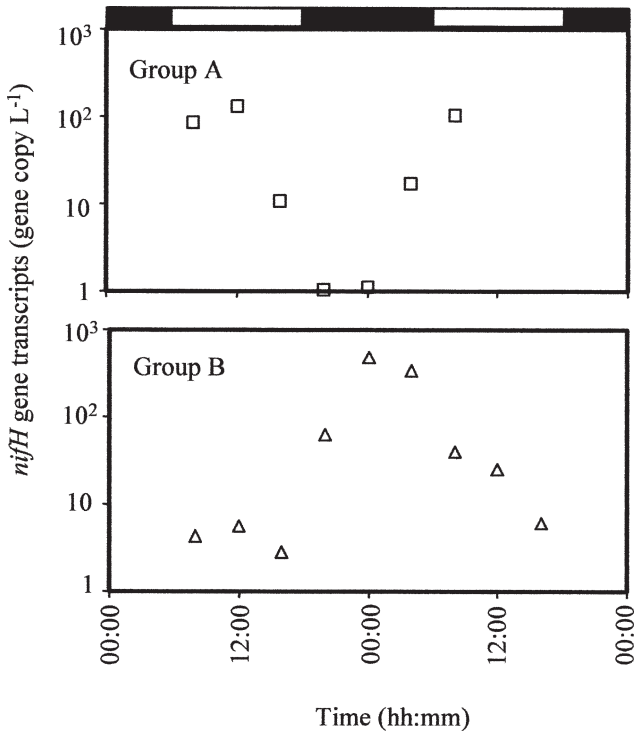


Fig. 5. Normalized *nifH* gene expression in experiment G6 (offshore of Kane'ohe Bay, February 2002) determined by QRT-PCR using Taqman primers and probes for the Group A and B unicellular cyanobacterial sequence types. *nifH* gene copy numbers were determined by QPCR and transcripts by QRT-PCR using Taqman primers and probes.

to diatom symbionts (Foster and Zehr, unpubl. data). G4 samples probed for the het-2 and het-3 groups (Fig. 1) (Foster and Zehr, unpubl. data) showed that these symbionts were present in G4, but at an order of magnitude lower than the het-1 phylotype. The rates per cell in the light might be slightly lower considering the contribution of the het-2 phylotype (expression of het-2 and het-3 were not determined because they were so much less abundant than the het-1 phylotype) and more in agreement with the cell-specific rates in the light in experiment G6. There were very few *Richelia* (het-1, het-2, or het-3) or *Trichodesmium* present in G6 (water for the G6 experiment had been prefiltered with a 10-μm mesh to remove the larger diazotrophs), so light N_2 fixation was likely dominated by unicellular cyanobacteria, which were more abundant in experiment G6 than in G4.

We found no consistent effect of PO_4^{3-} addition on N_2 fixation rates. We tested for differences across all time points (data not shown) and treatment by nested ANOVA for experiments G4, G5, and G6. We found no significant differences in experiment G5. In contrast, experiment G4 showed significant experiment-wide differences among time

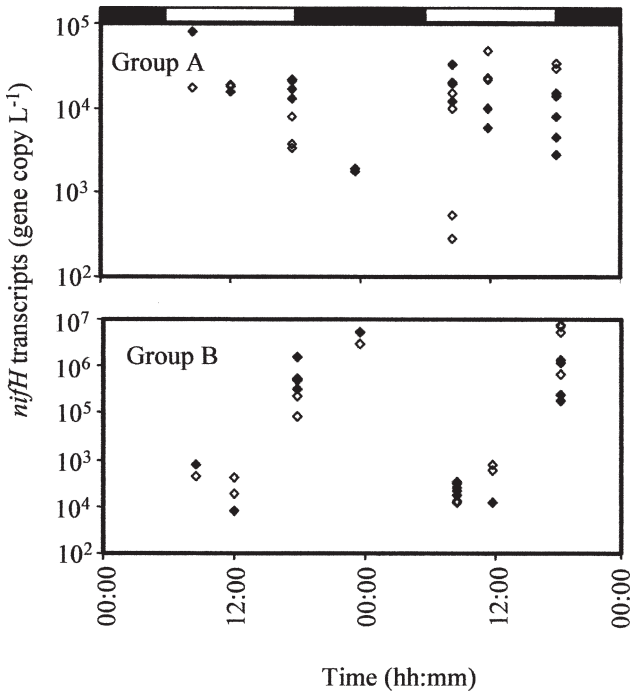


Fig. 6. *nifH* gene expression in experiment G4, showing expression in controls (open symbols) and phosphorus treatments (filled symbols). Experimental treatments were duplicated, and each sample was run at least twice by QRT-PCR, when possible. All replicates (analytical and experimental) are shown.

points ($p = 0.0025$) but not between the control and PO_4^{3-} treatments ($p = 0.2001$). Finally, experiment G6 showed significant differences among time points ($p < 0.0001$) as well as treatments ($p = 0.0161$). A multiple comparison test (Tukey's honestly significant different test) revealed a complex relationship between the control and PO_4^{3-} treatments: the PO_4^{3-} incubations had a lower rate of N_2 fixation than the controls at the first two time points, a pattern that was reversed for the last three time points (which extended beyond the time periods analyzed for gene expression). The complex pattern of effect of P on N_2 fixation will be discussed elsewhere (Montoya, unpubl. data).

Discussion

nifH Diversity

All of the *nifH* sequences detected in RNA samples clustered with sequence groups previously reported. The major groups of diazotrophs found in this study by degenerate RT-PCR cluster with sequences obtained in studies from the Pacific Ocean (Zehr et al. 2001; Falcón et al. 2004*a*; Church et al. 2005*a*), the Atlantic Ocean (Falcón et al. 2004*a*) and the Arabian Sea (Mazard et al. 2004; Bird et al. 2005). Most of the diazotrophic groups identified to date appear to be widely distributed throughout the ocean basins.

γ Proteobacteria *nifH* sequences have been reported from several oceanic environments, including the Arabian Sea, the Atlantic Ocean, and the Pacific Ocean (Zehr et al. 2001; Bird et al. 2005; Church et al. 2005*a*). These Proteobacteria appear to be oligotrophic diazotrophic bacteria and are likely heterotrophic or photoheterotrophic. It remains unclear whether these bacterial *nifH* sequences are linked to 16S rRNA phylotypes usually found in oceanic ribosomal RNA surveys. The apparently low abundances of these Proteobacterial diazotrophs suggest they may not contribute substantially to N_2 fixation relative to the cyanobacteria.

Several clusters of oceanic Proteobacteria *nifH* have been reported from oceanic samples (Zehr et al. 1998; Falcón et al. 2004*a*), but we have shown that at least one of the α Proteobacteria groups can be present as a contaminant in PCR reagents (Group 4, Fig. 1A) (Zehr et al. 2003*a*). There are Proteobacterial *nifH* genes in many reagents at low levels, which can be problematic when nested PCR or RT-PCR approaches are used (Zehr et al. 2003*a*). The problem with such phylotypes is that they could be present in the samples and the reagents, and it can be difficult to distinguish false positives from the phylotype being present in both reagents and the environment. These sequences can be confirmed as real environmental sequences by QPCR, like we have done with the AO15 phylotype, but the rest of the Proteobacterial *nifH* sequences reported from the oceans should be interpreted with caution.

There are only a few major groups of oceanic cyanobacterial *nifH,* but there are distinct subclusters within the groups of unicellular and heterocystous cyanobacteria. Other cyanobacteria that thrive in the open ocean, such as the non–N_2-fixing *Prochlorococcus* strains, have relatively small variations between 16S rRNA sequences that correspond to major differences between genome composition and genome sizes (Rocap et al. 2003). Closely related strains of *Prochlorococcus* that differ substantially in genome size and content are adapted to different habitats within the water column (Partensky et al. 1999; Hess et al. 2001; Rocap et al. 2003). Similarly, genomic variability within closely related 16S rRNA phylotypes of *Vibrio* has recently been documented (Thompson et al. 2005). It is possible that the *nifH* sequence clusters in the diazotrophic cyanobacteria groups also represent different strains or ecotypes that occupy distinct environmental niches. The genomic variability of the diazotrophic cyanobacterial groups has yet to be determined.

The *nifH* sequence types found in our experiments were similar to those found in freshly collected samples from Sta. ALOHA (G1, Table 2). *Trichodesmium*, diatom symbionts, and unicellular cyanobacterial diazotrophs were present in the first experiment in July 2000 and were also found in the subsequent experiments in 2001 and 2002. These results suggest similar diazotrophs are present in the North Pacific subtropical gyre from year to year. The composition of the clone libraries and the abundance of individual groups of diazotrophs as assayed by QPCR of *nifH* genes remained relatively constant across the 36-h period of the experiments. This indicates that population abundances were stable and that the experimental design did not result in significant population shifts during the 36-h incubations. Thus, the experimental design should be adequate for

Table 4. Summary of rates of $^{15}N_2$ fixation measured (mean±SD) in experiments using water from Sta. ALOHA and outside Kane'ohe Bay. Time of sampling shown in brackets. Rates were measured cumulatively during dark (D), dark-light (DL) or dark-light-dark (DLD) periods. Since the rates are integral over successive time points, similar rates measured during D, DL, or DLD indicate continuous N_2 fixation during both light and dark periods.

Experiment	Dates	Treatment	*n*	T1 : D N_2 fixation rate (nmol N L^{-1} h^{-1}) [Time of sampling]	T1 : DL N_2 fixation rate (nmol N L^{-1} h^{-1}) [Time of sampling]	T1 : DLD N_2 fixation rate (nmol N L^{-1} h^{-1}) [Time of sampling]	Average N_2 fixation rate (nmol N L^{-1} h^{-1}) (*n*) = No. of replicates
ALOHA							
				[11:00 h]	[20:20 h]		
G1	28 Jul 00–30 Jul 00	Control	3	0.0088±0.0029	0.012±0.00027		0.014±0.005 (6)
		+ PO_4^{3-}	3	0.017±0.017	0.017±0.0060		0.014±0.005 (6)
				[08:15 h]	[19:15 h]	[08:25]	
G4	10 Aug 01–13 Aug 01	Control	3	0.15±0.02[a]	0.090±0.0053[a]	0.11±0.027[a]	0.095±0.047 (15)
		+ PO_4^{3-}	3	0.26±0.15[b]	0.14±0.02[ab]	0.12±0.03[a]	0.136±0.091 (15)
Kane'ohe Bay							
				[08:20 h]	[16:35 h]	[09:00 h]	
G2	30 Jul 01–01 Aug 01	Control	2	0.0094±0.077	0.040±0.020	0.043±0.0069	0.029±0.017 (6)
		+ PO_4^{3-}	2	0.0070±0.0012	0.0045±0.027	0.042±0.031	0.030±0.002 (6)
				[08:40 h]	[17:20 h]	[08:45 h]	
G5	13 Aug 01–15 Aug 01	Control	3	0.028±0.0031[a]	0.038±0.0085[a]	0.042±0.016[a]	0.036±0.011 (9)
		Prefiltered	3	0.061±0.029[b]	0.043±0.015[ab]	0.055±0.013[ab]	0.053±0.019 (9)
				[08:40 h]	[16:40 h]	[04:40 h]	
G6	17 Feb 02–19 Feb 02	Control	3	0.036±0.021[a]	0.073+0.0088[b]	0.044+0.011[a]	0.048±0.017 (15)
		+ PO_4^{3-}	3	0.017±0.0048[ac]	0.058±0.015[bd]	0.057±0.011[ad]	0.050±0.020 (15)

[a, b, c, d] Indicates results of 2-way ANOVA. Treatments and time points with same letter are not significantly different.

examining short-term effects on diazotroph population abundance, gene expression, and N_2 fixation activity.

Size class of diazotrophs and N_2 fixation

The fact that the estimates of Group B unicellular cyanobacterial diazotroph abundance by epifluorescence microscopy agreed well with the Group B QPCR results suggests that Group B *nifH* gene copy numbers provide a reasonable proxy for Group B cell abundances (Table 3). However, a six-fold increase in the QPCR-derived estimates of Group A abundances (in G6 compared to G4) was not reflected in microscopy-derived counts of 3–8-μm diameter unicellular cyanobacteria (Table 3). Although our microscopic enumeration of cells had a high error rate because of the relatively low abundance of some of the unicellular cyanobacteria (relatively few cells per field of view), these data indicate that the Group A unicellular cyanobacteria differ from the Group B cyanobacteria in cell size (presumably smaller) or that the Group A phylotype lacks phycoerythrin. If these cells are smaller than Group B, they would likely be similar in size and therefore indistinguishable from non-N_2–fixing *Synechococcus* by microscopy and flow cytometry.

We prefiltered all of our experimental samples through coarse nylon mesh (110 μm) to reduce the abundance of large diazotrophs. In the G5 and G6 experiments, we carried out a second prefiltration through a 10-μm nylon mesh to reduce the abundance of individual filaments of *Trichodesmium* or other large diazotrophs from our incubations. We did this to maximize the ability to detect rates of activity by small diazotrophs and to ensure that observed ^{15}N incorporation was not attributable to transfer from large cells to small cells during the incubation. Even a small number of larger diazotrophs could potentially transfer ^{15}N to the small size fraction of PN by exudation of $DO^{15}N$ and/or $DI^{15}N$. There was no significant difference in measured rates of N_2 fixation regardless of whether we carried out the second prefiltration step (experiment G5, Table 4). This further supports the conclusion that measured N_2 fixation rates in the small class in the other experiments was caused by activity by microorganisms that passed through the 10-μm filter and was not because of transfer of fixed N from larger diazotrophs to smaller microorganisms.

Clone libraries and QPCR results (Tables 2, 3; Fig. 3) show that a fraction ($\sim$10%) of the genes from the larger diazotrophs can pass through mesh, and some small diazotrophs are caught on large mesh (experiment G6, Table 3). *Trichodesmium* filaments are often $<$10 μm wide, so it seems likely that some of these free trichomes might pass through the filter. It is possible that the heterocystous symbionts have a free-living stage, but the symbiont filaments themselves are larger than 10 μm in diameter, and the presumed diatom hosts are much larger. Since the clone libraries and QPCR measure genes, not cells, it is possible that materials passing through the filter were not active cells, but cell fragments or extracellular DNA. These results have implications for activity measurements and pigment assays, since it is difficult, if not impossible, to obtain a pure separation of the different groups of plankton. Although the size fractionation worked moderately well for distinguishing between the larger diazotrophs (*Trichodesmium* and the heterocystous symbionts) and the unicellular cyanobacteria, the results confirm that size fraction results must be interpreted with caution.

nifH gene expression in enclosed populations confirms in situ diel cycles

The quantitative RT-PCR results show that the daily patterns of gene expression were stable across the experiments, which along with the abundance data (discussed above) confirms that populations were stable and remained active for the course of the experiments. However, there was no detectable effect of PO_4^{3-} on diazotroph abundance (Fig. 3) or *nifH* gene expression. We anticipated that diazotrophs, since not limited by N availability, would be stimulated by the addition of PO_4^{3-}. The lack of a consistent response suggests that during the course of these experiments ($\sim$36 h) the diazotrophs were likely limited by the availability of other nutrients, trace metals, or other factors. Mills et al. (2004) found that PO_4^{3-} and Fe colimit N_2 fixation in the Eastern Tropical North Atlantic.

Because daily patterns of gene expression generally coincide with N_2 fixation (Colón-López et al. 1997; Chen et al. 1998), it appears that the Group B unicellular cyanobacterial phylotypes were fixing N_2 at night, and the heterocystous and Group A unicellular cyanobacterial phylotypes are fixing N_2 during the day. An isolate of the Group A cyanobacterium was previously reported from the Pacific Ocean (Falcón et al. 2004*b*). However, our testing of isolates from these strains yielded only *nifH* sequences identical to the Group B phylotypes, and these cultures did not test positive with the Group A QPCR probes. The Group A daily expression pattern in the present study and that of Church et al. (2005*b*) contradict the pattern described by Falcón et al. (2004*b*), who reported that a Pacific Ocean unicellular cyanobacteria isolate was a Group A type and fixed nitrogen during the dark period. It could be that culture or environmental conditions (light, nutrients, and temperature) affect the daily cycle of nitrogenase gene expression and N_2 fixation. Because the original cultures reported in Falcón et al. (Falcón et al. 2004*b*) are no longer available, the original isolation of a Group A phylotype cannot be confirmed. Resolution will have to await the successful cultivation of the Group A phylotype.

It is assumed that the Group A phylotype is a unicellular cyanobacterium because of its phylogenetic affiliation and its diel *nifH* gene expression. However, this has yet to be confirmed conclusively by cultivation or experimentation. The results of this study and that of Church et al. (2005*b*), may present a new enigma: simultaneous N_2 fixation and photosynthesis by unicellular cyanobacteria that cannot have cellular level spatial separation of the two processes. However, culture experiments with unicellular cyanobacteria have previously shown that N_2 fixation can occur in the light period under the right conditions of irradiance and growth rate (Ortegacalvo and Stal 1991; Stal 2003). In fact, experiments with unicellular cyanobacterial cultures show

that cultures often exhibit daily rhythms of N_2 fixation under constant illumination (Mitsui et al. 1987; Schneegurt et al. 1994). Although the mechanisms are not understood, it may be that different cells within the population are in different physiological states. Our data suggest that natural, open ocean populations of unicellular cyanobacteria may have similar behavior. However, we are currently attempting to confirm that this phylotype is derived from a cyanobacterium using cultivation and environmental genomics approaches.

N_2 fixation rates by different diazotroph groups

The rate of N_2 fixation varied by roughly an order of magnitude among the five experiments (Table 4) along with differences in the presence and abundance of different diazotroph groups (Tables 2, 3; Fig. 3). For example, the rate of N_2 fixation in experiment G4 was roughly twice the rate in experiment G6 (Table 4), whereas overall diazotroph abundance, measured as the concentration of *nifH* gene copies in all size fractions, was about ten-fold higher in experiment G4 (Table 3) than in experiment G6. (Note that the N_2 fixation rate measurements were in the small size fraction in both experiments, but that experiment G6 was prefiltered through 10 μm mesh before the experiment). The measured rates in G4 are consistent with those reported for Sta. ALOHA by Dore et al. (2002). Interestingly, the composition of the diazotroph community was very different in these two experiments, with the G4 population strongly dominated by Group B cyanobacteria and the G6 population dominated by Group A cyanobacteria.

N_2 fixation rates with very strong diel phasing should show clear differences between rates measured in incubations terminated at the end and beginning of the light period. The rates were similar although in some cases significantly different for dark, dark + light, or dark + light + dark incubations, indicating that N_2 fixation was occurring during both light and dark periods (Table 3). This was consistent with the presence of cyanobacteria, which typically express nitrogenase during the day (*Trichodesmium, Richelia*, and Group A unicellular cyanobacteria) and night (Group B unicellular cyanobacteria) (Figs. 4, 5; Table 3). These experiments demonstrated conclusively that there are different patterns of gene expression in the different cyanobacterial groups, as previously suggested from diel sampling of natural populations at Sta. ALOHA in December 2002 (Church et al. 2005*b*). It is significant that the bottle incubation experiments in our study exhibited diel gene expression patterns equivalent to those in freshly collected samples. Our results show that the previously documented expression patterns detected in situ (Church et al. 2005*b*) were not the result of sampling different water masses at different times of day and that the populations continue to express nitrogenase even under experimental conditions.

The N_2 fixation rates measured in experiment G4 were higher than in experiment G6 and were coupled with higher abundances of Group B unicellular cyanobacteria and γ Proteobacteria in the small size fraction and *Trichodesmium* and *Richelia* in the large size fraction (Table 3). The number of gene copies of the Group B phylotype exceeded those of all other diazotrophs combined (in both size fractions) in experiment G4. Rates per cell (gene copy) in the light (*Trichodesmuim, Richelia*, and Group A unicellular cyanobacteria) exceeded those in the dark (Group B unicellular cyanobacteria). However, the total rates were not significantly different between light and dark (because of the high abundance of Group B cyanobacteria in experiment G4), indicating that the Group B unicellular cyanobacteria contribute significantly to total N_2 fixation. This is consistent with the finding of Montoya et al. (2004) that the small size fraction plays a significant role in N_2 fixation in oceanic waters.

Total rates of N_2 fixation were between 0.03 nmol N L^{-1} h^{-1} and 0.05 nmol N L^{-1} h^{-1} in experiments G5 and G6 (Table 4). These experiments were initiated with water obtained offshore of Kane'ohe Bay and the N_2 fixation rates were lower than in experiment G4. *Trichodesmium* and *Richelia* were present in very low concentrations in experiment G6, likely partly because of the prefiltration of this experiment. Although the G6 experiment had been prefiltered, the numbers of *Trichodesmium* and *Richelia* were in even lower abundances than in the small size fraction of experiment G4, which had not been prefiltered. Group B unicellular cyanobacteria were present, but also were in lower concentrations than in experiment G4.

The most abundant *nifH* gene in experiment G6 was the Group A unicellular cyanobacterial phylotype (Table 3). The rates per cell (assuming a single gene copy per cell, which is true of *Trichodesmium* and *Crocosphaera*) of the light phase N_2-fixers in experiment G6 (*Trichodesmium, Richelia*, and the Group A unicellular cyanobacteria) were an order of magnitude lower than in experiment G4 (Table 3). The higher rates per cell of the light phase N_2-fixers in experiment G4 may be attributable to the presence of undetected N_2-fixers in this experiment, but it is more likely that the rates per cell are due to differences in community composition and physiology. Although, it is possible that het-2 or het-3 filamentous, heterocyst-forming, symbiotic cyanobacteria were present in experiment G4 (Foster and Zehr, unpubl. data), which would account for the calculated differences in cell-specific rates in the light. However, subsequent to this study we designed probes to the het-2 and het-3 cyanobacteria, and they were found to be an order of magnitude lower than het-1 in experiment G4 (data not shown). It is also possible that the Group A unicellular cyanobacteria fix N_2 at lower rates per cell than the larger diazotrophs (*Trichodesmium* and *Richelia*), which were much more abundant in experiment G6. The gene expression results are consistent with this hypothesis, because the het-1 phylotype has a much higher number of transcripts per gene copy than do the group A phylotypes (Fig. 4).

The N_2 fixation rates per cell in experiment G6, where the unicellular Group A cyanobacteria were the most abundant N_2-fixer that expressed *nifH* in the light phase, indicate that the Group A unicellular cyanobacteria phylotype might be important in N_2 fixation, but only when present at substantially higher abundance than the larger diazotrophs, which have higher N_2 fixation rates per cell. This is consistent with the hypothesis that the Group A

diazotrophs are smaller cells than the Group B unicellular cyanobacteria. However, more data are needed under different physiological conditions to determine the variability in cell-specific rates of the Group A *nifH* phylotype. In contrast, rates per cell of the dark phase N_2-fixers (Group B unicellular cyanobacteria) were much higher than in G6. The Group B *nifH* gene expression per gene copy was much higher than in G6 (Figs. 4, 5), which is consistent with higher dark rates per cell in this experiment. This indicates that *nifH* gene expression may serve as an indicator of the per cell rate of fixation, but also that gene copy abundance is not a sufficient measure of the relative contribution to total N_2 fixation.

The diazotrophic γ Proteobacteria were present in experiments G4 and G6, and it appeared there was a diel cycle in gene expression in these bacteria as well as the cyanobacteria. Presumably the diel cycling by heterotrophic bacteria might be linked to the excretion of organic carbon from phototrophs or perhaps reflects a phototrophic or endosymbiotic physiology by the Proteobacteria. We did not attempt to assess the contribution of bacteria other than cyanobacteria to total N_2 fixation, because we assumed that these are smaller cells and likely to have lower rates of N_2 fixation per cell.

Lack of stimulation of N_2 fixation by addition of PO_4^{3-}

We found no consistent evidence for stimulation of N_2-fixing microorganisms, *nifH* gene expression, or N_2 fixation rates by P addition in the experiments (Table 4; Figs. 3, 4, 6). Although the composition of the diazotroph community varied between experiments, PO_4^{3-} addition did not substantially affect the composition of clone libraries during the relatively short time scales of these incubations. The addition of PO_4^{3-} did not result in a statistically significant increase in diazotroph abundance (Fig. 3). There was a complex response to PO_4^{3-} addition with significant increases in N_2 fixation at later time points that will be discussed elsewhere (J. P. Montoya, unpubl. data). There was no consistent effect of PO_4^{3-} on N_2 fixation during the time period examined for the gene expression or in the averaged light-dark period N_2 fixation rate data presented in Table 4.

We expected to see stimulation of gene expression in response to PO_4^{3-}, even if not detected as increased rates. None of the diazotroph groups showed stimulation in the G4 experiment where both large and small diazotrophs were present. The G6 experiment focused on the effect of PO_4^{3-} on unicellular cyanobacterial diazotrophs, and most of the larger diazotrophs had been removed, but no effect on gene expression was observed. However, it is conceivable that the populations became P limited during the course of the incubation, and the addition of PO_4^{3-} simply prevented decline of the populations over time.

Since diazotrophs have access to an essentially unlimited supply of nitrogen to support growth, phosphorus availability can potentially limit their growth and production. We anticipated that our PO_4^{3-} amendments would lead to enhanced N_2 fixation relative to our unamended controls by releasing the diazotrophs from P limitation. *Trichodesmium* and *Crocosphaera* appear to differ in their abilities to use different P compounds, with *Trichodesmium* able to use phosphonates (organic compounds containing C–P bonds) whereas *Crocosphaera* (Group B) lacks the genes for the phosphonate lyase (Orchard et al. 2003; Dyhrman et al. 2006; Dyhrman and Haley 2006). We would have expected the addition of inorganic P to stimulate *Crocosphaera* (experiments G4 or G6) as well as *Trichodesmium* (experiment G4), or the other cyanobacteria (Group A). Although we did not measure P in the experiments, the experiments were handled with clean techniques, and the additions were significantly above background levels (0.5 μmol L^{-1}), so it is unlikely that the water or bottles were systematically contaminated with P.

Future experiments in which PO_4^{3-} concentration and alkaline phosphatase activity are monitored could help to determine whether populations became more P limited during the experiment. It is not possible to target specific diazotroph groups using these approaches until they have all been cultivated and more genomic information is available.

This is the first study that simultaneously examines *nifH* gene abundance, gene expression, and N_2 fixation rates in the oligotrophic open ocean. These experiments have provided links between specific diazotroph populations and rates of N_2 fixation in the North Pacific subtropical gyre. Using controlled conditions, we found that diazotrophic cyanobacteria and bacteria maintain daily cycles of gene expression and activity for at least 36 h in bottle experiments. Consistent with the Church et al. (2005*b*) study, we observed different diel patterns of *nifH* expression by different groups of diazotrophs.

The Group A cyanobacterial *nifH* phylotype, which are believed to be unicellular cyanobacteria because of the phylogenetic affiliation of the *nifH* gene, appear to express nitrogenase and thus, probably fix N_2 during the day. Microscopy and gene abundance data indicate that the Group A phylotype differs in size or fluorescent properties from the Group B *Crocosphaera*-like cyanobacteria. These cyanobacteria may be difficult to identify if they are smaller than the Group B cells, since they will be similar in size to non–N_2-fixing *Synechococcus*.

Cell-specific rates, calculated as N_2 fixation divided by gene copy number, varied between the experiments, but generally agreed with abundance of the different groups of diazotrophs and the levels of *nifH* expression of each group.

Phosphate additions had very little effect on the abundance or gene expression of diazotrophs during the course of the 36-h experiments, indicating that other factors may limit the growth gene expression and activity of diazotrophs in the North Pacific subtropical gyre.

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