Limnol. Oceanogr., 53(1), 2008, 63–77

Regional distributions of nitrogen-fixing bacteria in the Pacific Ocean

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Abstract

We evaluated the regional distributions of six nitrogen (N_2)-fixing bacteria in the North Pacific Ocean using quantitative polymerase chain reaction amplification of planktonic *nifH* genes. Samples were collected on four oceanographic research cruises between March 2002 and May 2005 that spanned a latitudinal range from 12°S and 54°N between 152°W and 170°W. Samples were collected throughout the upper ocean (<200 m) in the northern regions of the South Pacific Subtropical Gyre (SPSG), equatorial waters, the North Pacific Subtropical Gyre (NPSG), the North Pacific Transitional Zone (NPTZ), and within the Pacific Sub Arctic Gyre (PSAG). There were distinct spatial gradients in concentrations of nutrients, chlorophyll, and the abundances of N_2-fixing bacteria within the various oceanic biomes. In general, *nifH*-containing bacteria were most abundant in the midregions of the NPSG (latitudes between ~14°N and 29°N), where unicellular cyanobacterial phylotypes dominated *nifH* gene abundances. The abundances of all *nifH*-containing groups declined within the northern and southern regions of NPSG. Although *nifH*-containing groups were detectable in the northern regions of the SPSG, throughout the equatorial waters, and within the NPTZ, gene copy abundances of most groups were lower in these regions than those found the in the NPSG. In the NPSG, surface water abundances of the various *nifH* phylotypes examined ranged from <50 copies L^{-1} to ~10^5 *nifH* copies L^{-1}. Overall, the abundances of an uncultivated, presumed unicellular *nifH* sequence-type (termed Group A) were the most abundant and widely distributed of the phylotypes examined. Our results indicate that the distributions of N_2-fixing plankton were largely restricted to the subtropical regions of the North and South Pacific Oceans.

In large areas of the world's oceans, the activities of nitrogen (N_2)-fixing microorganisms (diazotrophs) provide an important source of nitrogen to the marine ecosystem. Planktonic diazotrophs provide a major fraction of the exogenous nitrogen supporting both productivity and carbon export in many tropical and subtropical ocean environments (Capone and Carpenter 1982; Michaels et al. 1996; Karl et al. 2002). Time-series measurements of particulate nitrogen export in the oligotrophic North Pacific Ocean indicate that N_2-fixing microorganisms contribute more than half of the new nitrogen supporting particulate matter export, rivaling or surpassing the contribution by nitrate in supporting new production in this ecosystem (Karl et al. 1997; Dore et al. 2002).

The biological reduction of N_2 gas to ammonia (NH_3) is catalyzed by the enzyme nitrogenase. Nitrogenase consists of two conserved proteins: an iron (Fe) containing dinitrogenase reductase (or Fe protein), encoded by the *nifH* gene, and a molybdenum iron (MoFe) dinitrogenase (or MoFe protein) that is encoded by the *nifDK* genes. Developments of molecular-based techniques to interrogate *nifH* gene sequences in marine environments have provided unique tools for assessing the diversity of organisms with the genetic capacity for N_2 fixation (Zehr and McReynolds 1989; Zehr et al. 1996).

Much of our understanding of the ecology and biogeochemistry of oceanic diazotrophy has derived from studies on the filamentous cyanobacterium *Trichodesmium* spp., a cosmopolitan cyanobacterium in tropical and subtropical marine systems, including oligotrophic regions throughout the Atlantic and Pacific Oceans (Capone and Carpenter 1982; Carpenter and Romans 1991; Letelier and Karl 1996), the Red Sea (Post et al. 2002), and the Arabian Sea (Capone et al. 1998). However, use of the *nifH* gene as a genetic marker for N_2-fixing plankton has identified greater diversity among the N_2-fixing marine microorganisms than previously recognized. In particular, several

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Acknowledgments

We thank the captain and crew of the R/V *Kilo Moana* for facilitating this research and for skillful seamanship through occasionally unfriendly seas. The dedicated efforts of the staff of the Biogeochemical & Ecological Analysis of Complex Habitats (BEACH) program helped with the collection and analyses of nutrient and chlorophyll samples during the COOK-BOOK I, II, and BEACH-BASH cruises. We thank the anonymous reviewers for their constructive comments on this manuscript. Funding for this project came via grants from the National Science Foundation to J.PZ. and M.J.C. (OCE 04-25363), M.A.S. (OCE 03-27225), and D.M.K. (OCE 03-26616). Additional support from the Gordon and Betty Moore Foundation to D.M.K. and J.P.Z. helped make this study possible.

groups of cyanobacteria, members of the Proteobacteria, and various anaerobic microorganisms all appear to have the capacity to fix N_2 in the oceans (Zehr et al. 1998, 2001; Braun et al. 1999). Despite increased awareness of this potential diversity, few studies have provided quantitative information on the abundances or distributions of specific diazotroph assemblages in the sea.

The Pacific Ocean contains a number of distinct plankton habitats whose boundaries are determined largely by wind-driven circulation (Longhurst 1998). In the northern regions of the Pacific, prevailing westerly winds and associated Ekman divergence form a large cyclonic gyre termed the Pacific Sub Arctic Gyre (PSAG). Further south, the North Pacific Transitional Zone (NPTZ) demarcates the boundary between cool, relatively fresh waters of the PSAG and warmer, saltier waters of the North Pacific Subtropical Gyre (NPSG). The northeastern trade winds and associated Ekman transport establish the massive anticyclonic convergent circulation pattern throughout the NPSG. The southern end (~10°N) of the NPSG is marked by the Intertropical Convergence Zone, which denotes the northern boundary of North Equatorial Counter Current (NECC). This region develops along the boundary where northeastern and southeastern trade winds intersect, creating the transitional boundary between westward-flowing Northern Equatorial Current and the eastward-propagating NECC. Along the equator, trade wind–induced stress forms the region of the Pacific Equatorial divergence, where vertical shoaling of isopycnal surfaces entrains nutrients in the upper ocean. South of the equatorial regions, the South Pacific Subtropical Gyre (SPSG) lies within the large anticyclonic gyre found throughout the low and midlatitudes of the South Pacific.

In this study, we used a quantitative polymerase chain reaction (QPCR)–based approach (Short et al. 2004) to determine the vertical and meridional distributions of N_2-fixing bacteria in the upper ocean of the Pacific Ocean. We report the results from four cruises spanning a latitudinal range between ~12°S and 55°N, transecting plankton habitats in the North Pacific, Equatorial Pacific, and northern South Pacific. By combining information on the abundances and diversity of N_2-fixing bacteria, we sought to identify the biogeographical distributions of planktonic N_2 fixers in these regions and to evaluate how biogeochemical gradients between these habitats influenced the distributions of N_2-fixing bacteria. The results suggest that oceanic diazotrophs of the Pacific are restricted largely to midlatitude regions of the subtropical gyres. Moreover, our study also suggests that temporal variations in the locations of the transitional boundaries that define the subtropical gyres partly control the areal extent of diazotrophs in the Pacific.

Methods

Sample collections—Samples were collected on four oceanographic research cruises in the North and South Pacific Oceans (Fig. 1) aboard the R/V *Kilo Moana*. The first of these cruises (KM0308) occurred in April 2003, and

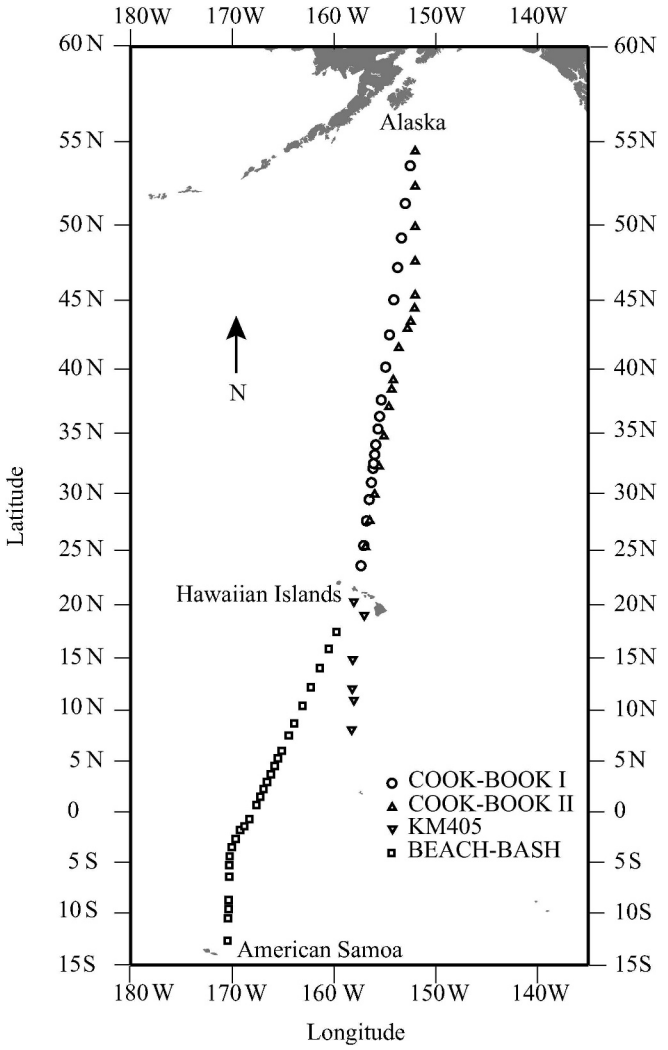

Fig. 1. Cruise tracks and station positions (symbols) sampled during the three research cruises of this study. COOK-BOOK I, April 2003; COOK-BOOK II, October 2003; KM0405, March 2004; and BEACH-BASH, May 2005.

samples were collected from the upper ocean (<200 m) along 152–157°W between Honolulu, Hawaii (21°18′N) and Kodiak, Alaska (57°49′N). In October 2003 (KM0314), seawater samples were collected along the return transect (KM0314) from Kodiak, Alaska, to Honolulu, Hawaii, along 152–156°W. Together, these cruises were termed the Comprehensive Oligotrophic Ocean Knowledge–Biogeochemical Observations Oahu-Kodiak (COOK-BOOK I and II) transits. In March 2004, samples were collected during a research cruise (KM0405) between ~20°N and 8°N latitude along 158°W. In May 2005, seawater was collected in the northern regions of the SPSG during a cruise transect from American Samoa to Honolulu, Hawaii (15°S–17°N, KM0507) along 159–170°W. This cruise was termed Biogeochemical and Ecological Analysis of Complex Habitats–Between American Samoa and Hawaii (BEACH-BASH).

Water samples were collected using a 24-bottle conductivity–temperature–depth (CTD) rosette sampler or subsampled from the ship's flow-through seawater intake system. Seawater samples for subsequent extraction of planktonic DNA were collected at six depths (10, 25, 50, 75, 100, 150 m) on COOK-BOOK I and II. Samples for subsequent extraction of planktonic RNA were collected from near-surface waters (10 m) during COOK-BOOK I, and depth profiles were sampled at the same six depths where samples for subsequent DNA extraction were collected on COOK-BOOK II. During KM0405, seawater samples were collected from nine depths in the upper ocean (5, 25, 50, 75, 100, 125, 150, 175, 200 m) for subsequent extraction of both DNA and RNA. During the BEACH-BASH cruise, seawater samples for extraction of plankton DNA were collected from near-surface water (5–15 m) either using a CTD rosette sampler or via the ship's seawater intake system from a depth of ~5 m.

Planktonic biomass was concentrated onto 0.2-μm-porosity filters, and nucleic acids were extracted from the filter concentrates. During COOK-BOOK I, seawater was subsampled into 10-liter polyethylene carboys and filtered using a peristaltic pump and acid-washed silicon tubing onto inline filters. Samples for planktonic DNA were collected onto 0.2-μm Sterivex® filter capsules (Millipore), while RNA samples were filtered onto inline 25-mm-diameter 0.2-μm-pore-size Supor® filters (Pall Gelman Inc.). Between 7 and 10 liters were filtered from each depth onto the Sterivex® filter capsules for DNA collections, and 1–2 liters were filtered for RNA samples. On completion of filtration, residual water remaining in the Sterivex® filter capsules was purged with a syringe, and the capsules were capped and immediately frozen in liquid N_2. Filters for RNA analyses were placed in 2.0-mL bead beater microcentrifuge tubes containing 0.2 g of 0.1 mm zirconium beads and 500 μL of Qiagen® RLT buffer with 0.1% β-mercaptoethanol (Sigma-Aldrich).

During COOK-BOOK II, KM0405, and BEACH-BASH, approximately 1 liter of seawater was filtered onto 25-mm-diameter, 0.2-μm-pore-size Supor® filters for subsequent extraction of DNA and RNA. On completion of filtration, filters for DNA analyses were immersed in 500 μL of Tris-EDTA (TE: pH 8.0) in 2.0-mL microcentrifuge tubes. Filters for subsequent RNA analyses were placed in 2.0-mL bead beater microcentrifuge tubes containing an identical RLT buffer and zirconium bead solution as previously described. Microcentrifuge tubes for both DNA and RNA samples were immediately frozen in liquid nitrogen until shore-based laboratory analyses were performed.

Nucleic acid extractions—Extraction of planktonic DNA and RNA from the 25 mm diameter 0.2-μm-pore-size filters followed the protocols described in Church et al. (2005*a*,*b*). Extraction of DNA from the Sterivex® filter capsules followed a modification of the Tillet and Neilan (2000) potassium xanthogenate protocol as described by Short et al. (2004). Extracted DNA was stored at −20°C until analyzed. DNA concentrations were quantified by PicoGreen® DNA quantification kit (Molecular Probes) following the manufacturer's specifications.

Amplification, cloning, and sequencing—To identify diazotroph population diversity, a ~360-base-pair fragment of the *nifH* gene was amplified by reverse-transcriptase PCR (RT-PCR) from planktonic RNA following the nested PCR approach described in Zehr and Turner (2001). Amplified products were cloned into a pGEM T-easy vector (Promega), and clones were sequenced on an automated capillary sequencer (Applied Biosystems 3100). Edited sequences were translated and imported into an aligned ARB database for phylogenetic analyses (Zehr et al. 2003).

QPCR reaction conditions—Reaction conditions and thermal cycling protocols used for QPCR amplification of *nifH* gene abundances are described elsewhere (Short et al. 2004; Church et al. 2005*b*). All the phylotypes targeted by QPCR had been reported from *nifH* RT-PCR clone libraries in the North Pacific Ocean (Zehr et al. 1998, 2001; Church et al. 2005*b*). Six *nifH* phylotypes were targeted by QPCR, including *Crocosphaera* spp. (termed Group B), an uncultivated phylotype termed Group A that is presumed to be a unicellular cyanobacterium, *Trichodesmium* spp., and two *nifH* sequence types clustering among heterocystous cyanobacteria (termed Het_1 and Het_2); in addition, a single *nifH* sequence type clustering among *nifH* containing γ-Proteobacteria was targeted by QPCR. With one exception, description of the TaqMan primers and probes are described in Church et al. (2005*a*); for this study, we developed an additional set of primers and probe specific to the heterocystous *nifH* phylotype (termed Het_2) retrieved from RT-PCR clone libraries at Station ALOHA (Church et al. 2005*b*). The oligonucleotide sequences of the primers and probe used to target the Het_2 phylotype were forward (5′-TCCTGA GCCTGGTGTTGGTT-3′), reverse (5′-CCGTTTTCC TCCAAGAAGTTGA-3′), and probe (5′-TGCTGGTC GCGGCATTATTACCG-3′). Primers and probe for this phylotype were checked for specificity and cross reactivity following the protocols described in Short et al. (2004). Standards for the QPCR reactions consisted of serial dilutions of plasmids containing the target *nifH*-sequence; standardization procedures were identical to those described in Short et al. (2004).

Hydrography, nutrients, and chlorophyll—Near-surface (~5 m) measurements of temperature and salinity were determined using a flow-through thermosalinograph (SeaBird) attached to the ship's seawater intake system. Vertical profiles of temperature, salinity, and fluorescence were determined from a SeaBird CTD package and profiling Seapoint fluorometer attached to the sampling rosette. Concentrations of chlorophyll *a* (Chl *a*) and phaeophytin were determined from discrete seawater samples (125 mL) collected from the CTD rosette and the underway flow-through seawater system. These discrete samples were used to calibrate chloropigment concentrations based on fluorescence measurements derived from the CTD fluorometer.

Chlorophyll and phaeopigment concentrations were determined fluorometrically (Yentsch and Menzel 1963). For these analyses, 125 mL of seawater were filtered onto 25-mm glass-fiber filters (Whatman), and the filters were immersed in 100% high-performance liquid chromatography–grade acetone, and stored at 4°C in the dark (Letelier et al. 1996). After transport to the shore-based laboratory, extracted chlorophylls and phaeophytins were analyzed using a Turner AU-10 fluorometer.

Water samples for determinations of nitrate + nitrite ($NO_3^- + NO_2^-$) and soluble reactive phosphorus (SRP) were collected at each of the stations where *nifH* gene abundances were measured. Nutrient samples were collected from the CTD rosette, trace metal–clean Go-Flo bottles (KM0405), or the ship's flow-through seawater system into 125- or 500-mL acid-washed polyethylene bottles and frozen. During COOK-BOOK I and II and BEACH-BASH cruises, concentrations of $NO_3^- + NO_2^-$ were analyzed on a Braun and Luebbe autoanalyzer (AAIII) following an adaptation of the colorimetric methods described in Armstrong et al. (1967). Samples with $NO_3^- + NO_2^-$ concentrations below detection (<0.03 μmol L^{-1}) by the AAIII were analyzed using a modification of the high-precision chemiluminescence method developed by Garside (1982) with modifications described by Dore and Karl (1996). Concentrations of $NO_3^- + NO_2^-$ from samples collected on KM0405 were analyzed on an Alpkem Rapid Flow Analyzer using standard colorimetric methods. Concentrations of SRP were determined on COOK-BOOK I and II and BEACH-BASH cruises using the high-sensitivity MAGnesium Induced Coprecipitation method (Karl and Tien 1992).

Statistical evaluation of data—Correlation analyses and model II linear regression analyses were used to examine relationships among surface water biogeochemical measurements (temperature, $NO_3^- + NO_2^-$, SRP) and QPCR-derived gene copy abundances. QPCR-derived *nifH* gene copy abundance estimates were $\log_{10}$ transformed to achieve normality, and statistical testing was determined based on the transformed data. Regression significance was determined at the 95% confidence level ($p < 0.05$).

Results

Distributions of nutrients, chlorophyll, and nifH phylotypes—The study area included a wide range of surface water temperatures (4.5–32°C) and highly variable nutrient concentrations (e.g., $NO_3^- + NO_2^-$ ranged from <0.01–19 μmol L^{-1}). During the first three cruises (COOK-BOOK I and II and KM0405), vertical profiles of temperature, density, fluorescence, and nutrients revealed distinct latitudinal gradients in both physical and biogeochemical properties (Fig. 2). During the BEACH-BASH cruise, malfunctioning of the CTD winch precluded high-resolution vertical sampling through several regions of the cruise transect; however, gradients in near-surface water hydrographic and biogeochemical properties are described below.

During April 2003 (COOK-BOOK I), the northern boundary of the NPSG extended to ~30°N, north of which the NPTZ was demarcated by strong latitudinal gradients in density, temperature, nutrients, and fluorescence (Fig. 2). Within the NPSG, upper ocean temperatures ranged from 20°C to 25°C, concentrations of $NO_3^- + NO_2^-$ varied between 0.004 and 0.25 μmol L^{-1}, and chlorophyll concentrations peaked between 100 and 125 m (Figs. 2, 3A). Near-surface SRP concentrations in the NPSG varied between 0.046 and 0.087 μmol L^{-1}, increasing sharply along the transitional region of the NPTZ (Fig. 3).

In October 2003 (COOK-BOOK II), the position of the NPTZ had migrated north, extending the northern boundary of the oligotrophic NPSG to ~39°N. The waters of the NPSG were warmer (21–27°C) and concentrations of nutrients ($NO_3^- + NO_2^-$ and SRP) lower than those measured in April (Fig. 2). Near-surface water concentrations of $NO_3^- + NO_2^-$ and SRP in the NPSG ranged ~0.002–0.050 and ~0.005–0.020 μmol L^{-1}, respectively (Fig. 3).

The position of the NPTZ, which marks the northern boundary of the NPSG, varied considerably between the COOK-BOOK I and II cruises; during COOK-BOOK I (April), the NPTZ extended from ~30°N to ~35°N, and during COOK-BOOK II (October), the NPTZ migrated northward and expanded, extending from ~39°N to near 45°N. Accompanying the sharp hydrographic transitions within the NPTZ, concentrations of nutrients and chlorophyll increased across the frontal boundary. During COOK-BOOK I, both $NO_3^- + NO_2^-$ and fluorescence were elevated in the upper 75 m of the NPTZ (Fig. 3E), while peak fluorescence during COOK-BOOK II ascended from >100 m in the waters of the NPSG to a near-surface water enrichment along the northern edge of the NPTZ (Fig. 2E). In addition, near-surface water concentrations of nutrients increased rapidly through this transitional region (Fig. 3).

North of the NPTZ, the PSAG was characterized by cool surface waters (ranging 4.5–13°C in April and 11–13°C in October), elevated concentrations of $NO_3^- + NO_2^-$ and SRP, and generally enhanced concentrations of chlorophyll (Fig. 2A,B,D,E).

In March 2004, vertical profiles through the southern regions of the NPSG (14–20°N) and the near-equatorial waters (8–12°N) revealed a relatively warm (26–27°C) and well-stratified upper ocean (Fig. 2C; Table 1). South of ~14°N, upward sloping of isopycnal surfaces brought cooler, nutrient-enriched waters closer to the sea surface (Fig. 2C) and resulted in enhanced upper ocean inventories (0–125 m) of $NO_3^- + NO_2^-$ (Table 1). In contrast, north of ~14°N, near-surface water concentrations of $NO_3^- + NO_2^-$ remained near or below analytical detection limits (<0.03 μmol L^{-1}; Fig. 2F).

In May 2005, near-surface waters along a cruise transect between 15°S and 17°N revealed distinct latitudinal gradients in temperature, Chl *a*, and nutrients (Fig. 3). Near-surface waters along the northern regions of the South Pacific (5–13°S) were warm (~30–32°C) and nutrient poor (concentrations of $NO_3^- + NO_2^-$ and SRP ranged 0.005–0.017 and 0.142–0.197 μmol L^{-1}, respectively).

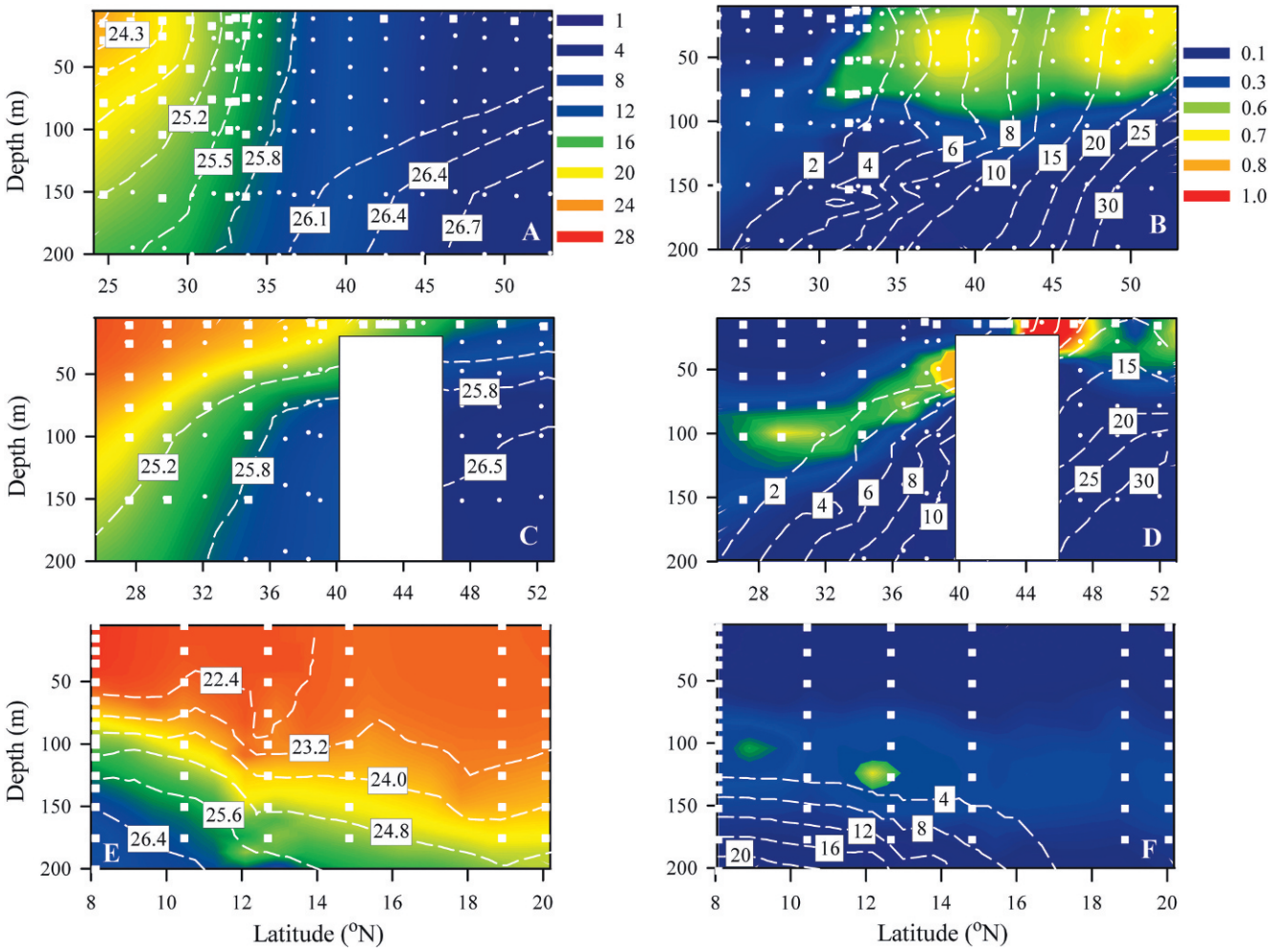

Fig. 2. Contour plots depicting vertical distributions of temperature, potential density, fluorescence, and $NO_3^- + NO_2^-$ as a function of latitude during three cruises sampled for this study. For hydrographic plots (A,C,E) colors represent temperature contours (°C), and labeled white contour lines are isopycnal surfaces (kg m^{-3}); for the biogeochemical plots (B,D,F), colors represent fluorescence (relative fluorescence units), and labeled white contour lines depict nutrient concentrations (μmol L^{-1}). White circles represent locations and discrete depths where samples were collected and analyzed; white squares are stations and depths where DNA and RNA samples were collected to evaluate diazotroph *nifH* gene abundances and gene expression. (A,B) COOK-BOOK I (April 2003); (C,D) COOK-BOOK II (October 2003); and (E,F) KM0405 (March 2004). During COOK-BOOK II (C,D), inclement weather prevented the collection of vertical profiles in the region indicated by the white box.

Within the equatorial regions (5°S–8°N), concentrations of Chl *a*, $NO_3^- + NO_2^-$, and SRP all increased (Fig. 3). In the equatorial waters, $NO_3^- + NO_2^-$ concentrations increased rapidly south of the equator (~5°S) and decreased along the northern side of the equator (~2°S; Fig. 3). SRP concentrations also increased south of the equator (~5°S) but remained elevated further north of the equator (Fig. 3). Chl *a* concentrations also increased in the equatorial waters with near-surface ocean Chl *a* concentrations peaking north of the equator (~5–8°N; Fig. 3). North of the equatorial waters, concentrations of nutrients and Chl *a* were typical of the oligotrophic NPSG, and near-surface temperatures ranged ~24–28°C.

nifH *diversity and phylotype distributions*—RT-PCR amplification, cloning, and sequencing of *nifH* gene transcripts collected during COOK-BOOK I and II and KM0405 revealed several distinct phylogenetic sequence types, including four phylotypes clustering among *nifH*-containing cyanobacteria and eight phylotypes clustering among *nifH*-containing γ-Proteobacteria (Fig. 4; Table 2). Sequences sharing >95% identity were grouped as a phylotype; such grouping retains known phenotypic clustering of these organisms and is consistent with previously reported phylogenetic grouping of these *nifH* sequence types (Zehr et al. 2001; Foster and Zehr 2006). Among the cyanobacteria, three of the phylotypes had been previously identified from RT-PCR amplified clone libraries in the NPSG, including a *nifH* sequence 99% identical to *Crocosphaera* spp. (termed Group B), the Group A phylotype, and a group of sequences closely related (>98% identical *nifH* DNA sequences) to *Katagnymene spiralis* and *Trichodesmium thiebautti*. In addition, a single *nifH* sequence type clustering among heterocyst-forming *nifH*

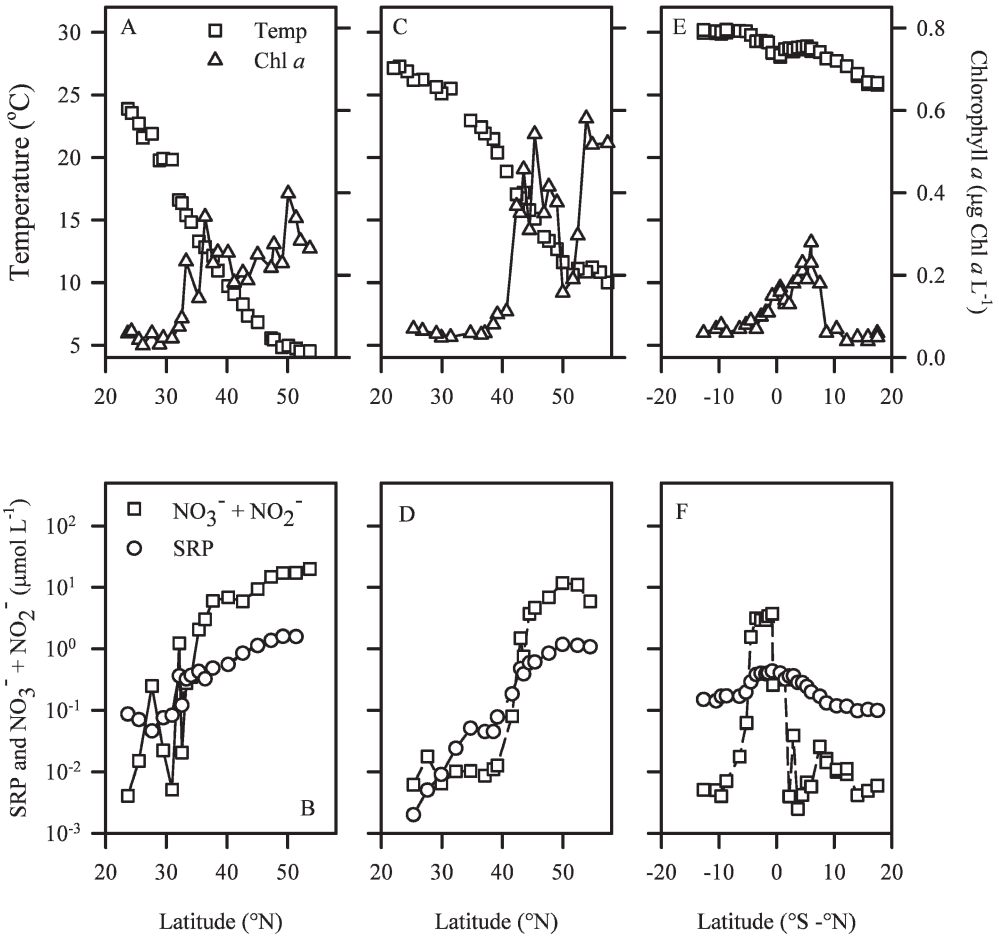

Fig. 3. Near-surface ocean biogeochemical properties along cruise tracks (A,B) COOK-BOOK I, (C,D) COOK-BOOK II, and (E,F) BEACH-BASH. Note: KM0405 cruise transect not shown because no discrete measurements of Chl *a* were measured and near-surface nutrient concentrations were below analytical detection (<0.06 μmol L^{-1}).

sequence types was retrieved from the near-surface ocean (10 m depth) at 27°N during the COOK-BOOK II cruise (Table 2). This sequence type (termed Het_3) phylogenetically clustered among two previously reported heterocystous phylotypes (termed Het_1 and Het_2) retrieved in RT-PCR clone libraries from the NPSG (Fig. 4); this phylotype appears to derive from cyanobacteria of the genera *Calothrix* (Foster and Zehr 2006).

Among the eight *nifH* phylotypes that clustered among γ-Proteobacteria, two of the γ-Proteobacteria phylotypes (termed γ-Proteo_1 and γ-Proteo_5) had been previously reported in RT-PCR clone libraries from the Pacific, the Atlantic, and the Arabian Sea (Bird et al. 2005; Church et al. 2005*a*; Langlois et al. 2005). The remaining sequences that clustered with *nifH*-containing γ-Proteobacteria formed novel groups only distantly related to cultivated microorganisms (Fig. 4).

QPCR derived estimates of nifH phylotype abundance—The vertical and meridional distributions of six *nifH* phylotypes were examined using QPCR. In general, the *nifH* phylotypes were most abundant in the oligotrophic regions of the NPSG, where surface water temperatures ranged 24–27°C and concentrations of $NO_3^- + NO_2^-$ and SRP were <0.02 and <0.08 µmol L^{-1}, respectively. During both COOK-BOOK I and II, *nifH* gene abundances were greatest in the oligotrophic waters of the central NPSG and declined rapidly toward the northern boundary of the NPTZ. North of the NPSG (within the NPTZ), unicellular cyanobacterial phylotypes (both Group A and Group B) were detected but at gene copy abundances approaching the limits of detection by the QPCR assays (~100 gene copies L^{-1}; Figs. 5, 6). Similarly, during both KM0405 and BEACH-BASH, *nifH* phylotype abundances were consistently greatest in the NPSG, with abundances decreasing rapidly south of ~14°N, coinciding with the approximate boundary of the NECC (Figs. 7, 8).

Correlation and regression analyses were used to identify possible relationships between the Group A phylotype abundances and several of the measured biogeochemical properties (temperature, $NO_3^- + NO_2^-$, and SRP). Such analyses were restricted to the Group A phylotype because of the low data densities (due to gene copy abundances below the limit of detection in many samples) for other phylotypes examined. Correlation analyses indicated a weak but positive relationship between near-surface water

Table 1. Depth-integrated (0–150 m) *nifH* gene abundances and inventories of nitrate + nitrite and chlorophyll *a* (Chl *a*) in various ecological regions of the North Pacific. Note stations are arranged by latitudinal position rather than chronologically. Data were included from COOK-BOOK I and II and KM0405 cruises; no vertical profiles of *nifH* gene abundances were collected during BEACH-BASH cruise.

Region* and date	Latitude	MLD† (m) temp. (°C)	$NO_3^- + NO_2^-$ (mmol N m^{-2})	Chl *a* (mg m^{-2})	Group A (*nifH* copies m^{-2})	Group B (*nifH* copies m^{-2})	Tricho (*nifH* copies m^{-2})	Het_1 (*nifH* copies m^{-2})	Het_2 (*nifH* copies m^{-2})	γ-Proteo (*nifH* copies m^{-2})
NECC										
Feb 2004	8°5′N	51 27.0	1,006	36	4.3×10^7	1.6×10^7	Not dect.‡	Not dect.	Not dect.	Not dect.
Feb 2004	10°47′N	49 26.2	411	32	1.1×10^7	Not dect	5.6×10^6	Not dect.	Not dect.	Not dect.
Feb 2004	12°42′N	84 26.2	108		9.5×10^6	Not dect.	Not dect.	Not dect.	8.5×10^7	Not dect.
NPSG										
Feb 2004	14°53′N	50 25.4	98	28	1.7×10^9	1.0×10^8	3.1×10^7	Not dect.	6.3×10^7	Not dect.
Feb 2004	18°58′N	47 25.6	No data	36	3.5×10^9	6.2×10^7	Not dect.	2.2×10^5	6.4×10^7	Not dect.
Feb 2004	20°15′N	73 25.6	No data	24	4.4×10^9	5.0×10^6	4.8×10^7	Not dect.	6.6×10^7	Not dect.
Apr 2003	23°38′N	28 23.7	9.4	12.2	4.3×10^9	6.4×10^7	1.3×10^7	1.9×10^5	Not dect.	Not dect.
Oct 2003	25°15′N	62 26.0	41	16.5	3.2×10^8	3.0×10^9	3.2×10^8	9.2×10^8	3.3×10^8	3.2×10^6
Apr 2003	27°17′N	54 21.6	45	10.6	5.7×10^9	3.5×10^6	9.8×10^6	1.1×10^5	2.8×10^4	Not dect.
Oct 2003	29°52′N	51 24.2	95.6	15.3	1.1×10^9	1.2×10^8	1.5×10^7	1.9×10^5	1.8×10^8	Not dect.
NPTZ										
Apr 2003	32°32′N	46 16.3	168	12.6	1.7×10^7	Not dect.	Not dect.	Not dect.	Not dect.	Not dect.
Apr 2003	33°15′N	43 14.7	218	27.1	2.8×10^6	2.2×10^6	Not dect.	Not dect.	Not dect.	Not dect.
Oct 2003	34°42′N	42 22.7	277	15.8	Not dect.	Not dect.	Not dect.	Not dect.	1.9×10^6	Not dect.
PSAG										
Apr 2003	47°14′N	91 5.1	2,553	25.2	Not dect.	Not dect.	Not dect.	Not dect.	Not dect.	Not dect.
Oct 2003	47°38′N	35 13.1	2,358	16.6	Not dect.	Not dect.	Not dect.	Not dect.	Not dect.	Not dect.

* Regional designations taken from Longhurst (1998); abbreviations for the provinces are North Equatorial Counter Current (NECC), North Pacific Subtropical Gyre (NPSG), North Pacific Transition Zone (NPTZ), and Pacific Sub Arctic Gyre (PSAG).
† Mixed layer depth (MLD) determined on the basis of the 0.125 change in potential density criteria (Monterey and Levitus 1997).
‡ Stations where *nifH* gene copy abundances below the limits of detection (~100 *nifH* copies L^{-1}) indicated as "not dect."

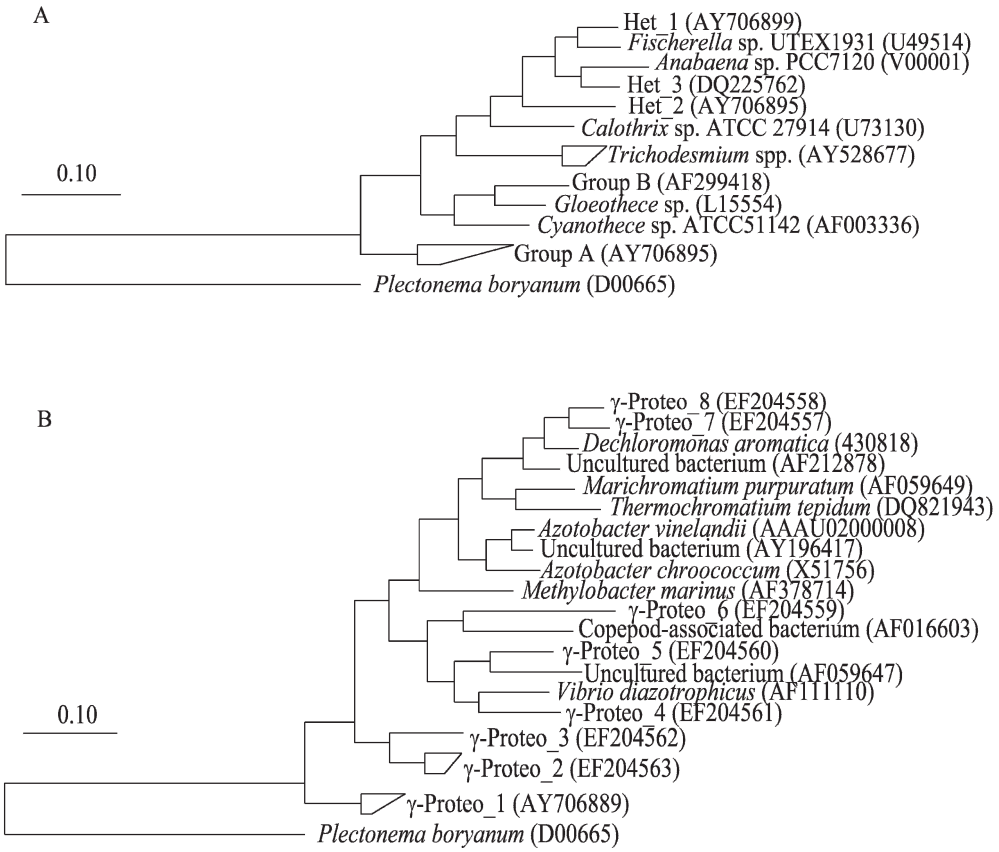

Fig. 4. Neighbor-joining phylogenetic trees depicting relationships of RT-PCR amplified *nifH* gene transcripts. Sequences >95% identical were grouped (as shown by open polygons). GenBank accession numbers are shown in parentheses. (A) Relationships of *nifH* cyanobacterial sequences retrieved during this study; also depicted are several cultivated organisms and environmental sequences obtained from other studies. (B) Phylogenetic relationships of *nifH* sequences clustering among γ-Proteobacteria obtained during this study; also shown are closest cultivated microorganisms and environmental clones.

Table 2. Phylogenetic affiliations, locations, cruises, and depths where *nifH* sequences were retrieved from RT-PCR clone libraries during this study. *nifH* sequence groups are shown in Fig. 3. Cruise abbreviations are COOK-BOOK I (CBI, April 2003), COOK-BOOK II (CBII, October 2003), and KM0405 (March 2004).

Phylogenetic affiliation	*nifH* sequence group	Cruise, location: depth
Cyanobacteria	Group A	CBI, 23°N: 10, 25, 50, 75, 100
		CBI, 27°N: 10, 25, 75
		CBII, 27°N: 10 m
	Group B	CBII, 27°N: 10 m
	Trichodesmium	CBI, 23°N: 10, 25
		CBII, 27°N: 10 m
	Het_3	CBII, 27°N: 10 m
Proteobacteria	Proteo_1	CBII, 27°N: 10 m
		CBII, 37°N: 10 m
		KM0405, 8°N: 25 m
	Proteo_2	KM0405, 8°N: 15, 50 m
	Proteo_3	KM0405, 8°N: 50 m
	Proteo_4	CBI, 32°N: 10 m
	Proteo_5	CBII, 41°N: 10 m
	Proteo_6	CBII, 41°N: 10 m
		CBII, 44°N: 10 m
	Proteo_7	CBII, 41°N: 10 m
	Proteo_8	KM0405, 8°N: 125 m

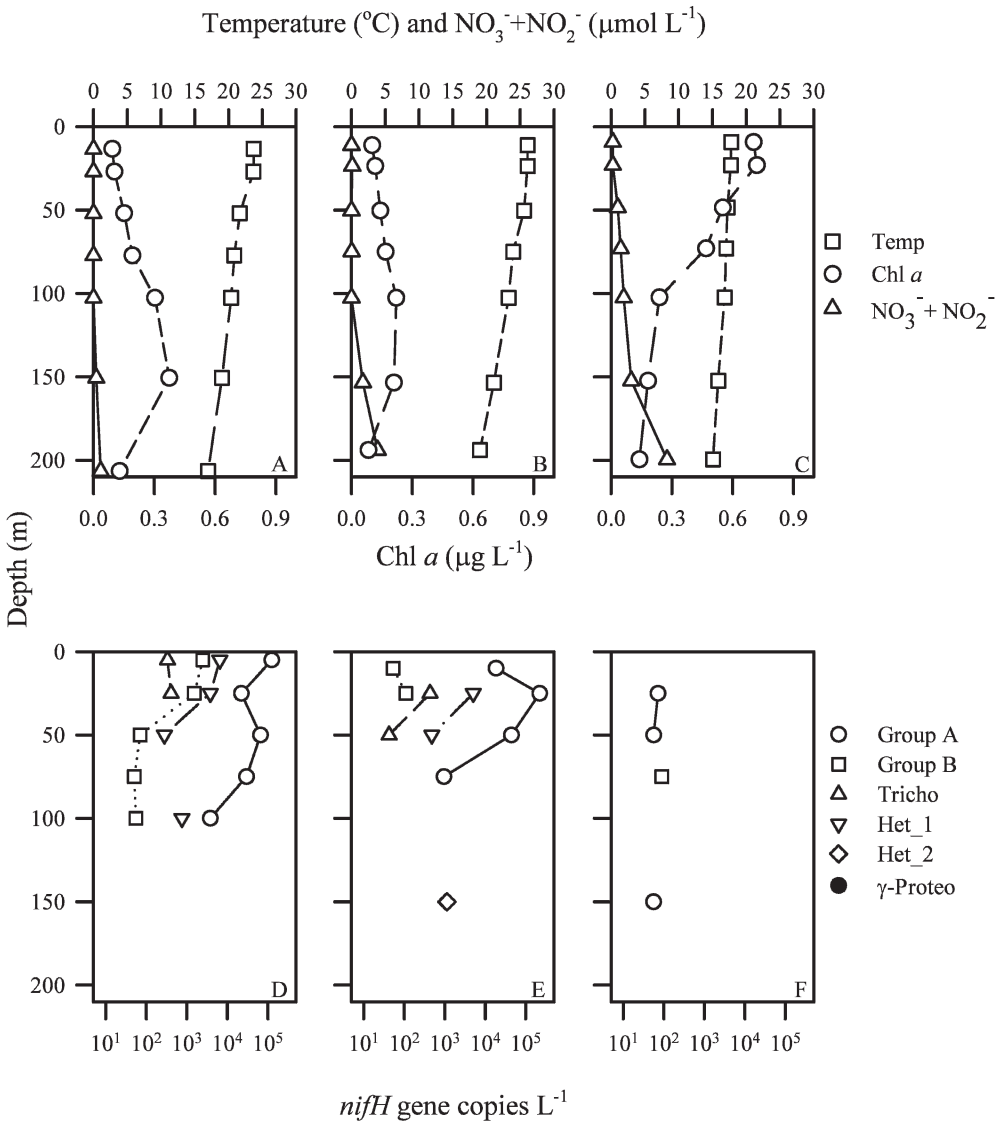

Fig. 5. (A–C) Vertical profiles of temperature, Chl *a*, NO_3^- + NO_2^-, and (D–F) QPCR determinations of *nifH* gene abundances of six *nifH* phylotypes at 23°N (A,D), 27°N (B,E), and 33°N (C,F) during COOK-BOOK I. Note that samples for QPCR determinations of gene abundances were collected from the same depths as depicted in the upper panels; however, in many cases, *nifH* gene abundances were below the limit of detection.

temperatures and the Group A *nifH* phylotype abundances (Pearson correlation coefficient = 0.02), while Group A abundances demonstrated a weak but inverse correlation with near-surface nutrient concentrations (Pearson correlation coefficients with NO_3^- + NO_2^- = −0.12 and SRP = −0.20, respectively). Model II linear regression analyses revealed no significant ($p > 0.05$) relationships between the near-surface water Group A *nifH* phylotype gene copy abundances and surface water nutrient concentrations (SRP and NO_3^- + NO_2^-) or temperatures.

During COOK-BOOK I and II and KM0405, *nifH* phylotype abundances were generally greatest in the well-lit upper ocean waters of the NPSG (Figs. 5–7). During COOK-BOOK I, unicellular Group A phylotypes dominated *nifH* gene abundances within the NPSG (Fig. 5). In October 2003 (COOK-BOOK II), *nifH* gene distributions were also restricted largely to the NPSG (south of ~30°N); only the Het_2 phylotype was observed in the southern regions of the NPTZ at relatively low abundances (10^2 *nifH* copies L^{-1}; Fig. 6). Several of the *nifH* phylotype abundances were greater in October (COOK-BOOK II) than April (COOK-BOOK I); for example, inventories (0–100 m) of Group B, *Trichodesmium*, and Het_1 *nifH* genes increased more than an order of magnitude near 25°N in comparison to the previous spring (Fig. 8; Table 2).

In March 2004 (KM0405), *nifH* gene copy abundances were restricted largely to the waters of the NPSG, and gene copy abundances were dominated by the Group A phylotype (Figs. 7, 8). South of ~14°N, where surface ocean temperatures increased to 26–27°C and NO_3^- + NO_2^- inventories (0–125 m) increased to 0.11–1 mol N

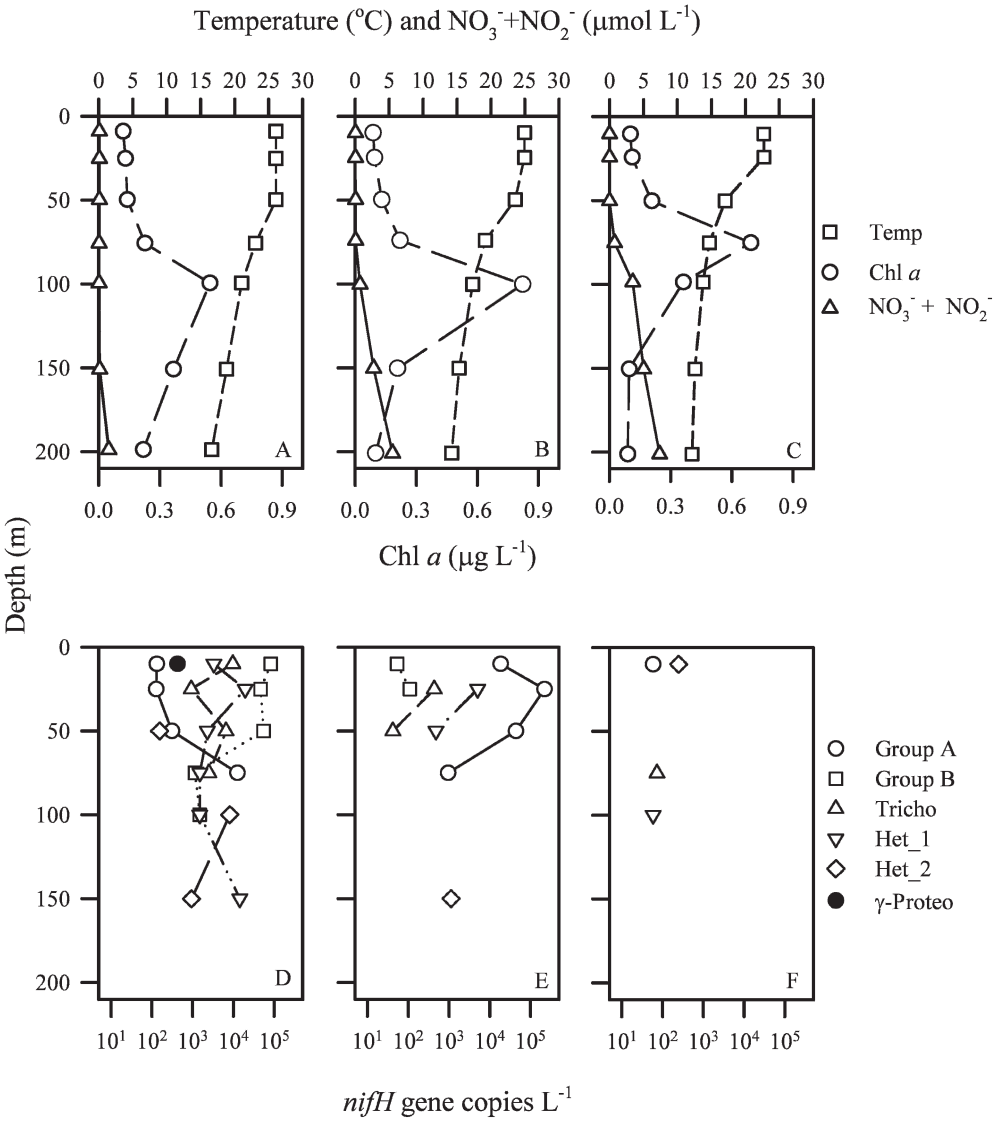

Fig. 6. (A–C) Vertical profiles of temperature, Chl *a*, and $NO_3^- + NO_2^-$; (D–F) *nifH* gene abundances at 25°N (A,D), 29°N (B,E), and 34°N (C,F) during COOK-BOOK II. Note that samples for QPCR determinations of gene abundances were collected from the same depths as depicted in the upper panels; however, in many cases, *nifH* gene abundances were below the limit of detection.

m^{-2}, abundances of all the *nifH* phylotypes decreased. Only the Group A and *Trichodesmium* phylotypes were detected south of the NPSG (Table 2).

Quantification of near-surface water abundances of *nifH* phylotypes during the BEACH-BASH cruise (May 2005) transect from the SPSG to the NPSG provided insight into the distributions of N_2-fixing microorganisms in SPSG, equatorial waters, and the NPSG (Fig. 8). Throughout the northern SPSG (5–13°S), the Het_2, *Trichodesmium*, and Group A *nifH* phylotypes were all detectable at relatively low abundances (10^2–10^3 *nifH* copies L^{-1}). In the equatorial waters (8°S–14°N), concentrations of *Trichodesmium*, Group A, and Group B phylotypes were all detectable but at low copy abundances (~10^2–10^3 *nifH* copies L^{-1}). Neither of the heterocystous phylotypes (Het_1 and Het_2) were detectable in the equatorial waters. Within the southern NPSG (14–17°N), the abundances of the Group A and Het_2 phylotypes increased sharply. In particular, the abundances of the Het_2 group increased from undetectable levels south of ~14°N to concentrations ranging from 10^4 to 10^5 *nifH* copies L^{-1} toward the northern end of the cruise transect (17°N). In addition, the abundances of the Group A unicellular phylotype increased more than three orders of magnitude into the NPSG (increasing from ~10^2 *nifH* copies L^{-1} south of 14°N to >10^6 copies L^{-1} north of 14°N).

Discussion

We evaluated the distributions, abundances, and diversities of upper ocean (<200 m) planktonic diazotrophs during four research cruises spanning a latitudinal range

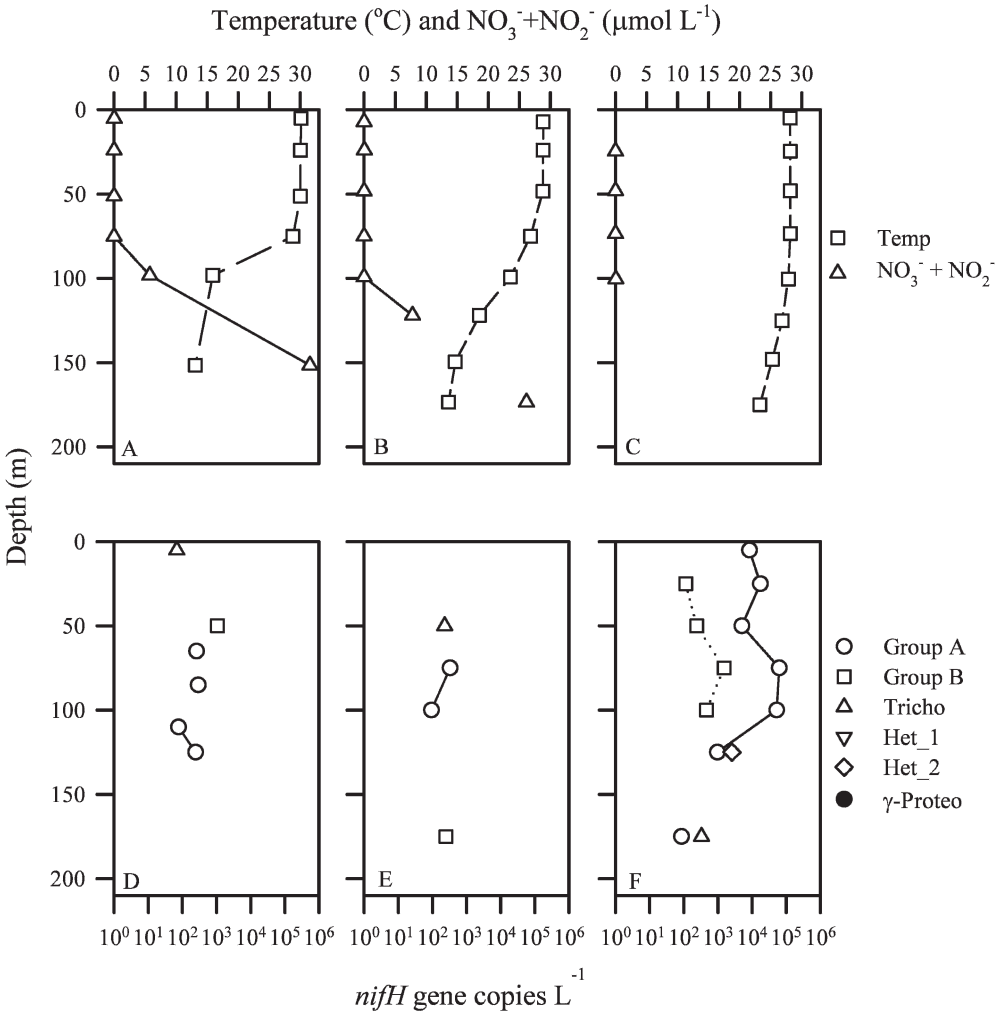

Fig. 7. (A–C) Vertical profiles of temperature, NO_3^- + NO_2^-, and (D–F) *nifH* gene abundances at 8°N (A,D), 10°N (B,E), and 18°N (C,F) during KM0405. Note that samples for QPCR determinations of gene abundances were collected from the same depths as depicted in the upper panels; however, in many cases, *nifH* gene abundances were below the limit of detection.

between 12°S and 54°N in the Pacific Ocean. These cruises transected several prominent biogeochemical gradients and distinct planktonic habitats. Based on QPCR amplification of *nifH* gene abundances, our results indicate that N_2-fixing bacteria in the Pacific Ocean were restricted largely to the well-lit, nutrient-poor waters of the NPSG (~14–32°N). The rapid decline in *nifH* gene abundances to the north and south of the NPSG suggested that the distributions of N_2-fixing bacteria in the Pacific Ocean may be partly controlled by spatial variability in the physical position of the frontal systems bounding the NPSG, including the NPTZ to the north and the NECC to the south. As a result, temporal variations in the latitudinal locations of these transitional boundaries could modify the geographic boundaries of the habitat suitable for diazotrophic bacteria in the North Pacific Ocean.

To examine the regional distributions of N_2-fixing microorganisms, we identified six phylotypes frequently retrieved from RT-PCR amplified *nifH* clone libraries in the open sea. RT-PCR amplification, cloning, and sequencing of *nifH* gene transcripts from the three cruises in the North Pacific Ocean confirmed the presence of several of the phylotypes targeted by QPCR (including *Trichodesmium*, *Crocosphaera* spp., Group A, and a γ-Proteobacteria phylotype) and revealed active transcription of the *nifH* gene by these phylotypes. In addition to detecting active expression by previously identified phylotypes, we also retrieved six *nifH* sequence types that clustered among γ-Proteobacteria and one *nifH* sequence that clustered among sequences from heterocystous cyanobacteria (termed Het_3). The Het_3 phylotype has recently been shown to be derived from the diatom symbiont *Calothrix* sp. (Foster and Zehr 2006). For this study, we did not use QPCR to evaluate the distributions of the Het_3 or the six novel γ-Proteobacterial sequence types recovered in the RT-PCR clone library; however, we did evaluate *nifH* gene distributions of two heterocyst-forming cyanobacteria (Het_1 and Het_2) frequently retrieved from RT-PCR clone libraries in the NPSG (Church et al. 2005*b*; Foster and Zehr 2006; Zehr et al. 2007).

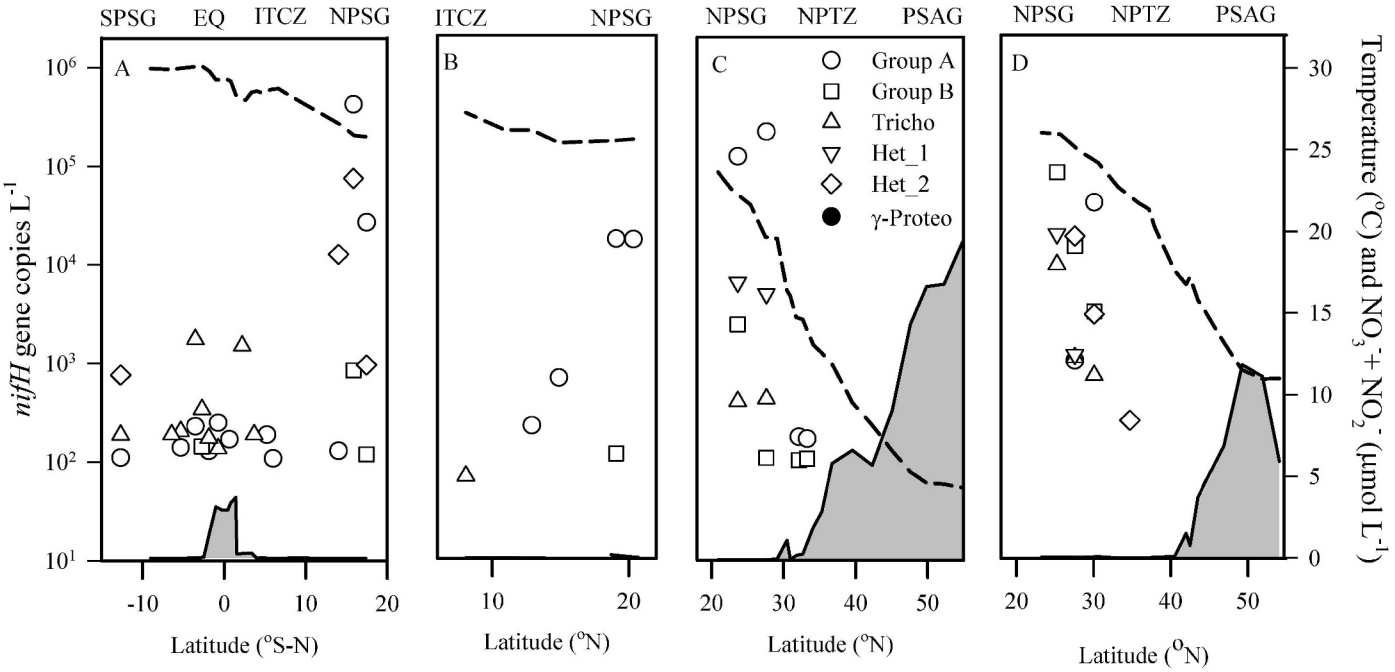

Fig. 8. Latitudinal distributions of *nifH* gene abundances in near-surface waters (~10 m) during (A) BEACH-BASH, (B) KM0405, (C) COOK-BOOK I, and (D) COOK-BOOK II. Also shown are near-surface ocean concentrations of $NO_3^- + NO_2^-$ (gray area) and temperature (dashed line). Boundaries between biogeochemical provinces are labeled above each graph; abbreviations are NPSG (North Pacific Subtropical Gyre), EQ (Equatorial region), NPTZ (North Pacific Transition Zone), PSAG (Pacific Sub Arctic Gyre), and SPSG (South Pacific Subtropical Gyre).

The *nifH* gene distributions of all six phylotypes had several consistent patterns. In particular, the abundances of all the *nifH* phylotypes were greatest in the well-lit portion of the upper ocean (<100 m) where $NO_3^- + NO_2^-$ was low. In addition, the latitudinal distributions of the *nifH* phylotypes revealed elevated abundances and greater overall N_2-fixing microorganism diversity in the NPSG. Finally, the unicellular cyanobacterial diazotroph assemblages (predominantly the Group A phylotype) demonstrated the greatest latitudinal range, with detectable gene copies found in the SPSG, equatorial waters, and within the NPSG. In the NPSG, the unicellular *nifH*-containing cyanobacteria (Group A and B) were generally the most abundant of the phylotypes examined, with near-surface water abundances ranging between $\sim 10^2$ and 10^5 *nifH* copies L^{-1} (Fig. 8). In comparison, abundances of the filamentous cyanobacterial phylotypes (*Trichodesmium*, Het_1, and Het_2) were more spatially variable with concentrations ranging from undetectable to $\sim 5 \times 10^5$ gene copies L^{-1} within the NPSG (Fig. 8). Together, these results provide important information regarding the suitability of specific ocean habitats for these groups of diazotrophs.

Few studies have examined the distributions of N_2-fixing microorganisms in the Pacific Ocean, and to our knowledge, the present study is among the first to characterize basin-scale distributions and diversity of selected diazotrophs using a QPCR approach. In a prior study that occupied a similar latitudinal region in the Pacific, Marumo and Asaoka (1974) identified and quantified N_2-fixing cyanobacteria using light microscopy. A transect between 50°N and 15°S along 155°W in the North Pacific provided insight into the distributions of several N_2-fixing cyanobacteria including *Trichodesmium thiebautii*, *Trichodesmium erythraeum*, *Oscillatoria* sp., *Katagnymene spiralis*, and *Richelia intracellularis* (Marumo and Asaoka 1974). During their study, *T. thiebautii* had the greatest geographical range, extending from 15°S to 40°N, and none of the species were observed north of 40°N or at water temperatures below 20°C (Marumo and Asaoka 1974).

Venrick (1974) described the distributions of the endosymbiotic heterocystous cyanobacteria *Richelia intracellularis* during several cruises in the central North Pacific, including two cruises between Honolulu, Hawaii, and Kodiak, Alaska (following nearly identical cruise tracks as the COOK-BOOK I and II cruises). *Richelia* sp. was almost always observed as an endosymbiont of diatoms of the genera *Rhizosolenia* spp., and the abundances of *Richelia* sp. were highly variable in space (Venrick 1974). A recent study combining epifluorescent microscopy and genetic techniques identified the Het_1 and Het_2 *nifH* sequence types reported in the present study as deriving from two species of *Richelia* (Foster and Zehr 2006).

Letelier and Karl (1996) described the highly variable contribution of free trichomes and colonies of *Trichodesmium* to upper ocean plankton biomass. These authors observed elevated abundances of *Trichodesmium* in the NPSG during the summer months when sea surface temperatures were warm and the upper ocean was well stratified. In the present study, abundances of several of the *nifH* phylotypes (including *Trichodesmium*) were greater in

October cruises when surface ocean temperatures were warm and mixed layer depths had shoaled.

A number of previous studies have examined the distributions of *nifH*-containing organisms in both the Atlantic Ocean (Langlois et al. 2005) and the Arabian Sea (Mazard et al. 2004; Bird et al. 2005). Mazard et al. (2004) concluded that unicellular N_2-fixing cyanobacteria were largely segregated to warm (>29°C), nutrient-depleted regions of the upper ocean, suggesting that temperature likely played an important role in constraining the distributions of unicellular diazotrophs. A study in the tropical and subtropical North Atlantic noted that unicellular cyanobacterial *nifH* genes were more widely distributed (both with depth and with latitude) than *Trichodesmium nifH* genes (Langlois et al. 2005). These authors also concluded that temperature likely plays an important role in constraining the types (filamentous vs. unicellular) of cyanobacteria in marine ecosystems; filamentous cyanobacteria were observed between 26.5°C and 30°C, while unicellular diazotrophs were observed at temperatures ranging from 15°C to 30°C. In the present study, we found that N_2-fixing microorganisms were most abundant in ocean waters ranging between ~24°C and 28°C; however, both unicellular and filamentous cyanobacteria were found at lower abundances in waters as cool as 17°C and as warm as 32°C (Fig. 8).

Based on laboratory experiments with *Trichodesmium* and 21 strains of heterocyst-forming cyanobacteria, Staal et al. (2003) concluded that heterocystous cyanobacteria are outcompeted by *Trichodesmium* in tropical ecosystems where water temperatures exceed ~25°C; they hypothesize that at warmer temperatures, decreased diffusion of oxygen and N_2 through the glycolipid layer surrounding heterocysts reduced cellular respiration and N_2 fixation by heterocyst-forming cyanobacteria. In our study, we did not observe either of the heterocystous cyanobacteria targeted by QPCR in the near-equatorial waters; however, *Trichodesmium* was observed at low abundances in the warm, nutrient-enriched equatorial waters.

Although temperature has been hypothesized to play an important role in constraining cyanobacterial abundances, the results of the present study indicate factors other than temperature must also limit the accumulation of diazotroph biomass in the oceans. Group A *nifH* phylotype abundances did not appear to significantly covary with temperature during our study (Model II linear regressions, $p > 0.05$); however, such statistical analyses are hampered by low data density (e.g., number of paired determinations of Group A *nifH* gene abundance and temperature = 17) and complicated because the samples were collected at different times of the year (and in different years). During both COOKBOOK I and II, *nifH* gene abundances declined sharply toward the northern boundaries of the NPSG, approximately coincident with the southern boundary of the NPTZ where temperatures declined to <20°C. However, abundances of the *nifH* phylotypes also declined south of the NPSG, approximately coincident with the northern boundary of the NECC, where temperatures increased toward the equator. These results suggest that factors other than temperature, such as nutrients, light, predation, or lysis, also modulate N_2-fixing bacterial abundances throughout the Pacific Ocean.

One feature common to both the NPTZ and the equatorial waters was increased upper ocean nutrient concentrations. Depth-integrated $NO_3^- + NO_2^-$ inventories averaged 508, 58, 221, and 2,455 mmol N m^{-2} in the NECC, NPSG, NPTZ, and PSAG, respectively (Table 1), while total depth-integrated *nifH* gene copy abundances in the NECC, NPSG, and NPTZ were 6×10^7, 4×10^9, and 8×10^6 *nifH* copies m^{-2}, respectively. All the various *nifH* groups examined were below detection within the PSAG. Diazotrophic cyanobacteria have been shown able to assimilate combined nitrogen (including NO_3^-, NO_2^-, NH_4^+, and amino acids) in the laboratory; however, it remains unclear how nitrogen availability may influence the activity and growth of natural assemblages of N_2-fixing microorganisms. Various strains of unicellular and filamentous cyanobacterial diazotrophs can assimilate reduced forms of nitrogen; however, NO_3^-, NO_2^-, and NH_4^+ can also inhibit nitrogenase synthesis and N_2 fixation (Thomas et al. 1982; Cheng et al. 1999; Mulholland et al. 1999). The inhibition of N_2 fixation by NO_3^- appears to occur to a lesser extent than by NH_4^+ or NO_2^- (Cheng et al. 1999). Laboratory studies with *Trichodesmium* spp. indicate that could account for a substantial fraction of the total nitrogen demands of actively growing populations and that *Trichodesmium* spp. assimilation of NO_3^- inhibits N_2 fixation (Mulholland and Capone 2000; Holl and Montoya 2005). However, none of the *nifH* phylotypes were significantly related to variations in near-surface $NO_3^- + NO_2^-$ concentrations (Model II linear regressions, $p > 0.05$).

It was notable that in all the biogeochemical regions examined (SPSG, equatorial regions, NPSG, NPTZ, and PSAG), concentrations of Chl *a* closely followed changes in the availability of $NO_3^- + NO_2^-$. Throughout the subtropical gyres, the vertical position of the deep chlorophyll maximum tends to co-occur with the top of the nitracline (Letelier et al. 2004). Outside the subtropical gyres, concentrations of Chl *a* are greater in the near-surface waters coincident with increased availability of $NO_3^- + NO_2^-$ where light fluxes are elevated. Based on these observations, we suggest that more rapidly growing, nondiazotrophic photoautotrophic plankton may outcompete slower-growing diazotroph assemblages for nutrients such as iron or phosphate in those regions of the ocean where $NO_3^- + NO_2^-$ concentrations are elevated. As a result, competition for limiting nutrients would segregate N_2-fixing microbes to areas where bioavailable nitrogen limits the growth of non–N_2-fixing plankton.

One of the perplexing observations from the present study was the low abundance of *nifH* phylotypes observed in the northern regions of the SPSG. At the time of our sampling of the SPSG (May 2005), upper ocean temperatures in the SPSG were elevated relative to the NPSG (~30°C in the SPSG and ~26°C in the NPSG). Another notable difference between the NPSG and SPSG was the apparent asymmetrical distribution of SRP. In the northern regions of SPSG (8–12°S), SRP concentrations ranged ~140–170 nmol L^{-1}, while SRP concentrations in the near-surface ocean in the

southern NPSG (8–17°N) were slightly lower, ranging ~100–120 nmol L^{-1}. Although the reasons for this remain unknown, the moderate accumulation of SRP in the SPSG could reflect limitation of diazotroph growth by other nutrients (e.g., iron) in the SPSG. Such results would be consistent with previous studies describing iron limitation of plankton growth in the South Pacific (Behrenfeld and Kolber 1999) and extremely low atmospheric flux of iron to this region of the SPSG (Duce and Tindale 1991).

Overall, *nifH* abundances of the γ-Proteobacterial phylotype (γ-Proteo_1) were low compared to the groups of cyanobacteria examined. *nifH* genes deriving from the γ-Proteo_1 were found at only one station in the NPSG (25°N) during COOK-BOOK II (Fig. 6); however, gene transcripts from this phylotype were recovered from stations sampled in April and October 2003. In addition, we found seven other γ-Proteobacterial phylotypes actively expressing at stations as far north as 44°N and as far south as 8°N.

Several other studies examining the spatial variability in *nifH*-containing γ-Proteobacteria also noted that these phylotypes are often observed in waters that are cooler and richer in nutrients than the cyanobacterial diazotrophs. In particular, Bird et al. (2005) observed *nifH* expression by the γ-Proteo_1 phylotype in samples collected from the Arabian Sea, including samples collected below the thermocline where concentrations of $NO_3^- + NO_2^-$ increased to >5 μmol L^{-1}. Similarly, Langlois et al. (2005) described the distributions of various *nifH* phylotypes in the subtropical Atlantic Ocean; these authors retrieved *nifH* sequences clustering among γ-Proteobacteria from samples collected near the top of the nitracline.

Based on the latitudinal constraint of *nifH* groups to within the oligotrophic NPSG, we suggest that temporal changes in the meridional position of the frontal systems that bound the NPSG likely influence the distributions of planktonic diazotrophs. Seasonal and interannual fluctuations in wind strength and direction substantially modify the latitudinal position of the NPTZ (Niiler and Reynolds 1984). In addition, the latitudinal position of the subtropical convergence and equatorial countercurrent can be strongly modified by variations in basin-scale forcing, such as the El-Niño Southern Oscillation and the Pacific Decadal Oscillation (Chai et al. 2003). Our results suggest that contraction and expansion of these permanent frontal systems likely play an important role in controlling the distributions of N_2-fixing marine microbes.

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Received: 2 January 2007
Accepted: 15 September 2007
Amended: 27 September 2007