

Nitrogen fixation by unicellular diazotrophic cyanobacteria in the temperate oligotrophic North Pacific Ocean

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Abstract

N₂ fixation has been understudied in marine environments outside of the subtropical and tropical oceans and where water temperatures are typically below 20–25°C. We identified *nifH* phylotypes and measured N₂ fixation rates under ambient conditions (maximum of 19°C) in water collected 750 km off the coast of California in oligotrophic waters of the North Pacific Ocean (34°N, 129°W). Near-surface N₂ fixation rates averaged 0.25 ± 0.05 nmol N L⁻¹ d⁻¹ for 24 incubation bottles. Despite low ambient concentrations of iron (<0.1 nmol L⁻¹) and phosphorus (<0.3 μmol L⁻¹), N₂ fixation rates were unaffected by iron and phosphorus amendments. Using reverse transcription–quantitative polymerase chain reaction (RT-QPCR) methodology, we estimated transcript abundance and patterns of expression for several unicellular diazotrophs, including the group A phylotype, which showed the highest daily mRNA abundances. The N₂-fixing assemblage extended to 60–80 m depth, well below the seasonal thermocline (40 m). The calculated areal N₂ fixation rate (15 μmol N m⁻² d⁻¹) was small compared with estimates from other regions of the Pacific; however, the estimated fixation rate was similar to other published results, suggesting that processes other than cellular growth rate may determine the abundance of unicellular diazotrophs. Despite the low N₂ fixation rates, the new nitrogen added to the euphotic zone by N₂ fixation could account for at least 10% of new production during the study period.

The euphotic zone of the oceanic gyres is frequently characterized by nitrogen limitation of phytoplankton growth, rapid nutrient recycling rates, and low rates of

nutrient inputs from terrestrial or deep-ocean sources. Therefore, localized phytoplankton CO₂ use and potential carbon export to the deep ocean is small compared with the coastal oceans. However, when considering the vastness of ocean gyres, the slow or seemingly insignificant processes that operate within them can contribute significantly to both the carbon and nitrogen biogeochemical cycles at a global scale (Karl et al. 1995). In this context, marine microbial N₂ fixation is recognized as an important component of the marine nitrogen cycle (Arrigo 2005). From a geochemical perspective, N₂ fixation acts to balance the oceanic nitrogen inventory by offsetting the loss of fixed nitrogen that occurs through denitrification in the ocean's interior and seafloor. Recent estimates of fixed-N budgets in the ocean reveal a substantial imbalance, implying that the oceans are losing up to 200 Tg N yr⁻¹ (Codispoti et al. 2001; Codispoti 2006). Geological evidence suggests that the nitrogen cycle is in balance on glacial/interglacial timescales (Gruber and Sarmiento 1997); therefore, the modern-day imbalance is either

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temporary or the contribution of N_2 fixation and other N-cycle processes is not fully appreciated (Codispoti et al. 2001). If N_2 fixation is a more important term in the oceanic nitrogen cycle than current measurements suggest, then its role as a long-term (millennial scale) supply of biologically available nitrogen to the oceans becomes pivotal for describing oceanic feedback mechanisms that contribute to the regulation of atmospheric CO_2 levels and global climate change (Michaels et al. 2001). However, the contribution of N_2 fixation is poorly constrained outside of bloom conditions, owing to few reports that are available.

The colonial cyanobacteria *Trichodesmium* spp. are considered largely responsible for marine N_2 fixation. Direct measurements of N_2 fixation by *Trichodesmium* are in the range of $239 \pm 38 \mu\text{mol } N_2 \text{ m}^{-2} \text{ d}^{-1}$ (Capone et al. 2005), which is equivalent to $11.2\text{--}33.6 \text{ Tg } N \text{ yr}^{-1}$ (depending on the areal extent of N_2 fixation) in the Atlantic Ocean. These rates are similar in magnitude to nitrate input from below the euphotic zone (Galloway et al. 2004) and show that N_2 fixation is an important source of new nitrogen to the Atlantic Ocean. Until recently, it was unclear how ecological or physiological limitations contributed to the geographical range of diazotrophs, and marine N_2 fixation was only identified in warm regions of the oligotrophic ocean. Evidence from shipboard and laboratory experiments has revealed roles for phosphorus supply (Mills et al. 2004), iron limitation (Kustka et al. 2002; Mills et al. 2004), and temperature limitation (Staal et al. 2003; Breitbarth et al. 2006) as growth constraints on *Trichodesmium* spp. and other diazotrophs in the ocean gyres.

New approaches to the study of marine N_2 fixation have revealed a potentially important role for unicellular microbes in oceanic N_2 fixation. In particular, Montoya et al. (2004) found that N_2 fixation rates by organisms $<10 \mu\text{m}$ were comparable with or exceeded rates of larger organisms such as *Trichodesmium*. Characterization of planktonic *nifH* genes (the gene that encodes the iron subunit of nitrogenase, the enzyme responsible for N_2 fixation) has proven useful for identifying uncultivated diazotrophic microorganisms (Zehr et al. 2003). In the open ocean, *nifH* from the $<10\text{-}\mu\text{m}$ fraction has been reported from various locations (Zehr and Ward 2002; Falcon et al. 2004b; Mazard et al. 2004), and *nifH* mRNA has also been detected in similar environments (Zehr et al. 2001; Church et al. 2005b). Several studies have identified the types and temporal dynamics of mRNA production by unicellular diazotrophs in subtropical regions (e.g., Zehr and Turner 2001; Falcon et al. 2004a; Church et al. 2005b). These studies suggest that unicellular diazotrophs may have an important role as contributors to the nitrogen—and potentially carbon—fluxes of organic matter in marine food webs. Thus, the presence of marine unicellular N_2 fixers as ordinary and active components of oceanic ecosystems warrants a better understanding of their biogeography, biodiversity, and activity, especially outside the regions where N_2 fixation by *Trichodesmium* is dominant.

Here we report the characterization of the N_2 -fixing assemblage in an oligotrophic, nitrate-depleted region of the North Pacific with an annual sea surface temperature

$<19^\circ\text{C}$. The proximity to the Eastern Tropical North Pacific denitrification zone and the ambient oligotrophic conditions imply a potential for N_2 fixation. We used trace-metal clean techniques and isotope tracer assays to determine N_2 fixation rates, and real-time polymerase chain reaction (PCR) assays were used to quantify the expression of *nifH* for specific phylotypes. The results demonstrate that N_2 fixation extends well beyond the typical temperature boundaries ($20\text{--}25^\circ\text{C}$) of the current estimates for ocean gyre N_2 fixation, and the assemblage was dominated by unicellular cyanobacterial diazotrophs. The N_2 fixation rates were low compared with tropical and subtropical environments; however, owing to the low overall primary productivity, the rates contributed at least 10% of the nitrogen required to support the estimated new production.

Materials and methods

Study site—We sampled a region near 34°N , 129°W from 19 to 27 October 2005. This region of the Pacific is north of the subtropics (32°N) and outside of the continental margins and can be characterized as an ecological transition zone between the Subtropical Front and the Subarctic Front (Longhurst 1998). During much of the year, the presence of a deep pycnocline and a shallow thermocline result in mixed-layer water temperatures that are approximately $5\text{--}10^\circ\text{C}$ cooler than the subtropical areas south of 32°N . The macronutrient and iron concentrations are typical of oligotrophic ocean waters, and the chlorophyll *a* (Chl *a*) distribution has a deep maximum and an average surface concentration of less than $0.1 \mu\text{g } L^{-1}$.

Samples for nitrate, nitrite, phosphate, and silicic acid were collected with a rosette sampler, subsampled into 20-mL high-density polyethylene scintillation vials, and frozen at -40°C . On shore analysis was performed with a modified Alpkem Series 300 rapid flow analyzer (Sakamoto et al. 1990). Detection limits for NO_3^- , NO_2^- , PO_4^{3-} , and $Si(OH)_4$ were 0.03, 0.01, 0.08, and $0.06 \mu\text{mol } L^{-1}$, respectively, based on $3\times$ the standard deviation of low nutrient seawater that was measured in conjunction with the samples. Ammonium was measured immediately after collection (1–2 h) using an ion conductivity method (Hall and Aller 1992) modified with an improved diffusion cell and conductivity detector to achieve a detection limit of $0.02 \mu\text{mol } L^{-1}$ (Plant unpubl. data). Dissolved iron ($<0.2\text{-}\mu\text{m}$ filter) was measured as soon as possible after collection (0–2 d) in samples acidified to pH 3 following the methods described in Johnson et al. (2003). The detection limit for Fe^{3+} was $0.01 \text{ nmol } L^{-1}$.

Chl *a* and pheopigments were collected in 1-liter bottles and then vacuum filtered ($<10 \text{ mm Hg}$) through a 25-mm GF/F filter. Filters were placed in 30-mL scintillation vials, and pigments were extracted with 90% acetone for 24 h inside a freezer. Chlorophyll concentrations were determined based on the fluorescence technique (Parsons et al. 1984) using a Turner Designs 10-005R fluorometer.

N_2 and C fixation—Rates of N_2 fixation were measured with the $^{15}N_2$ label method (Montoya et al. 1996). All

Table 1. Types of additions (columns 2–5) and the final Fe³⁺ concentrations (column 6, pmol L⁻¹) in the N₂ fixation incubation bottles for the different treatments and the control (column 1). Y = added, N = not added.

	HCO ₃ ⁻	N ₂	H ₂ PO ₄	FeSO ₄	Final [Fe ³⁺]
Control	Y	Y	N	N	89
Phosphorus (P)	Y	Y	Y	N	115
Iron (Fe)	Y	Y	N	Y	1,240
P + Fe	Y	Y	Y	Y	1,140

collection and incubation steps were carried out using trace-metal cleaned equipment, following the protocols for low-iron experiments in the ocean (Fitzwater et al. 1982, 1996). Seawater from a depth of 10 m was collected in 2.5-liter trace-metal clean Niskin bottles and dispensed into an acid-cleaned 25-liter carboy, then distributed to trace-metal clean 4-liter polycarbonate incubation bottles (Nalgene) inside a Class 100 laminar flow hood. Three replicates of the time zero, control, FeSO₄ addition (2 nmol L⁻¹ Fe, GFS chemicals), H₂PO₄ addition (1 μmol L⁻¹ P, GFS chemicals), and FeSO₄ + H₂PO₄ additions (*see Table 1*) were created by making the appropriate additions prior to the bottles being completely filled, then the bottles were capped with a septum enclosure (Nalgene). Time-zero samples were immediately filtered onto combusted GF/F filters (25 mm Whatman, precombusted at 450°C for 4.5 h), dried at 60°C, and stored in a desiccator. The tracer ¹⁵N₂ (Cambridge Isotope Laboratories) was added to each incubation bottle by injection (8 mL, or 14.3% of ambient N₂) through the septum using a gas-tight syringe (Alltech) and a stainless steel needle (Alltech) that was sheathed in PEEK tubing (*see below*). Samples were incubated for 47–49 h (Montoya et al. 2004) at 40% of surface irradiance and ambient surface water temperature in an on-deck incubator. All experiments were run from dusk to dusk. Incubations were terminated by filtration through GF/F filters, dried at 60°C, and stored in a desiccator.

Primary productivity was measured simultaneously with N₂ fixation by adding 8.1% of the ambient TCO₂ as 99% ¹³C HCO₃⁻ (Cambridge Isotope Laboratories). This isotope tracer method gives a reliable measure of net C fixation when compared with the ¹⁴C method (Chavez et al. 1996). The relatively long incubations can cause tracer recycling that would result in an underestimate of the net productivity (Marra 2002); therefore, the measurements represent a minimum estimate. The technique allowed for analysis of the stable carbon isotope abundance on the same sample as the stable nitrogen isotope abundance.

The entire experiment was repeated once (exp 1 and exp 2), but for exp 2 one extra day of incubation time was allowed after the nutrient additions before measuring the ¹⁵N₂ fixed.

Prior to the cruise, laboratory analyses were carried out to determine whether the experimental manipulations (described above) introduced iron (Fe³⁺) to the incubation bottles. Four of the trace-metal clean bottles were filled with MilliQ (MQ) water and capped with the septum enclosures. The Fe³⁺ concentration was determined for one

clean bottle, one bottle that was injected with N₂ using a stainless steel syringe needle, one with the H₂PO₄ addition, and one with the HCO₃⁻ addition. The Fe³⁺ concentrations were below detection, 258 ± 28, 19 ± 20, and 28 ± 14 pmol L⁻¹, respectively. Therefore, the injection process with the metal needle was the only treatment that added significant Fe³⁺ to the bottle. To minimize this during the experiments, PEEK tubing (1.6 mm × 0.76 mm inner diameter, Alltech) was cut to fit over the metal syringe needle and then soaked in 10% trace-metal cleaned HCl for several days. The PEEK tubing was strong enough to pierce the septum and fit snugly over the needle of the gas-tight syringe. Therefore, the needle did not contact the seawater inside the incubation bottle during the ¹⁵N inoculation. After injection of the gas, the needle was withdrawn, but the PEEK tubing was allowed to remain in the septum until the bottle equilibrated with the atmospheric air pressure by ejecting water out through the tubing. The concentrations of Fe³⁺ in the incubation experiments without an iron addition was <115 pmol Fe³⁺ (Table 1), which was similar to ambient Fe³⁺ of seawater, illustrating that the contamination associated with the gas injection process was decreased.

Both ¹⁵N and ¹³C (atom %), and the mass of the particulate organic nitrogen and particulate organic carbon (μg), were determined with a Europa Integra continuous flow mass spectrometer at the University of California, Davis, Stable Isotope Facility. Prior to analysis, samples were sealed in a desiccator containing an open beaker of 100% HCl for 36 h to remove inorganic carbon on the filters. The samples were redried at 60°C, then packaged for the combustion analysis. Fixation rates were calculated as a function of the change in the tracer concentration of the particulate organic pool relative to the size of the pool between time zero and the 48 h time point, as described in detail elsewhere (Chavez et al. 1996; Montoya et al. 1996; Mulholland et al. 2006). Instrument accuracy and precision were estimated at 0.36773 ± 0.0016 ¹⁵N atom % and 1.07404 ± 0.00021 ¹³C atom % based on the mean and standard deviation of glycine standards measured in conjunction with the samples. The range in the difference between the time-zero samples and the incubation experiment samples was 0.01315–0.03179 ¹⁵N atom % and 0.65936–1.09815 ¹³C atom %.

DNA and RNA collection—Seawater was collected from discrete depths using 10-liter Niskin bottles arranged on a conductivity–temperature–depth (CTD) rosette. To evaluate the temporal pattern of *nifH* expression, depth profiles of planktonic RNA were collected every 3 h from six discrete depths (10, 20, 40, 60, 80, and 100 m) from midday (12:00 h) to sunrise (06:00 h). Seawater was collected from the CTD rosette into 2-liter, bleach-rinsed polycarbonate bottles and immediately filtered using a peristaltic pump through a 0.2-μm pore-size Supor filter (Pall) held within a 25-mm diameter Swinnex filter holder (Millipore). The filters were removed; placed into 2-mL bead beater tubes (Biospec Products) containing 350 μL of RLT buffer (Qiagen RNeasy), 1% β-mercaptoethanol, and 30 μL of 0.1-mm glass beads (Biospec Products); frozen in

liquid nitrogen; and stored at -80°C until processed in the laboratory. In parallel with the RNA samples, 2-liter samples were collected for DNA and filtered, frozen in liquid nitrogen, and stored at -80°C . In addition to the depth profiling for RNA and DNA, 10-liter samples were gravity filtered onto a 47-mm diameter 5- μm membrane filter (Pretics, GEOsmotic) held within a 47-mm diameter Swinnex directly from the CTD Niskin bottle. Using blue (450–490 nm) and green (510–560 nm) excitation filters, cyanobacteria populations were identified with epifluorescence microscopy.

A final set of seawater samples was collected from the incubation experiments, included as a fourth bottle in the experimental design described above. One 2-liter subsample was filtered for RNA and another separate sample for DNA, and the remainder was collected for microscopy counts. Each subsample was filtered and preserved as described above. For microscopy, the filtered sample was fixed in a 4% paraformaldehyde, then rinsed three times in 0.1 mol L^{-1} phosphate buffered saline and stored at -20°C .

RNA and DNA extraction—The bead beater tubes containing RNA samples were placed inside a bead beater machine and agitated for 2 min. The tubes were centrifuged for 1 min at $8,000 \times g$, and supernatants were transferred to clean 1.5-mL microcentrifuge tubes. Three hundred and fifty microliters of 90% ethanol was added, samples were inverted, liquid was applied to Qiagen RNeasy minicolumns, and the RNA was purified and eluted following the Qiagen RNeasy protocols. To avoid DNA contamination, samples were treated for 1 h at room temperature with DNase I using Qiagen on-column DNase I RNA extraction protocol. RNA was eluted in 35 μL of Rnase-free water provided by the kit and stored frozen at -80°C .

Nucleic acids were extracted following the protocols based on the xanthogenate method (Tillett and Neilan 2000). Briefly, 500 μL of $2\times$ xanthogenate buffer was added to bead beater tubes containing filter samples and beads. The tubes were agitated for 2 min using a bead beater, incubated for 2 h at 70°C , and placed on ice for 30 min. The tubes were then centrifuged briefly at $8,000 \times g$, and the supernatant was transferred to a clean 1.5-mL centrifuge tube with an equal volume of 70% ethanol. DNA was precipitated for 30 min at $20,000 \times g$ in a bench top centrifuge, and the pelleted nucleic acids were rinsed with 500 μL of 90% ethanol and centrifuged again. Ethanol was removed, and samples were dried overnight and eluted in 30 μL of DNase-free 5-Kd-filtered water.

nifH PCR, cloning, sequencing, and sequence analyses—The six DNA extracts from the $^{15}\text{N}_2$ uptake incubation experiments and one from the DNA depth profiles (25 Oct 25, 12:00 h) were processed using a nested PCR method to amplify a 359 base pair (bp) fragment of the *nifH* gene following the protocols described in Zehr and Turner (2001). The PCR amplifications were performed with a Gene Amp 9700 thermal cycler (Applied Biosystems). Reaction mixtures included 2 μL of template DNA; 32.5 μL of nuclease-free water; 8.0 μL of 25 mmol L^{-1} MgCl_2 ; 5.0 μL of $10\times$ Dynazyme reaction buffer (Qiagen);

2.0 μL of 10 mmol L^{-1} each dNTPs (dATP, dCTP, dGTP, dTTP); 0.5 μL each of 100 $\mu\text{mol} \text{L}^{-1}$ *nifH3* and *nifH4* primers each (Zehr and Turner 2001); and 0.5 μL (0.5 U) of *Taq* polymerase (Qiagen) (total final reaction volume of 50 liters). Reaction conditions consisted of an initial denaturing step at 94°C for 4 min, then 25 cycles of 30 s each at 94°C , 57°C , and 72°C , followed by a final extension at 72°C for 7 min. In the second round of PCR the same reaction mixture and conditions were used; however, *nifH1* and *nifH2* primers (Zehr and Turner 2001) were used, and 1 μL of round 1 product was added. A nuclease-free water addition was used for negative controls in the PCR reactions, and no amplification in the negative controls was detected.

The 25 μL of the 50 PCR μL reactions were analyzed on a 1.2% agarose gel. The 359-bp amplification product was excised and purified using the QIAquick gel purification kit (Qiagen) according to the manufacturer's protocol. Purified PCR products were ligated and cloned with the PGEM_easy Vector system (Promega) following the manufacturer's protocol. Plasmids were purified using the Montage plasmid miniprep96 kit (Millipore) following the manufacturer's partial lysate protocol, and 3 μL of purified plasmid minipreps were sequenced using SP6 or T7 primers at the Berkeley DNA Sequencing Facility (University of California, Berkeley). Vector and primer sequences were removed using SeqLab of the Genetics Computer Group (GCG) Wisconsin package version 10.3. For each clone library, sequences were considered different if they differed by more than 1 bp. Each library contained two to nine identical clones, so only one representative sequence (4 *nifH* sequences total) were deposited in GenBank under accession numbers DQ789165–DQ789168.

The edited *nifH* sequences were imported into an ARB database (Ludwig et al. 2004) containing all *nifH* sequences available in the National Center for Biotechnology Information (NCBI) database as of April 2006. Sequences were aligned to a Hidden Markov Model (HMM) of the oxidoreductase PFAM (accession number PF00142; www.sanger.ac.uk) within the GCG software. For the phylogenetic reconstruction based on nucleotide *nifH* sequences reported here and representative sequences, a Jukes–Cantor correction factor was used and 1,000 bootstrap replicates were run in ARB.

Reverse transcription–quantitative polymerase chain reaction (RT-QPCR)—For RT-QPCR assays, total RNA was reverse transcribed using Super-Script III first strand cDNA synthesis kit (Invitrogen) following manufacturer's recommendations. The reaction mixtures contained 2 μL of RNA template, 0.5 $\mu\text{mol} \text{L}^{-1}$ of each reverse primers (*nifH2* and *nifH4*) (Zehr and Turner 2001), 1 mmol L^{-1} dNTP mixture, $1\times$ RT buffer, 5 mmol L^{-1} MgCl_2 , 10 mmol L^{-1} dithiothreitol, 1 U RnaseOUT (Invitrogen), and 1 U SuperScript III reverse transcriptase. A second set of reactions was set up as described above; however, these had no SuperScript III reverse transcriptase and served as negative controls (no RT). Two additional negative control reactions were set up with water. Reaction conditions were as follows: 55°C for 50 min, 85°C for 5 min, then tubes

were placed on ice. One unit of Rnase H was added to each reaction mixture, and tubes were incubated at 30°C for 20 min to eliminate residual RNA. The cDNA was stored at -20°C until used in the QPCR assays.

For the QPCR assays, we used the TaqMan® primers and probes described by Church et al. (2005b) to evaluate the expression of group A (accession no. AF059642) and group B (accession no. AF299418) phylotypes. In addition, we analyzed for the expression of group C (accession no. DQ273169) with the TaqMan oligonucleotides designed by Foster et al. (2007). For all TaqMan PCR, the 25- μ L reactions contained 12.5 μ L of 2 \times TaqMan buffer (Applied Biosystems), 8.0 μ L of 5-kD-filtered nuclease-free water (Ambion), 0.5 μ mol L⁻¹ each of the forward and reverse primers, 0.25 μ mol L⁻¹ fluorogenic probe, and 2 μ L of template cDNA. Reactions were assayed in quadruplicate, with the fourth replicate used to estimate the reaction efficiency (*see below*). Two microliters of 5-kD-filtered nuclease-free water (Ambion) was added for the no template controls (NTCs) and the no-RT controls were run the same as the RT samples.

PCR amplifications were conducted in a 7500 real-time PCR system (Applied Biosystems) with the following parameters: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s followed by 60°C for 1 min. Gene copy numbers were calculated from the mean C_t value of three replicates and the standard curve for the appropriate primer and probe set (*see below*). In some samples only two of the three replicates produced an amplification signal, these were noted as detectable, but not quantifiable.

Standard curves—For each primer and probe set, duplicate standard curves were made from 10-fold dilution series ranging from 10⁸ to one gene copies per reaction. Standard curves were made using linearized plasmids of the target *nifH* sequence.

PCR efficiency—The efficiency of PCR reaction for each sample was determined as previously described by Short et al. (2004) where the following formula was used: $X_n = X_0 \times (1 + E_x)^n$, where X_0 is the initial number of target molecules and n is the number of cycles (C_t). The E_x is determined by using the calculated X_n with the C_t value from the fourth replicate of each sample, which contained 2 μ L of the plasmid, plus 2 μ L of sample DNA. The E_x values were above 95% efficiency for all samples in the study. The calibration curves for group A, group B, and group C diazotrophs were described by the equations $y = -3.22x + 40.2$ ($R^2 = 0.993$), $y = -3.44x + 40.1$ ($R^2 = 0.996$), and $y = -3.43x + 40.5$ ($R^2 = 0.998$), respectively.

Results and discussion

Environmental conditions—During the occupation of the study site (10 d), the upper 40 m of the water column was a uniform temperature of 19.2°C, indicating the extent of the seasonal thermocline. The temperature range between 40 and 110 m (Fig. 1A) was 19–14°C. In the upper 80 m, the ranges of nutrient concentrations were <0.04 μ mol L⁻¹ NO₃⁻ + NO₂⁻, <0.02–0.04 μ mol L⁻¹ NH₄⁺, <0.08–

0.3 μ mol L⁻¹ PO₄³⁻, 2.0–2.5 μ mol L⁻¹ Si(OH)₄, and 0.08–0.1 nmol L⁻¹ Fe³⁺. Based on an average phytoplankton nutrient requirement (N:Si:P:Fe = 16:16:1:0.0075; e.g., Ho et al. 2003), nitrogen was likely limiting growth (Fig. 1B). In most oligotrophic environments, 0.1 nmol L⁻¹ Fe³⁺ is not considered to limit growth rate of oceanic phytoplankton species (Sunda and Huntsman 1995); however, these low concentrations would prevent significant biomass increase of larger phytoplankton if nitrogen became available. The potential for nitrogen limitation was indicated by the relationship between the dissolved inorganic nitrogen (NO₃⁻, NO₂⁻, and NH₄⁺) and PO₄³⁻ in the top 300 m, which showed a deviation from a 16:1 relationship (i.e., Redfield ratio) toward a nitrogen-limited environment as nitrogen and phosphorus concentrations approached lower values (Fig. 1C). The temporal variability in phosphate in the top 100 m indicated that phosphorus drawdown occurred in the absence of measurable NO₃⁻, NO₂⁻, or NH₄⁺ (Fig. 1D). The N:P relationship in Fig. 1D can be created if other sources of nitrogen are available for growth, such as dissolved organic nitrogen or N₂ fixation (e.g., Karl et al. 2001).

N₂ fixation rates and primary productivity—N₂ fixation rates were measured in triplicate for each of the four experimental treatments, and the entire experiment was repeated once; therefore, a total of 24 separate incubations was performed. The N₂ fixation rates ranged from 0.15 to 0.31 nmol N L⁻¹ d⁻¹ in exp 1 and from 0.15 to 0.26 nmol N L⁻¹ d⁻¹ in exp 2 (Fig. 2A). There was no statistical difference in rates across treatments in either exp 1 or exp 2 (SigmaStat, one-way ANOVA $p = 0.35$ and $p = 0.5$, respectively). There were lower N₂ fixation rates in the second experiment throughout all treatments and the control. Primary productivity (PP) was measured in the same bottles as N₂ fixation rates and ranged from 0.16 to 0.21 μ mol C L⁻¹ d⁻¹ in exp 1 and from 0.10 to 0.14 μ mol C L⁻¹ d⁻¹ in exp 2 (Fig. 2B). As with the N₂ fixation rates, there was no response to addition of phosphorus, iron, or phosphorus and iron to growth rates, and there was a lower rate of PP in the second experiment compared with the first.

The particulate organic carbon (POC) and particulate organic nitrogen (PON) concentrations from all incubation bottles averaged 3.0 ± 0.3 μ mol L⁻¹ and 0.28 ± 0.02 μ mol L⁻¹, respectively. Normalized to the average Chl *a* concentration (0.08 μ g L⁻¹), the estimated POC and PON of primary producers (C:Chl *a* = 50 and C:N = 7) was 0.33 μ mol C L⁻¹ and 0.048 μ mol N L⁻¹. The remainder of the carbon (89%) and nitrogen (82%) collected on the filters was most likely composed of chemotrophic bacteria, heterotrophic protists, and zooplankton. Based on these estimates and the average PP of 0.15 μ mol C L⁻¹ d⁻¹, the turnover time of the autotrophic organic carbon was 2.2 d. The required nitrogen to sustain this growth rate is 22 nmol N L⁻¹ d⁻¹. Since the measured N₂ fixation rates averaged 0.25 nmol N L⁻¹ d⁻¹, N₂ fixation accounted for about 1% of the daily autotrophic nitrogen requirement. The C:Chl *a* value is probably the most variable aspect of this calculation; however, since the ecological range in C:Chl

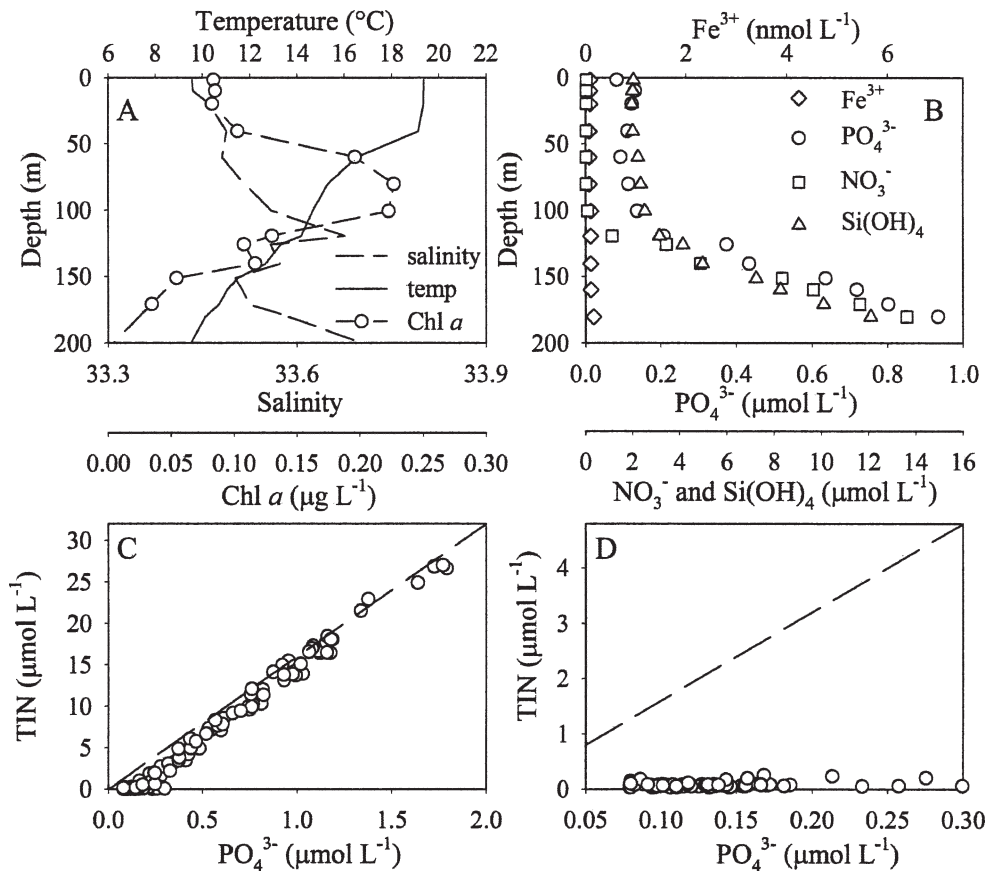


Fig. 1. (A) Average temperature, salinity, and Chl *a* during the period 16–24 October, 2005 at 34°N, 129°W, (B) average nutrient concentration plotted on a scale relative to a nutrient ratio of N:Si:P:Fe of 16:16:1:0.003, (C) inorganic nitrogen (TIN) versus PO_4^{3-} concentration in the upper 300 m of the water column, and (D) TIN versus PO_4^{3-} in the upper 100 m of the water column. TIN includes NO_3^- , NO_2^- , and NH_4^+ . The dashed line indicates an N:P of 16.

a is 10–90, the maximum range in the estimate of N_2 fixation to the autotrophic nitrogen requirement is between 0.2% and 2.5%. Another technique to compare the two rate measurements is to convert the N_2 fixation rates to units of carbon (e.g., if C:N = 7, then 0.25 nmol N L^{-1} can account for 1.7 nmol C L^{-1}). From this exercise, the

contribution of N_2 fixation to the PP in all the incubation measurements range between 0.7 and 1.4%. Therefore, the majority of the primary productivity was supported by other forms of biologically available nitrogen. The standing stock of nitrate, nitrite, and ammonium (about 70 nmol L^{-1}) could support the measured growth rates for

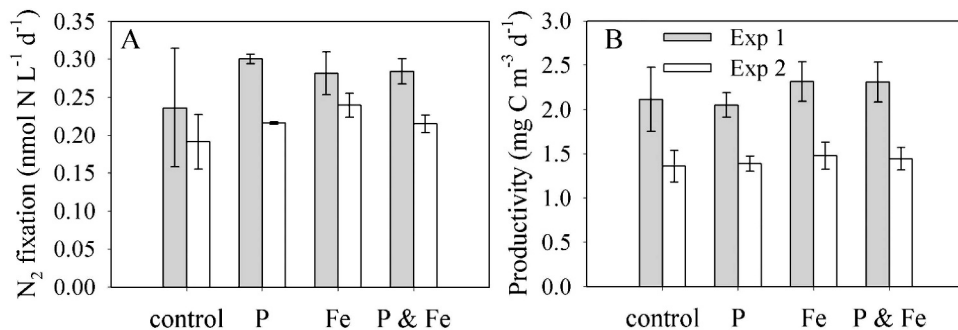


Fig. 2. (A) N_2 fixation rates of the plankton assemblage at 10 m under treatments of: ambient conditions (control), 1 $\mu\text{mol L}^{-1}$ H_2PO_4 addition (P), 2 nmol L^{-1} FeSO_4 addition (Fe), and the two additions combined (P & Fe). No statistical differences were detected between treatments (SigmaStat one-way ANOVA $p = 0.25$ and $p = 0.115$). (B) Net primary productivity of the plankton assemblage at 10 m under the same treatments as (A). No statistical differences were detected between treatments (SigmaStat one-way ANOVA $p = 0.50$ and $p = 0.73$).

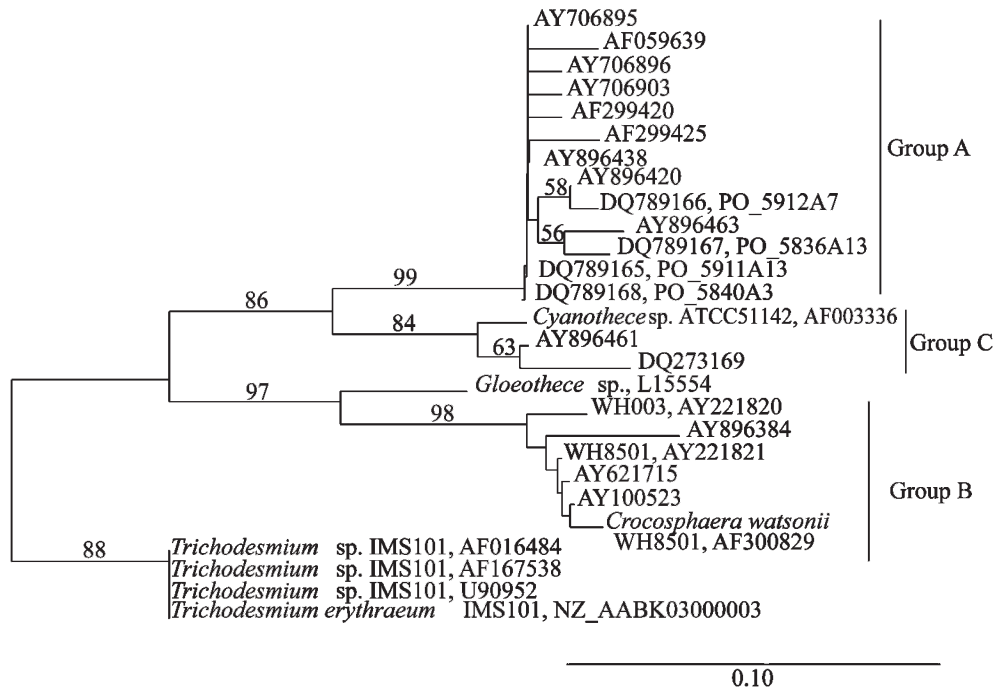


Fig. 3. Neighbor-Joining phylogenetic reconstruction inferred from amino acid *nifH* sequences from this study and selected sequences. The sequences and GenBank accession numbers indicated by “PO_” were found in this study. Other selected sequences are used for comparison and are labeled with genus and species names, accession numbers, and the values indicated at the nodes represent the percentage from 1,000 bootstrap replicates.

approximately 3 d. Therefore, significant nitrogen recycling must occur to support the measured autotrophic carbon doubling time of 2.2 d. However, since N₂ fixation represents a new source of nitrogen—as opposed to recycled nitrogen—then it’s valid to compare the N₂ fixation rate with new production only. The average estimate of new production in the oligotrophic Pacific is approximately 10% (or less) of total productivity (Dugdale and Wilkerson 1992), which amounts to <15 nmol C L⁻¹ d⁻¹. Therefore, in a similar manner as calculated for total productivity above, a N₂ fixation rate of 0.25 nmol N L⁻¹ d⁻¹ fueled >10% of the new production. For comparison, an estimate of nitrate flux via diffusion into the stratified upper ocean is approximately 0.14 mmol N m⁻² d⁻¹ (Lewis et al. 1986), or 1.4 nmol N L⁻¹ d⁻¹ in a 100-m mixed layer. With this value in the calculation above, NO₃⁻ flux could account for 65% of the new production at our study site.

The lack of response in the diazotroph community to phosphorus and iron concentration can be examined in relation to the measured C fixation rates and ambient nutrient concentrations in the water column. Based on the average rate of N₂ fixation, the phosphorus requirement to support the diazotroph community was 0.02 nmol P L⁻¹ d⁻¹, assuming N:P = 16. The average PO₄³⁻ concentration was 80 nmol L⁻¹; therefore, the standing stock of PO₄³⁻ could support the N₂ fixers for more than 10 yr at the measured rate. For N₂ fixation to supply the nitrogen that caused the “extra” PO₄³⁻ drawdown observed during the occupation of the study region (Fig. 1D),

rates of at least 160 nmol L⁻¹ d⁻¹ would be required. Similarly, iron requirements (P:Fe = 0.0075) are <0.1 pmol L⁻¹ d⁻¹, while the standing stock is approximately 100 pmol L⁻¹. Even if iron requirements were 10 times higher, the standing stock could support N₂ fixation for several months. The diazotroph population was not limited in their ability to acquire nutrients at the ambient concentrations (i.e., below the *K_m* concentration), since the additions of 1 μmol L⁻¹ P and 2 nmol L⁻¹ Fe should be sufficient to relieve the uptake-related limitation imposed by low concentrations.

N₂ fixation rate measurements for the unicellular microbes have been reported in numerous studies in tropical and subtropical regions, and typical values are 0.38 nmol N L⁻¹ d⁻¹ (Zehr et al. 2001), 0.55 nmol N L⁻¹ d⁻¹ (Dore et al. 2002), and 0.48 nmol N L⁻¹ d⁻¹ (Falcon et al. 2004a). In addition, a survey of unicellular microbial N₂ fixation rates across several regions of the Pacific detected rates between 0.12 and 22.2 nmol N L⁻¹ d⁻¹ (Montoya et al. 2004). Therefore, our results (average of 0.25 nmol N L⁻¹ d⁻¹) are within the range of those previously reported.

Characterization of the diazotroph assemblage—NifH amplified from DNA templates in five out of six incubation experiments. Four unreported sequence types were retrieved from a total of 15 cloned sequences and the translated amino acid sequences cluster with and are 96–98% similar to unicellular group A *nifH* sequences previously reported from samples collected in the sub-

tropical North Pacific Ocean (Zehr et al. 2001) (Fig. 3). The *nifH* nucleotide sequences were 98–99% similar to representative unicellular group A sequences.

In situ *nifH* expression for unicellular groups A, B, and C was estimated using a TaqMAN quantitative reverse transcription PCR (RT-QPCR) assay for samples collected from CTD depth profiles. The *nifH* expression by group A was detected throughout the upper 60 m at all time points, and concentrations ranged from 2.4×10^2 to 3.1×10^4 *nifH* copies L^{-1} (Fig. 4A). Modest numbers of *nifH* cDNA copies were estimated for group A at 80 m (Table 2). Group A nitrogenase expression was highest during the daytime samples, and minimum values were recorded during the dark period (20:00, 23:00, and 02:00 h). The depth profiles showed one distinct maximum during the day, and all depths showed a general increase in mRNA synthesis starting at dawn (Fig. 4A). In contrast to group A, group C was the least abundant phylotype, where expression was detected in only 4 of the 42 samples and ranged from 2.5×10^2 to 4.3×10^3 *nifH* copies L^{-1} (Table 2). Group B was detected in at least two time points of six of the profiles. Highest estimates for group B *nifH* expression were from 40 m at 23:00 h (7.6×10^3 *nifH* copies).

The distribution of *nifH* mRNA phylotypes is comparable with the vertical and temporal patterns of the group A and group B organisms reported at Sta. ALOHA, but absolute abundances were approximately 10 times smaller (Church et al. 2005a,b). Group C has only been recently identified and reported from the Western Tropical North Atlantic (WTNA) (Langlois et al. 2005; Foster et al. 2007). The similarity between studies with respect to the temporal and spatial distribution of group A *nifH* suggests that their distribution is not a local phenomenon and is determined by upper euphotic-zone properties. The group A diazotrophs have not been proven to be photosynthetic, but they do have a diel response in *nifH* expression (Fig. 4A), and the sequences cluster closest to autotrophic diazotrophs such as *Cyanothece* sp. and other cyanobacteria (Fig. 3). This contrasts with the strategy of other nonheterocystous photosynthetic diazotrophs such as the group B organisms measured here and elsewhere (Falcon et al. 2004a; Mazard et al. 2004; Church et al. 2005b).

Trichodesmium was not detected by microscopy in water column depth profiles or from incubation bottles. Its absence is likely attributed to temperature limitation (Staal et al. 2003; Breitbarth et al. 2006) since it is found in high abundances during the same time of year in warmer regions of the North Pacific (Fennel et al. 2002). In addition, *Trichodesmium* may have been limited by their inability to use iron at the low concentrations measured in this study (Kustka et al. 2002; Mills et al. 2004), and, therefore, may be an additional constraint on its geographical distribution. Microscopic cell counts identified one sample from 40 m that contained 0.6 *Rhizosolenia clevei* filaments L^{-1} with two symbiotic *Richelia* per filament, but no other instances of these organisms were found in the water column. Therefore, N_2 fixation was dominated by the unicellular microbes, of which we detected the highest gene expression activity in the group A phylotype.

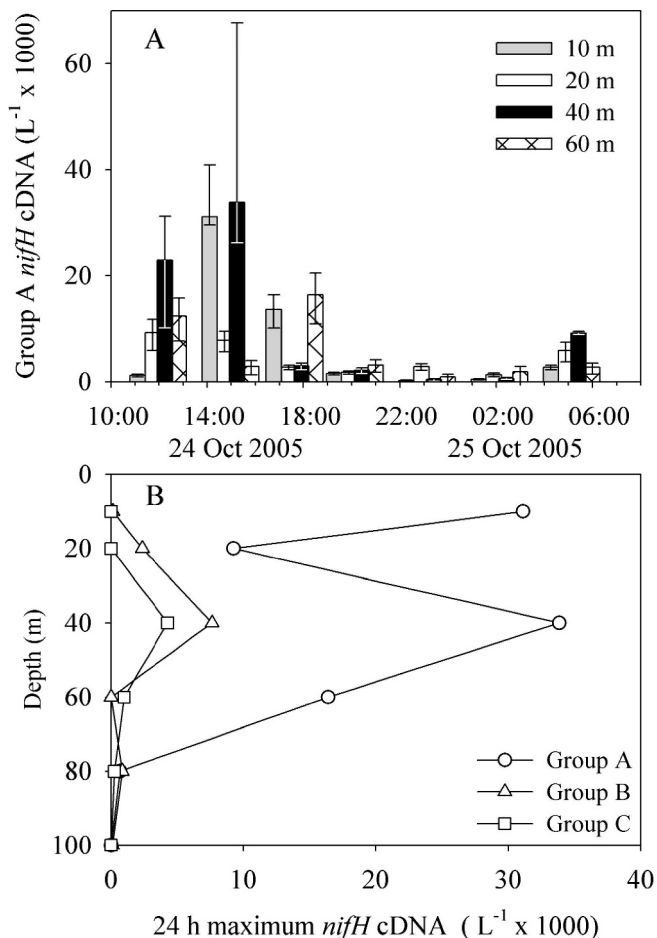


Fig. 4. (A) Temporal patterns of *nifH* transcription (*nifH* cDNA copies L^{-1}) by group A phylotype in the upper ocean (10, 20, 40, 60 m) at $34^{\circ}N$ $129^{\circ}W$ in October 2005. The bars show the mean cDNA concentrations from triplicate QPCRs, and error bars indicate one standard deviation. (B) The daily maximum mRNA concentration as a function of depth for the three groups of unicellular diazotrophs detected in this study.

Ecosystem-wide N_2 fixation—*NifH* gene expression was detected throughout the $19^{\circ}C$ region of the water column, and the group A *nifH* gene products were detected in abundance down to 60 m (Fig. 4B). This pattern suggests that N_2 fixation by group A also occurred in deeper and cooler waters than where the incubation experiments were performed. Applying the average N_2 fixation measurement evenly throughout the upper 40 m (i.e., the seasonal mixed layer, delineated by the thermocline illustrated in Fig. 1A and corresponding to the average seasonal mixed layer in this region in October; Longhurst 1998) gives an areal rate for N_2 fixation of $9.8 \pm 1.2 \mu mol N m^{-2} d^{-1}$. It is feasible that N_2 fixation is proportional to the concentration of *nifH* mRNA, since the presence of mRNA is commonly interpreted as an indication of N_2 fixation activity (e.g., Zehr et al. 2007). Therefore, N_2 fixation may have occurred wherever *nifH* mRNA was detected. Assuming that the daily maximum in the diel *nifH* expression is proportional to the daily N_2 fixation rate, than the mRNA normalized N_2 fixation rate at 10 m is $7.9 fmol N cDNA^{-1} d^{-1}$.

Applying this estimate to the water column profile measurements (Table 2) gives an integrated water column rate of 15.3 $\mu\text{mol N m}^{-2} \text{d}^{-1}$. This estimate represents an upper boundary on the N₂ fixation rate for this region since it integrates across deeper depths. However, this estimate should be considered speculation, since there is not yet any precedent for relating mRNA abundance to enzyme activity in the oceanic environment.

The N₂ fixation rates at our study site are relatively low compared with previous reports from the subtropical and tropical oceans. This is not unexpected, since the region is outside of the typical temperature range of marine N₂ fixation. However, other studies have reported even lower rates for unicellular diazotrophs, and evidence from size-fractionated measurements at ALOHA suggests that when N₂ fixation rates are high, the unicellular diazotrophs contribute less than *Trichodesmium* (Dore et al. 2002), implying that unicellular diazotrophs may rarely achieve high rates of N₂ fixation. Our highest daily mRNA abundances for group A and B were approximately 10 times lower than the highest numbers reported for Sta. ALOHA, and attempts to quantify *nifH* genes from the DNA samples were unsuccessful; probably because of the low abundance of the organisms in the samples. Church et al. (2005b) measured a daytime average of two *nifH* mRNA copies per cell for the group A phylotypes at ALOHA. Based on this measurement and our mRNA normalized rate measurements (above), the daily cellular rate of N₂ fixation for the group A organisms is 15.8 fmol N. Interestingly, Falcon et al. (2005) reported that laboratory measurements of cultured unicellular diazotrophs (2×10^7 cells ml⁻¹) had a daily N₂ fixation rate of 15 fmol cell⁻¹, and Tuit et al. (2004) reported a range for *Crocospaera* in culture of 3.2–14.2 fmol cell⁻¹, with the lower measurements attributed to low Fe conditions. Other estimates include 7.3 fmol N cell (Zehr et al. 2001) and 0.12–22.2 fmol N cell (Montoya et al. 2004). Therefore, even though the areal rates reported in this study were relatively low, the estimated cell specific rates are within the range of previously reported measurements. While temperature limitation may cause lower fixation rates (Mazard et al. 2004; Falcon et al. 2005), these results suggest that it is important to consider the additional constraints on the population size by processes such as grazing and virus infection that can determine abundances within the microbial food web (Suttle 2005).

Contribution to the global fixed-N budget—Recent reports have concluded that (1) the magnitude of marine N₂ fixation may be substantially higher than current measurements predict (Codispoti 2006) and (2) large regions of the Pacific Ocean outside of the subtropics are responsible for the missing marine N₂ fixation component (Codispoti 2006; Deutsch et al. 2007). It is important to determine what contribution the unicellular diazotrophs make to the global marine N₂ fixation budget. The areal rates measured in this study can be extrapolated to all regions of the ocean where the unicellular diazotrophic groups may be the dominant N₂ fixers. We have constrained our estimates to include only the months of

Table 2. Summary of cDNA *nifH* abundance (copies L⁻¹) estimated during the diel sampling period using the RT-QPCR method. Nd indicates samples that were below detection (<1 gene copy), and detected represents samples where only one of three replicates had the target phylotypes.

Depth (m)	Time (h)	Group A mRNA <i>nifH</i> copies	Group B mRNA <i>nifH</i> copies	Group C mRNA <i>nifH</i> copies
10	11:50	1.21 × 10 ³	Nd	Nd
	14:50	3.11 × 10 ⁴	1.26 × 10 ²	Nd
	17:30	1.36 × 10 ⁴	detected	Nd
	20:00	1.56 × 10 ³	Nd	Nd
	23:00	2.35 × 10 ²	detected	Nd
	02:00	4.45 × 10 ²	Nd	Nd
	05:00	2.71 × 10 ³	Nd	Nd
20	11:50	9.27 × 10 ³	detected	Nd
	14:50	7.82 × 10 ³	Nd	Nd
	17:30	2.73 × 10 ³	detected	Nd
	20:00	1.78 × 10 ³	detected	Nd
	23:00	2.85 × 10 ³	Nd	Nd
	02:00	1.30 × 10 ³	Nd	Nd
	05:00	5.86 × 10 ³	2.20 × 10 ³	Nd
40	11:50	2.29 × 10 ⁴	Nd	Nd
	14:50	3.38 × 10 ⁴	Nd	Nd
	17:30	2.97 × 10 ³	Nd	Nd
	20:00	2.18 × 10 ³	1.30 × 10 ³	Nd
	23:00	3.78 × 10 ²	7.64 × 10 ³	4.27 × 10 ³
	02:00	6.06 × 10 ²	1.94 × 10 ³	Nd
	05:00	9.20 × 10 ³	Nd	Nd
60	11:50	1.24 × 10 ⁴	1.98 × 10 ²	9.78 × 10 ²
	14:50	2.93 × 10 ³	Nd	Nd
	17:30	1.64 × 10 ⁴	detected	Nd
	20:00	3.20 × 10 ³	1.58 × 10 ²	Nd
	23:00	8.94 × 10 ²	8.65 × 10 ¹	Nd
	02:00	1.86 × 10 ³	2.24 × 10 ²	Nd
	05:00	2.71 × 10 ³	8.40 × 10 ²	2.57 × 10 ²
80	11:50	9.07 × 10 ¹	Nd	detected
	14:50	9.50 × 10 ¹	Nd	Nd
	17:30	6.67 × 10 ²	Nd	Nd
	20:00	detected	Nd	Nd
	23:00	Nd	Nd	Nd
	2:00	Nd	Nd	Nd
	5:00	detected	Nd	Nd
100	11:50	Nd	Nd	Nd
	14:50	Nd	Nd	Nd
	17:30	Nd	detected	Nd
	20:00	Nd	9.86 × 10 ¹	Nd
	23:00	Nd	Nd	Nd
	02:00	Nd	Nd	Nd
	05:00	Nd	Nd	Nd

an average year when sea surface temperatures range between 19°C and 25°C (Fig. 5). The temperature constraints were chosen based on the lower temperature boundaries measured in this study and delineated at the upper limit by the occurrence of active unicellular N₂ fixers in the Pacific Subtropics as reported in Zehr et al. (2007) and Church et al. (2005b). As argued above, waters above 25°C are also occupied by dominant N₂ fixers such as *Trichodesmium* spp.; therefore, the regions depicted in Fig. 5 represent a potential N₂ fixation niche occupied primarily by unicellular diazotrophs. Applying the areal N₂

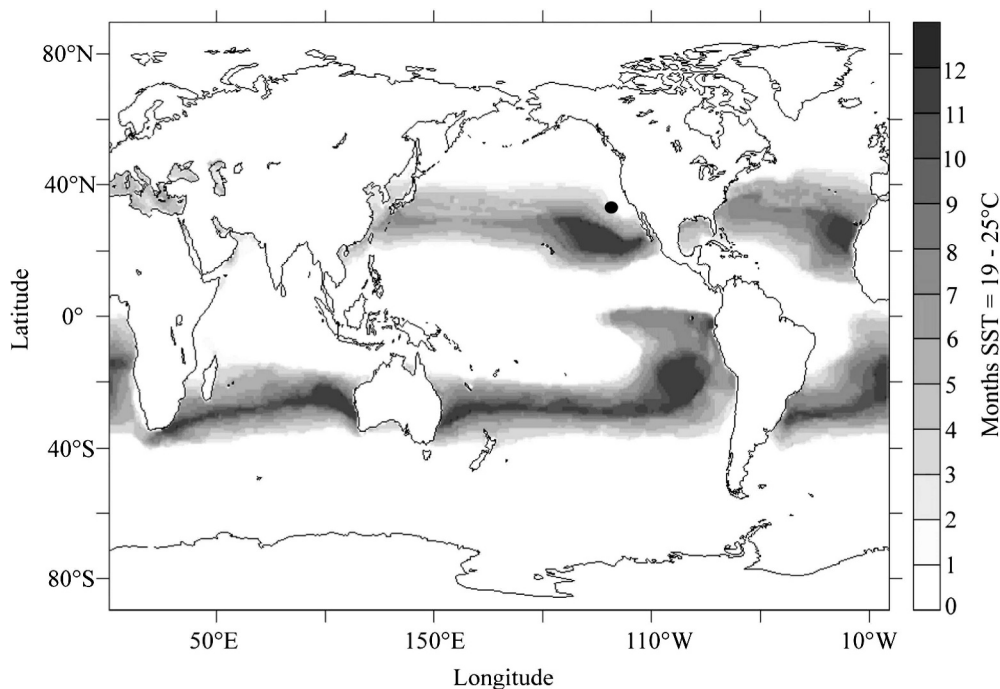


Fig. 5. Map of the number of months where regions of the global ocean have sea surface temperatures between 19 and 25°C. The study area (indicated with filled circle) is located in the eastern Pacific Ocean at 34°N, 129°W. Sea surface temperature data is from the World Ocean Atlas 2001 (Stephens et al. 2002).

fixation rate measured in this study to the spatial and temporal parameters illustrated in Fig. 5 produced an estimate of 3.6–5.6 Tg N yr⁻¹ of new nitrogen that is contributed by the nitrogen fixation assemblage described in this study. This estimate increases to approximately 12.6 Tg N yr⁻¹ if the areal rate measured by Dore et al. (2002) for the <10- μ m size fraction at Sta. ALOHA is used (35 μ mol m⁻² d⁻¹). While these values do not substantially change the estimated modern-day oceanic fixed-nitrogen deficit—which may be as high as 230 Tg N yr⁻¹ (Codispoti 2006)—they do provide insight into the importance of N₂ fixation in regions of the world's oceans where rates are seldom measured.

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