Latitudinal constraints on the abundance and activity of the cyanobacterium UCYN-A and other marine diazotrophs in the North Pacific

Mary R. Gradoville,1* Hanna Farnelid,1, a Angelique E. White,2 Kendra A. Turk-Kubo,1 Brittany Stewart,1,b François Ribalet,3 Sara Ferrón,2 Paulina Pinedo-Gonzalez,4, c E. Virginia Armbrust,3 David M. Karl,2 Seth John,4 Jonathan P. Zehr1

1Ocean Sciences Department, University of California Santa Cruz, Santa Cruz, California
2Department of Oceanography, University of Hawaii at Manoa, Honolulu, Hawaii
3School of Oceanography, University of Washington, Seattle, Washington
4University of Southern California, Los Angeles, California

Abstract

The number of marine environments known to harbor dinitrogen (N₂)-fixing (diazotrophic) microorganisms is increasing, prompting a reassessment of the biogeography of marine diazotrophs and N₂ fixation rates (NFRs). Here, we investigate the diversity, abundance, and activity of diazotrophic microorganisms in the North Pacific Subtropical Gyre (NPSG), a diazotrophic habitat, and the North Pacific Transition Zone (NPTZ), a region characterized by strong physical, chemical, and biological gradients. Samples were collected on two springtime meridional cruises during 2016 and 2017, spanning from 23.5°N to 41.4°N along 158°W. We observed an abrupt decrease in diazotrophic abundances near the southern edge of the NPTZ, which coincided with a salinity front and with a ∼10-fold increase in Synechococcus abundance, but without a concomitant change in phosphate or nitrate concentrations. In NPSG waters south of this diazotrophic boundary, nifH genes and NFRs were consistently detected and diazotrophic communities were dominated by UCYN-A, an uncultivated, symbiotic cyanobacterium (2.8 × 10³ to 1.0 × 10⁶ nifH gene copies L⁻¹). There was a significant positive relationship between quantitative polymerase chain reaction-derived UCYN-A nifH gene abundances and community NFRs in the NPSG, suggesting a large contribution of UCYN-A to community NFRs. In the NPTZ waters to the north, NFRs were low or undetected and nifH genes were rare, with the few detected sequences represented by UCYN-A and noncyanobacterial diazotrophs. The patterns we observed in UCYN-A abundance in the context of local biogeochemistry suggest that the environmental controls of this organism may differ from those of cultivated marine cyanobacterial diazotrophs.

*Correspondence: mgradovi@ucsc.edu

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Additional Supporting Information may be found in the online version of this article.

1Present address: Hanna Farnelid, Centre of Ecology and Evolution in Microbial Model Systems, Linnaeus University, Kalmar, Sweden
2Brittany Stewart, University of Southern California, Los Angeles, California
3Paulina Pinedo-Gonzalez, Lamont-Doherty Earth Observatory, Columbia University, New York, New York

The biological reduction of dinitrogen (N₂) gas to ammonia (N₂ fixation) provides nearly half of the planet’s bioavailable nitrogen (N) (Galloway et al. 2004) and can fuel a large fraction of productivity and export in N-limited ocean ecosystems (Karl et al. 1997). The process of N₂ fixation is performed by a select group of microorganisms, termed diazotrophs, that possess the enzyme nitrogenase. For the past half century, marine N₂ fixation has been thought to be primarily restricted to surface waters of the tropical and subtropical oceans, where N-limitation can select for diazotrophs (Gruber and Sarmiento 1997), cyanobacterial diazotrophs can photosynthesize, and temperatures are warm enough to facilitate the growth of the well-known diazotrophic cyanobacterium, Trichodesmium (Breitbarth et al. 2007). However, sequencing of the nitrogenase (nifH) gene has revealed unicellular cyanobacterial and diverse non-cyanobacterial marine diazotrophs with broad geographic ranges (Zehr et al. 1998; Moisander et al. 2010; Farnelid et al. 2011). Furthermore, recent surveys have reported diazotrophic genes and/or N₂ fixation rates (NFRs) in unexpected environments, including high-nutrient coastal systems (Mulholland et al. 2012; Bentzon-Tilia et al. 2015), oxygen minimum zones...
(Loescher et al. 2014; Jayakumar et al. 2017), polar regions (Blais et al. 2012; Harding et al. 2018; Shiozaki et al. 2017), and the deep sea (Hewson et al. 2007; Benavides et al. 2018). Thus, the biogeography and environmental controls of marine diazotrophs are still not well understood.

While much recent work has focused on understanding global patterns in diazotrophy, few studies have surveyed the distribution and activity of diazotrophs through the transition zones separating major biomes. These ecological transition zones are characterized by steep environmental gradients and often contain high diversity and species richness (Kark 2007). One such region is the North Pacific Transition Zone (NPTZ), which spans the Pacific basin between the North Pacific Subtropical Gyre (NPSG) and the Pacific Subarctic Gyre, comprising ~12% of the total surface area of the North Pacific. The NPTZ contains strong gradients in physical, chemical, and biological properties (Roden 1991; Polovina et al. 2001; Juranek et al. 2012). The NPTZ is bounded by thermohaline fronts: the subtropical frontal zone to the south (~32°N) is defined by a shift in surface salinity from ~35.2 to 34.8 (Roden 1991) and has been associated with surface chlorophyll blooms (Wilson 2003; Wilson et al. 2013), and the subarctic frontal zone to the north (~42°N) is defined by a shift in surface salinity from ~33.8 to 33.0 (Roden 1991) and has been associated with strong gradients in macronutrient concentrations (Park 1967; McGowan and Williams 1973). Within the NPTZ, a sharp, basin-wide gradient in surface chlorophyll termed the transition zone chlorophyll front (Polovina et al. 2001) moves seasonally ~1000 km north and south and has been associated with increased productivity and export compared to adjacent areas (Juranek et al. 2012). This chlorophyll front is likely caused by the southward Ekman transport of nutrient-rich subarctic surface waters during the winter and spring (Ayers and Lozier 2010). The multiple physical, chemical, and biological fronts within the NPTZ provide an opportunity to investigate the mechanisms that structure communities of microorganisms such as diazotrophs.

Theory, model output, and direct observations all suggest that diazotrophy should decrease through the transition from the NPSG to the NPTZ (Church et al. 2008; Ward et al. 2013). The warm surface waters of the NPSG are chronically limited in fixed N (<10 nmol nitrate L⁻¹; Karl and Church 2014) and comprise a known habitat for diazotrophs (Karl et al. 2002). Diazotrophic assemblages in the NPSG are dominated by cyanobacteria—unicellular groups (UCYN-A, Crocosphaera, and Cynothecce), filamentous Trichodesmium, and heterocystous groups associated with diatoms—but also include putative heterotrophs (Church et al. 2005b). The abundances of these diazotrophic taxa and the magnitude of N₂ fixation-supported carbon (C) export within the NPSG vary on a seasonal cycle (Karl et al. 1997, 2012; Church et al. 2009). Further north, NPTZ waters have lower temperatures, higher inorganic N concentrations, higher nitrate:phosphate (N:P) ratios, and higher nitrate:iron (N:Fe) supply ratios, all of which have been suggested to decrease the competitive ability of diazotrophs relative to other phytoplankton groups and/or inhibit N₂ fixation (Ohki et al. 1991; Stal 2009; Ward et al. 2013). A few samples collected across the NPTZ support the hypothesis that diazotrophs are rare or absent from this region (Marumo and Asaoka 1974; Church et al. 2008; Shiozaki et al. 2017). However, this area of the ocean has been extremely under-sampled for NFRs (Tang et al. 2019). Thus, the location of the boundary for diazotrophy within the NPTZ, and the biogeochemical drivers of that boundary have been largely unexplored.

The objective of this study was to determine how diazotrophic communities and NFRs vary within the NPSG and across the environmental gradients of the NPTZ. We present data from two springtime surveys from consecutive years spanning from the NPSG (~23°N) through the NPTZ and into the subarctic frontal zone (41.4°N). On both cruises, we investigated diazotrophic diversity, abundance, and activity using high-throughput sequencing and quantitative polymerase chain reaction (qPCR) of the nifH gene as well as ¹⁵N₂ fixation rate assays. We also used single-cell approaches to measure cell-specific NFRs by the dominant diazotroph in our samples—UCYN-A, a unique unicellular cyanobacterium living in a likely obligate symbiosis with a prymnesiophyte host (Zehr et al. 2016).

**Methods**

**Sampling strategy and seawater collection**

Samples were collected during two transect cruises in the North Pacific: a cruise aboard the R/V Ka’imikai-O- Kanaloa, 19 April 2016 to 3 May 2016 and a cruise aboard the R/V Marcus G. Langseth, 27 May 2017 to 13 June 2017 (Fig. 1).

Seawater was collected for DNA, RNA, and ¹⁵N₂ incubations using Niskin® sampling bottles attached to a rosette equipped with a conductivity, temperature, depth (CTD) and an in situ fluorometer. Seawater was sampled into 2- or 4-L acid-washed,
MilliQ®-rinsed polycarbonate bottles. Samples for RNA and NFR measurements were always collected before dawn, while samples for DNA were collected at different times of day. During the 2017 cruise, additional surface (10–15 m) seawater samples for DNA were collected using the trace metal sample collection setup (see below).

During the 2016 cruise, DNA samples were sequentially filtered through 25 mm, 3 μm pore size polyethersulfone membranes (Sterlitech, Kent) then onto 0.2-μm pore size Supor membranes (Pall Corporation, New York) using a peristaltic pump. During the 2017 cruise, both DNA and RNA samples were filtered onto 0.2 μm pore size Supor membranes. DNA filtration volumes differed between the two cruises. Maximum filtration volumes were 2 L in the 2016 cruise and 4 L in the 2017 cruise, but variable biomass at the different sampling sites resulted in final filtration volumes ranging from 1050 to 2000 mL during the 2016 cruise and from 700 to 4000 mL during the 2017 cruise (see Supporting Information Table S2). RNA samples were filtered for a maximum of 25 min; filtration volumes ranged from 970–3900 mL. For both cruises, filters were placed into microcentrifuge tubes containing mixtures of 0.1- and 0.5-mm glass beads (Biospec Products, Bartlesville), 396 μL of Buffer RLT (Qiagen, Venlo, the Netherlands), and 4 μL of beta-mercaptoethanol (RNA samples) or beads only (DNA samples), then flash-frozen in liquid N₂ and stored at −80°C until analysis.

**Biogeochemical and hydrological data**

Surface seawater samples for nitrate plus nitrite (N + N) and soluble reactive phosphorus (SRP, dominated by phosphate) were collected from Niskin® sampling bottles and the ship’s uncontaminated underway line into acid-cleaned high-density polyethylene bottles. The samples were immediately frozen and analyzed at the University of Hawaii following Foreman et al. (2019). Briefly, N + N and SRP were measured using a SEAL Analytical AutoAnalyzer III with high-resolution detectors following the colorimetric reactions described by Strickland and Parsons (1972) and Murphy and Riley (1962), respectively. The limit of quantification for these colorimetric methods is 75 nmol L⁻¹ for N + N and ~30 nmol L⁻¹ for SRP, and the average precision is 0.4% and 0.2%, respectively. Accuracies are within 2%, as determined with daily analyses of Wako CSK standard nitrate and phosphate solutions. For low N + N concentrations (<0.5 μmol L⁻¹), N + N was determined using a high sensitivity chemiluminescent method, described in detail in Foreman et al. (2016), which has a detection limit of 1 nmol L⁻¹. The precision of the high-sensitivity method ranges from 0.4% at 1000 nmol L⁻¹ to 1–7% at 2 nmol L⁻¹.

Surface seawater samples for trace metals were collected from depths of ~10–15 m while underway at speeds of 9–12 knots. The inlet tube was kept at depth using a trace metal clean surface tow fish system modified from that of Bruland et al. (2005). The water intake tube was attached to a PVC “vane” on a polypropylene line, with two PVC-encapsulated lead-weighted “torpedoes” and roughly 50 kg of steel weights mounted below the vane. The intake tubing was connected to a deck-mounted, compressed air-powered, polytetrafluoroethylene bellows pump (AstiPure II) to pump water at a rate of 5–10 L/min. Samples were filtered in-line using 0.2 μm syringe filters (PALL Acadrisc Syringe Filter Supor Membrane).

Seawater samples were analyzed for dissolved trace metal concentrations at the University of Southern California after preconcentration using an offline adaptation of the seaFAST-pico metal extraction system (Elemental Scientific Inc.) as described in Lagerström et al. (2013). Briefly, using the seaFAST, 10 mL aliquots of seawater were extracted onto Nobias PA1 chelating resin at pH ~6.5 with an ammonium acetate/acetic acid buffer, then eluted in 10% vol/vol nitric acid (HNO₃). Fe concentrations were measured by isotope dilution on a Thermo Fisher Element 2 High Resolution Inductively Coupled Plasma Mass Spectrometer. The accuracy of this analytical procedure was verified by analysis of a seawater reference material (GEOTRACES GS [BATS]), for which good agreement with the reported consensus value was obtained.

Picophytoplankton groups (*Prochlorococcus*, *Synechococcus*, and picoeukaryotes) were enumerated using a continuous SeaFlow instrument (Swallow et al. 2011). Additional discrete samples were collected alongside, fixed with glutaraldehyde (0.01% vol/vol final concentration), flash frozen, and stored at −80°C until analysis on land using an Influx flow cytometer (BD Biosciences, Seattle, WA) equipped with a 488-nm laser; emission at 692 nm (chlorophyll *a* fluorescence) and at 527 nm (phycoerythrin fluorescence) was triggered by forward light scatter. These two instruments provided comparable flow cytometric counts.

Sea surface temperature, salinity, and chloropigment fluorescence were measured using sensors that were integrated into the CTD rosette and sensors hooked into the ship’s underway uncontaminated seawater system.

**Nucleic acid extraction, nifH amplification, and sequencing**

Samples for DNA and RNA were extracted using the DNeasy and RNeasy Plant Mini Kits with the QIAcube instrument (Qiagen, Venlo, the Netherlands). DNA was extracted using the manufacturer’s protocol with additional steps of three flash freeze/thaw cycles, 2 minutes of bead-beating, and Proteinase K treatment, as described by Moisander et al. (2008). The final DNA elution volume was 100 μL. RNA was extracted according to the manufacturer’s protocol, with additional steps of bead-beating and DNase treatment (RNase-Free DNase Set, Qiagen, Venlo, the Netherlands). Complimentary DNA (cDNA) was generated with the Superscript III First-Strand Synthesis System (Thermo Fisher, Waltham), using the nifH3 gene-specific primer (Zani et al. 2000). Control reactions containing no reverse transcriptase enzyme were run for all samples to test for possible RNA contamination.

PCR was used to amplify *nifH* genes from DNA and cDNA samples using nested, degenerate *nifH* primers (Zehr and
McReynolds 1989; Zani et al. 2000). PCRs were performed using a Bio-Rad MyCycler thermocycler. The first round of PCR contained 1x Platinum Taq DNA polymerase PCR buffer (Thermo Fisher, Waltham), 250 μM deoxyribonucleotide triphosphates, 4 mM MgCl₂, 2 units Platinum Taq DNA polymerase (Thermo Fisher, Waltham), 0.5 μM nifH3 and nifH4 primers, and 3 μL DNA or cDNA, with a final reaction volume of 23 μL. This reaction was cycled at 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension of 72°C for 7 min. The second round of PCR had the same mixture components and thermocycling conditions (except the annealing temperature of 55°C) but included 2 μL of PCR product from the first reaction and primers consisting of gene-specific regions (nifH1 and nifH2) and common sequence tags, as previously described (Moonsamy et al. 2013). PCRs were performed in triplicate for each sample, and products were visualized using gel electrophoresis. For samples with three successful PCRs (visual nifH bands), products were pooled and used for sequencing. Negative control samples (eight no-template controls and two filter blanks) were included for sequencing despite the absence of visual bands after amplification. PCR amplicons were packaged in dry ice and shipped to the DNA Services Facility at the University of Illinois at Chicago. Here, a second PCR amplification was performed to add sequencing adaptors and barcodes, then amplicons were purified, pooled, and sequenced using MiSeq Standard v3, 2 × 300 bp paired-end sequencing according to the methods described in Gradoville et al. (2018). All raw sequence data are available from the NCBI (accession PRJNAS30276).

Bioinformatic analyses

Sequence reads from partial nifH amplicons were de-multiplexed using the Illumina MiSeq Reporter (MSR) version 2.5.1. Paired end reads were merged using Paired-End reAd mergeR (PEAR) (Zhang et al. 2014). Merged reads were screened for quality using the screen.seqs command in mothur (Schloss et al. 2009), and sequences with ambiguities or homopolymers of ≥2 bp were discarded. Primers were removed using the pcr.seqs command. Singleton sets were removed, operational taxonomic units (OTUs) were clustered at 97% nucleotide similarity, and a de novo chimera check was performed using Usearch v10 (Edgar 2010). The program Geneious v9.1.8 (www.geneious.com) was used to visualize a representative sequence from each OTU (assigned by Usearch) and OTUs with representative sequences containing frameshifts, stop codons, and non-nifH sequences were removed. Sequences that passed these quality control steps were subsampled to 6792 sequences per sample using the single_rarefaction.py command in the Quantitative Insights Into Microbial Ecology (QIIME) bioinformatic pipeline (Caporaso et al. 2010). All negative control samples contained ≤70 nifH sequences after quality control; these samples were removed during the rarefaction step and were excluded from further analyses.

The nifH OTUs were translated and classified via BLAST-p similarity to a custom reference database of 879 full-length nifH sequences derived from publicly available genomes (https://wwwzehr.pmc.ucsc.edu/Genome879/). Taxa were binned into previously assigned nifH groups, with the 1B (cyanobacterial) group further classified into the groups Trichodesmium, Crocosphaera, Cyanotheca, Nostocales (Nostoc/Trichomas), and UCYN-A. The group “other” was used to bin remaining 1B OTUs, OTUs with <85% similarity to any sequence in the reference database, and OTUs with equal similarity to database sequences from different nifH groups.

Quantitative PCR

The nifH genes from nine diazotrophic taxa were enumerated using qPCR assays with Taqman® chemistry. Primer/probe sets targeted the following diazotrophic groups: Trichodesmium (Church et al. 2005a), Crocosphaera (UCYN-B, Moisander et al. 2010), Cyanotheca-like organisms (UCYN-C, Foster et al. 2007), unicellular cyanobacterial group A1 (UCYN-A1, Church et al. 2005a), unicellular cyanobacterial groups A2 and A3 (UCYN-A2/A3, Thompson et al. 2014), Richelia associated with the diatom Rhizosolenia (Het-1, Church et al. 2005b), Hemiaulus associated with the diatom Rhizosolenia (Het-2, Foster et al. 2007), Calothrix associated with the diatom Chaetoceros (Het-3, Foster et al. 2007), and the gammaproteobacterial group “Gamma-A” (Langlois et al. 2008). All nine groups were quantified from the 2016 samples; UCYN-A1 and Trichodesmium were quantified from the 2017 samples.

Quantitative PCR assays were run in duplicate on an Applied Biosystems 7500 Real Time PCR System thermocycler. Details on the reaction mixtures, thermocycling conditions, preparation of standards, inhibition tests, and gene concentration calculations have been described previously (Goebel et al. 2010). The limit of detection (LOD) and limit of quantification (LOQ) for these qPCR assays have been previously determined to be 1 and 8 nifH copies per reaction, respectively (Goebel et al. 2010). Based on our DNA template additions (2 μL per reaction) and filtration volumes (800–4000 mL), this corresponds to LODs ranging from 13 to 63 nifH copies L⁻¹ and LOQs ranging from 100 to 500 nifH copies L⁻¹. For 2016 samples, qPCR abundances from the two size fractions were added together to estimate total (nonfractionated) abundances.

Community NFR and carbon fixation rates

Community (bulk) C fixation rates and NFRs were measured using the ¹³C method (Hama et al. 1983; Legendre and Gosselin 1997) and the ¹⁵N₂ tracer method of Montoya et al. (1996), with modifications to avoid problems associated with incomplete bubble dissolution (Mohr et al. 2010; Chang et al. 2019). All rate measurements were conducted using surface seawater (15 m) collected from predawn CTD casts. Measurements were performed at 12 stations during the 2016 cruise (~23.5–37°N) and at 8 stations during the 2017 cruise (~25.5–36°N). During both cruises, seawater was sampled into duplicate 4.4 L acid-washed, MilliQ-rinsed polycarbonate bottles. These bottles were spiked with ¹⁵N₂ (details varied between
cruises, see below) and 1.0 mL aliquots of a 47 mM 13C-labeled bicarbonate (99 atom% NaH13CO3, Cambridge Isotope Laboratories) using a separate, plunger-type syringe. Bottles were incubated on deck for 24 h in surface seawater-cooled incubators fitted with screening to mimic approximate in situ light conditions (~33% light level). Incubations were terminated by gentle vacuum filtration on precombusted 25-mm glass fiber filters (GF/F, Whatman). Time zero δ13C/δ15N natural abundance samples were also collected from each station where 15N2 assays were conducted; these samples were immediately filtered. Filters were frozen at −80°C and shipped to Oregon State University, where they were dried overnight at 60°C and packaged into tin and silver capsules. Particulate C, particulate N, and isotopic composition (δ13C, δ15N) were analyzed by continuous-flow isotope ratio mass spectrometry using a Carlo Erba elemental analyzer connected to a Thermo DeltaPlus isotope ratio mass spectrometer. All raw N yields were above 500 mV, which is the threshold where linearity effects are considered for this instrument.

For the 2016 cruise, the 15N2 tracer was added as 15N2-enriched seawater (Mohr et al. 2010) following the procedure described by Wilson et al. (2012). Briefly, to prepare the tracer, surface seawater from Station ALOHA (A Long-term Oligotrophic Habitat Assessment, 22° 45’N, 158° 00’W) was collected approximately 1 month prior to the cruise, filtered using a 0.2 μm in-line filter, and transported in the dark to the University of Hawaii. This seawater was degassed by bubbling with helium gas then applying vacuum pressure while heating and stirring. Next, ~13 mL 15N2 gas/L of degassed seawater was added and the sample was agitated to facilitate bubble dissolution. The 15N2 enriched seawater was transferred to glass serum bottles, which were crimp-sealed and stored at 4°C. At sea, after filling incubation bottles to capacity with collected seawater, 100–120 mL of seawater was removed and replaced with an equal volume of 15N2-enriched seawater. The 15N/14N ratio of the 15N2-enriched seawater tracer was measured via membrane inlet mass spectrometry (MIMS) according to Ferrón et al. (2016), yielding atom %15N2 values of 64.0 ± 4.5%, 74.2 ± 2.5%, 77.3 ± 1.9%, and 79.7 ± 2.7% for the four batches of 15N2-enriched seawater we produced. These values were used to calculate the initial atom %15N2 of incubation bottles. Measuring the 15N/14N ratio in the 15N2-enriched seawater instead of in the final sample may have been a source of error for the 2016 incubations (White et al. 2020).

For the 2017 cruise, in order to reduce the potential addition of nutrients from 15N2-enriched seawater (Klawonn et al. 2015; Chang et al. 2019), the 15N2 tracer was added by bubble injection and subsequent release. First, 4 mL of 15N2 gas (99 atom %, Cambridge Scientific, LOT # I-19197/AR0586172) was injected into each 4.4 L incubation bottle using a gas-tight syringe inserted through a silicone septum on the bottle cap. Incubation bottles were then rotated on a customized plankton wheel for 15 min at 17 rpm to facilitate 15N2 gas equilibration. Next, the cap was opened to release the residual 15N2 bubble, and a 1 mL of sample was removed and transferred to a pre-evacuated Exetainer® pre-loaded with 85% phosphoric acid for later analysis of the isotopic composition of dissolved inorganic C. A second subsample was transferred to a 20 mL serum vial using a peristaltic pump and a tube extending to the bottom of incubation bottles. This sample was collected for later measurement of the initial atom % of N2 via MIMS as per Ferrón et al. (2016), which averaged 2.66 ± 0.47% among all incubation bottles. The incubation bottle was then filled to capacity with seawater from the sampling site, capped, and incubated as described above.

15N2 fixation rates were calculated as described by Montoya et al. (1996). Limits of detection (LOD) and minimum quantifiable rates (MQRs) for N2 fixation measurements were calculated for each water mass, as described by Gradoville et al. (2017) (Table S1).

UCYN-A cell-specific NFRs

During the 2017 cruise, subsamples from community NFR incubation bottles were preserved in order to measure single-cell NFRs by the cyanobacterium UCYN-A. Immediately prior to filtration, 95 mL from both the 15N2 incubation bottles and the time zero 13C/15C natural abundance bottles were subsampled into amber bottles and fixed with 5 mL of 37% filtered formaldehyde. Fixed samples were stored at 4°C for up to 48 h, then gently vacuum-filtered onto a 25 mm, 0.6 μm pore size Isopore polycarbonate filter (EMD Millipore, Hayward) with a 25 mm, 0.8 μm pore cellulose acetate backing filter (Sterlitech, Kent). Filters were rinsed, dried, and stored at −80°C.

UCYN-A associations were visualized using a double-catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) assay. This assay involved two separate hybridizations, one to label UCYN-A, using the oligonucleotide probe UCYN-A 732 and helper oligonucleotides Helper A-732 and Helper B-732 (Krupke et al. 2013), and one to label the haptophyte host, using the probe UPRYM69 and helper oligonucleotides Helper A-PRYM and Helper B-PRYM (Cornejo-Castillo et al. 2016). The CARD-FISH assay was performed as described by Cabello et al. (2016), but without the use of competitor oligonucleotides. After the hybridizations, filter pieces were washed in MilliQ to remove mounting media, and cells were transferred to conductive, gridded silicon wafers (Pelco SFG12 Finder Grid Substrate, Ted Pella, Redding) by wetting filter pieces with 10 μL of MilliQ, placing the filter pieces face down on the silicon wafers, freezing at −80°C, and quickly removing the filter pieces while the sample was still frozen. UCYN-A1 associations were identified, imaged, and mapped (see Dekas and Orphan 2011) using a Zeiss Axioplan epifluorescence microscope (Santa Cruz, CA). Only intact associations containing fluorescent label for both the host and symbiont were used for analyses. Additionally, only those symbioses with diameters <4 μm were analyzed in order to target UCYN-A1 associations and not the larger UCYN-A3 associations (which were rare in our samples).

The C and N isotopic compositions of UCYN-A associations were measured using nanoscale secondary ion mass
Table 1. Ranges of physical, chemical, and biological conditions of seawater sampled from stations in the NPSG and the NPTZ during the 2016 and 2017 cruises.

| Cruise          | Region | Number of stations | Latitude (° N) | SST* (° C) | SSS† | N + N (μmol L⁻¹) | PO₄ (μmol L⁻¹) | Chl‡ (mg m⁻³) | Fe§ (nmol kg⁻¹) |
|-----------------|--------|--------------------|----------------|------------|-----|----------------|---------------|---------------|----------------|
| April–May 2016  | NPSG   | 6                  | 23.5–29.7      | 19.8–24.0  | 35.1–35.3 | 8d⁻¹-0.002 | 0.02–0.05 | 0.10–0.11 | 0.26–0.51 |
|                 | NPTZ   | 8                  | 32.6–37.3      | 11.4–17.1  | 34.1–34.7 | 0.06–0.87 | 0.07–0.51 | 0.16–0.37 | 0.06–0.18 |
| May–June 2017   | NPSG   | 11                 | 25.8–32.3      | 19.8–24.2  | 34.8–35.3 | 0.001–0.01 | 0.03–0.05 | 0.04–0.08 | 0.11–0.31 |
|                 | NPTZ   | 18                 | 32.9–41.4      | 11.0–18.0  | 33.0–34.4 | 0.002–1.99 | 0.07–0.51 | 0.05–3.33 | 0.11–0.51 |

*ST indicates sea surface temperature.
†SSS indicates sea surface salinity.
‡Calibrated concentrations of chlorophyll a (2016) and chloropigment (2017) from CTD fluorometer.
§Bd indicates below detection limits.

spectrometry (nanoSIMS). A total of 79 UCYN-A associations were analyzed (10–12 cells per sample). All measurements were performed using a Cameca NanoSIMS 50L at the Stanford Nano Shared Facilities laboratory (http://snsf.stanford.edu) at Stanford University. Cells were located and presputtered with a large diameter beam of cesium ions (Cs⁺) for 2 min to remove impurities and implant Cs⁺ on the cell surface, and the Cs⁺ was focused to a <100-nm spot diameter. For the analysis, cells were rastered (16 keV with a current between 2 and 4 pA) over a 25 × 25 μm area with a dwelling time of ~1 ms per pixel, producing a 256 × 256 pixel image. Secondary anions of ¹²C¹⁴N⁻, ¹²C¹⁵N⁻, ¹²C⁻, ¹³C⁻, ¹⁶O⁻, ²⁸Si⁻, and ³¹P⁻ were collected simultaneously, along with a secondary electron image (1 AU). Thirty frames were imaged for each individual cell. The look@nanoSIMS software (Polerecky et al. 2012) was used to accumulate the plane images and draw regions of interest (ROIs) around UCYN-A associations using the look@nanoSIMS automatic thresholding feature (both host and symbiont were analyzed together as a single association). The ¹⁵N/¹⁴N ratio was calculated for each ROI (as ¹²C¹⁵N/¹²C¹⁴N), and cell diameters were estimated from the perimeter of each ROI, assuming a spherical shape.

NFRs by UCYN-A associations (both host and symbiont together) were calculated according to Montoya et al. (1996), with the modification that the particulate N concentration ([PN]) term was replaced with the N content per UCYN-A association. This term was estimated by calculating the cell volume (assuming a spherical shape), estimating cellular C content using a previously derived equation relating cellular C content to biovolume in haptophytes (Strathmann 1967; Krupke et al. 2015), and converting cellular C content to cellular N content assuming a molar C:N ratio of 6.3 for UCYN-A associations (Martinez-Perez et al. 2016). For the initial atom% PN term (T_initial), we used the mean value of 32 analyzed unenriched symbioses (0.38 ± 0.0066 atom% ¹⁵N). Detection limits for cell-specific rates were determined by setting a minimum change in atom% ¹⁵N through the incubation (T_final - T_initial), which was calculated as 3 times the standard deviation of the atom% ¹⁵N of unenriched T_initial cells (0.0199 atom%, n = 32 cells).

Statistical analyses

Model II linear regressions (ordinary least squares method) were used to test the relationships between the abundances of diazotrophic taxa and community NFRs, and between UCYN-A1 abundances and environmental variables. Regressions were performed using the lmodel2 package version 1.7.3 (Legendre 1998) in the program R (version 3.4.1; http://www.r-project.org/).

Results

Oceanographic conditions

We collected samples from two springtime meridional surveys through the NPSG and the NPTZ. Both cruises sampled strong gradients in physical, chemical, and biological features (Table 1). Sea surface temperature decreased gradually with increasing latitude, ranging from 11.4 to 24.0°C in 2016 and from 11.0 to 26.2°C in 2017. Sea surface salinity ranged from ~34.8 to 35.3 in the NPSG, then abruptly decreased at ~30.5°N to reach ~34.3 at ~33°N (Fig. 2). This surface salinity gradient
is indicative of the southern boundary of the subtropical frontal zone (Roden 1991) and encompassed the 18°C isotherm during both cruises. Here, we have used the second derivative of surface salinity as a delineation between the NPSG and NPTZ, corresponding to 30.7°N in 2016 and 32.8°N in 2017 (Fig. 2). Sea surface salinity decreased below 33.8 north of 38°N during the 2017 cruise, indicative of the subpolar frontal zone (Fig. 2, Roden 1991). The vertical structure of salinity supports our regional delineations: stations south of ~32°N had a saline surface layer, indicating the subtropical domain, stations north of ~38°N had a low-salinity surface layer, reflecting the influence of the subarctic domain, and stations between ~32°N and 38°N show a mixture of these two water types, indicating the transition zone (Supporting Information Fig. S1, Roden 1991). Temperature/salinity curves from NPSG stations reflect typical curves from the subtropical gyre, while stations in the NPTZ progressively show characteristics more similar to the subarctic gyre (Supporting Information Fig. S2).

### Table 1

| Population | NPSG | NPTZ |
|------------|------|------|
| UCYN-A1    |      |      |
| UCYN-A2    |      |      |
| UCYN-A3    |      |      |

### Fig. 3

Abundances of nifH genes and biogeochemical conditions during the 2016 transect (~158°W). UCYN-A1 nifH gene abundances (via qPCR) are presented in (a), with x's and triangles representing samples below the limit of detection or quantification, respectively. The relative abundances of diazotrophic taxa (via nifH gene sequencing) from two size fractions are shown in (b) and (c), with vertical dashed lines indicating samples for which no nifH genes were recovered. Concentrations of picophytoplankton (via discrete flow cytometry samples [squares] and continuous SeaFlow measurements, (d), 13C primary production rates (e), and nitrate+nitrite concentrations (f) are shown for biogeochemical context. The division between the NPSG and NPTZ is presented in the upper panel.
Inorganic nutrient and dissolved iron concentrations varied with latitude. Concentrations of N + N and SRP were low in the NPSG (≤10 and ≤55 nmol L⁻¹, respectively), and increased sharply in the NPTZ during both cruises (Table 1, Figs. 3, 4). The latitudinal trend for dissolved iron concentrations differed between the two surveys (Table 1). In the 2016 cruise, dissolved iron concentrations were highest in the NPSG (0.26–0.51 nmol kg⁻¹) and decreased steadily with latitude to a minimum of ~0.06 nmol kg⁻¹. In contrast, during the 2017 cruise, the highest dissolved iron concentrations were observed in NPTZ stations south of the subpolar frontal zone (0.20–0.51 nmol kg⁻¹), while concentrations were lower in the NPSG (0.11–0.31 nmol kg⁻¹) and in the subpolar frontal zone (0.11–0.41 nmol kg⁻¹) (P. Pinedo, unpublished data).

The community structure of picophytoplankton had strong latitudinal patterns. Concentrations of picophytoplankton were relatively stable among NPSG stations, with high concentrations of Prochlorococcus (114.3 ± 9.3 and 137.9 ± 18.9 cells µL⁻¹ in 2016 and 2017, respectively) and lower concentrations of...
Synechococcus (8.9 ± 2.3 and 3.2 ± 1.2 cells μL⁻¹ in 2016 and 2017, respectively) and picoeukaryotes (5.5 ± 2.6 and 2.5 ± 1.8 cells μL⁻¹ in 2016 and 2017, respectively) (Figs. 3, 4). The concentrations of Synechococcus and picoeukaryotes increased 5–10-fold at the southern edge of the NPTZ (30.5°N in 2016 and 32.8°N in 2017), reaching >35 cells μL⁻¹ for Synechococcus and >27 cells μL⁻¹ for picoeukaryotes in both years (Figs. 3, 4). In the northern portion of the NPTZ, Prochlorococcus dropped by two orders of magnitude at around ~35°N in both years and concentrations of Synechococcus decreased ~two-fold north of ~33.5°N in 2016 (18.4 ± 11.3 cells μL⁻¹) and ~39.5°N in 2017 (9.6 ± 6.8 cells μL⁻¹), while concentrations of picoeukaryotes remained relatively high (22.8 ± 6.0 and 10.8 ± 4.4 cells μL⁻¹ in 2016 and 2017, respectively).

Chloropigment fluorescence and ¹³C primary production varied strongly with latitude. Chloropigment concentrations were low (<0.1 mg m⁻³) in the subtropical gyre and increased with latitude across the NPTZ; the transition zone chlorophyll front (0.2 mg m⁻³) was observed at ~32°N in 2016 and at ~36°N in 2017 (Table 1). ¹³C fixation rates ranged from 0.2 to 1.8 μmol C L⁻¹ d⁻¹ during the 2016 cruise and from 0.3 to 4.0 μmol C L⁻¹ d⁻¹ during the 2017 cruise; rates increased with latitude across the NPTZ during both cruises (Figs. 3, 4). The highest ¹³C fixation rates were observed in the subpolar frontal zone during 2017 (the northernmost stations sampled in this study). Because ¹³C samples were not fumed to remove inorganic carbon, we have compared our ¹³C fixation rates to ¹⁴C measurements conducted on the same cruise (A. White, unpublished data). The two sets of measurements correlate well (R² = 0.96 and 0.93 for 2016 and 2017 cruises, respectively) and show similar latitudinal patterns in the magnitude of production estimates (data not shown).

**Diazotrophic diversity, abundance, and distribution**

**Diversity of nifH genes and transcripts**

The diversity of marine diazotrophs was assessed via high-throughput sequencing of partial nifH genes and transcripts. Four different types of samples were sequenced: small size-fraction (0.2–3 μm) 2016 DNA samples, large size fraction (>3 μm) 2016 DNA samples, nonfractionated (>0.2 μm) 2017 DNA samples, and nonfractionated 2017 cDNA samples. A total of 140 samples containing 227 OTUs remained after quality control procedures and rarefaction. These OTUs clustered with all four nifH gene clades (Zehr et al. 2003), with the majority (94%) of sequences assigned to groups 1B (cyanobacteria) or 1G (mostly gamma-proteobacteria) (Figs. 2, 3). The diazotrophic group with the highest relative abundance in all sample types was the symbiotic cyanobacterium UCYN-A.

In the 2016 cruise, nifH genes were successfully amplified from 26/32 small size fraction NPSG samples and from 28/32 large size fraction NPSG samples but were not amplified from NPTZ samples of either size fraction (absence of visual band after gel electrophoresis). Within the NPSG, nifH gene sequences from surface waters were numerically dominated by UCYN-A in both the small and large size fractions (Fig. 3). Other cyanobacterial groups included Trichodesmium, Crocosphaera, Cyanothece and members of the order Nostocales. Of non-cyanobacterial nifH sequences, the most frequently detected group in surface samples was the gamma-proteobacterial group 1G. This group includes OTUs that match qPCR primer/probe sets designed to target several uncultivated diazotrophic phylotypes, including the globally distributed group “Gamma-A” (Church et al. 2005a; Langlois et al. 2008; Langlois et al. 2015), as well as phylotypes previously observed in the South Pacific (ETSP-2 and Gamma-3, Halm et al. 2011; Turk-Kubo et al. 2014). The community composition of diazotrophs shifted with depth; for example, the noncyanobacterial nifH group 3 (putative anaerobes) and the cyanobacterium Cyanothece displayed the highest relative abundances near the base of the euphotic zone (Supporting Information Fig. S3). There were higher relative abundances of genes from large cyanobacteria (Trichodesmium and members of the order Nostocales), Cyanothece, Crocosphaera, and noncyanobacterial groups in the large size fraction (Fig. 3, Supporting Information Fig. S3).

During the 2017 cruise, nifH genes were successfully amplified from 52/54 NPSG samples and from 22/110 NPTZ samples. Within the gyre, nifH gene sequences from surface seawater were dominated by UCYN-A, followed by group 1G and Trichodesmium (Fig. 3). In the NPTZ samples for which the nifH gene was amplified, UCYN-A and group 1G again had the highest relative abundances. However, there were higher relative abundances of group 1G and other noncyanobacterial groups in the NPTZ than in the NPSG. Furthermore, while cyanobacteria other than UCYN-A (Trichodesmium, Crocosphaera, Cyanothece, and the members of Nostocales) were detected in most surface NPSG samples, these groups were rarely or never detected in NPTZ samples. Likewise, overall diazotrophic species richness was lower in the NPTZ than in the NPSG (Supporting Information Fig. S4).

The community structure of active diazotrophs was assessed in 2017 using RNA samples collected from surface seawater. nifH transcripts were amplified from all NPSG samples but were not amplified from any NPTZ samples. Within the NPSG, the diazotrophic community composition of nifH transcripts was similar to that of surface nifH genes, with the majority of transcripts belonging to UCYN-A. Transcripts belonging to other cyanobacterial groups (Crocosphaera, Cyanothece, and the members of Nostocales) and to group 1G were also present in NPSG samples (Fig. 4).

**Abundances of marine diazotrophs**

Quantitative PCR was used to enumerate the nifH genes of nine diazotrophic groups from surface seawater samples in order to assess the quantitative significance of dominant diazotrophic taxa. In the 2016 cruise, nifH genes were detected in the six NPSG stations but were below the limit of detection or quantification in all samples from the NPTZ. Within the NPSG, the most abundant group was UCYN-A1, whose...
Community 15N2 fixation rates varied with latitude during both cruises. Different methods were used to introduce the 15N2 tracer on the two cruises (see Methods); LOD ranged from 0.27 to 0.90 nmol N L−1 d−1 in 2016 and from 0.33 to 0.59 during 2017 (Supporting Information Table S1). During the 2016 cruise, surface NFRs were detected in 4/6 NPSG stations and ranged from 1.75 to 10.8 nmol N L−1 (Fig. 5). Rates were below LOD in two northern NPSG stations as well as in all stations within the NPTZ. The NFR at one NPSG station during the 2016 cruise was above LOD but below MQR; this rate was retained for analyses (Stn. 14, Supporting Information Table S1). During the 2017 cruise, surface NFRs were detected at all stations within the NPSG. Rates in this region ranged from 10.2 to 20.8 nmol N L−1 d−1, approaching the upper bound of the range of ~0–20 nmol N L−1 d−1 reported at

![Image](https://example.com/image.png)

**Fig. 6.** Community N2 fixation rates (NFRs) (a), UCYN-A1 nifH gene abundances (b), and UCYN-A cell-specific NFRs (c) during the 2017 cruise. X’s denote below limit of detection; triangles denote below limit of quantification. The division between the NPSG and NPTZ is presented in the upper panel.

Abundances ranged from 2.8 × 10^3 to 7.3 × 10^5 nifH gene copies L−1 and from below quantification limits to 9.7 × 10^5 nifH gene copies L−1 in the small and large size fractions, respectively (Figs. 3, 5). The groups UCYN-B, UCYN-A2/A3, UCYN-C, het-1, het-2, and Gamma-A were also detected at low abundances during both cruises, this manuscript primarily focuses on the patterns observed for UCYN-A1.

![Image](https://example.com/image.png)

**Fig. 7.** Secondary electron image (a) and 15N enrichment (b) of two UCYN-A associations (indicated with white arrows) from the NPSG during the 2017 cruise. Images were produced from nanoSIMS measurements of UCYN-A symbioses after natural communities had been incubated with 15N2 gas.

In the 2017 cruise, only UCYN-A1 and *Trichodesmium* nifH gene abundances were quantified. UCYN-A1 nifH genes were detected in surface samples from all NPSG stations, with abundances ranging from 7.8 × 10^4 to 1.0 × 10^5 nifH gene copies L−1 (Figs. 3, 6). UCYN-A1 abundances were below either the limit of detection or the limit of quantification for most surface NPTZ stations, with the exception of two stations at the southern edge of the NPTZ and the northernmost station, which all had low UCYN-A1 abundances (<5 × 10^4 nifH gene copies L−1). *Trichodesmium* nifH genes were detected in surface waters from 11/12 NPSG stations (2.8 × 10^3–2.8 × 10^5 nifH copies L−1) but were below limits of detection or quantification at all NPTZ stations (Supporting Information Table S2). Since diazotrophs other than UCYN-A1 were detected sporadically and at low abundances during both cruises, this manuscript primarily focuses on the patterns observed for UCYN-A1.
Stations ALOHA (Fig. 6, Böttjer et al. 2017). In the NPTZ, NFRs were lower (1.35–5.52 nmol N L−1 d−1) but above LOD at all stations in which the assays were performed. The ratio of N2 fixation to C fixation ranged from 3.1 to 4.8% in the NPSG and from 0.20 to 0.67% in the NPTZ (Supporting Information Table S3). No N2 fixation assays were performed at stations north of 36°N during 2017.

**UCYN-A cell-specific NFRs**

UCYN-A cell-specific NFRs were measured during the 2017 cruise using CARD-FISH and nanoSIMS (Fig. 7). CARD-FISH assays were performed using samples from all eight stations in which 15N2 incubations were conducted. UCYN-A associations were only located on those filters from the five NPSG stations, presumably due to low cell abundances in the three NPTZ stations where UCYN-A1 nifH genes were below the limit of quantification. Therefore, UCYN-A cell-specific NFRs in the NPTZ could not be assessed. Within the NPSG, NFRs of individual UCYN-A associations ranged from 3.09 to 22.8 fmol N cell−1 d−1; average cell-specific rates from each station ranged from 5.7 to 10.9 fmol N cell−1 d−1 (Fig. 6). These rates are within the ranges of cell-specific rates by UCYN-A associations of this ecotype (UCYN-A1) previously reported in the North Atlantic Ocean and the Arctic Ocean (Martinez-Perez et al. 2016; Harding et al. 2018). All measured cell-specific NFRs exceeded detection limits, which ranged from 0.07 to 0.13 fmol N cell−1 d−1.

**Correlations among UCYN-A abundances, NFRs, and ocean biogeochemistry**

Community NFRs correlated strongly with surface UCYN-A1 nifH gene abundances on both cruises ($R^2 = 0.99, p < 0.001$ and $R^2 = 0.69, p = 0.01$ on the 2016 and 2017 cruises, respectively) and did not correlate significantly with the nifH gene abundances of any other quantified diazotrophic group (Table 2). The UCYN-A cell-specific NFRs (measured in 2017 only) did not correlate with UCYN-A1 nifH gene abundances ($R^2 = 0.05, p = 0.73$) or with community NFRs ($R^2 = 0.005, p = 0.92$).

There were significant correlations between UCYN-A1 nifH gene abundances and environmental parameters (Supporting Information Table S4). Using data from both cruises, UCYN-A1 nifH gene abundances correlated with sea surface temperature ($R^2 = 0.72, p < 0.001$) and inversely correlated with dissolved iron concentration in 2016 ($R^2 = 0.73, p = 0.002$) but inversely correlated with dissolved iron concentration in 2017 ($R^2 = 0.72, p < 0.001$). When restricting regression analyses to each year separately, UCYN-A1 nifH gene abundances correlated with dissolved iron concentration in 2016 ($R^2 = 0.73, p = 0.002$) but inversely correlated with dissolved iron concentration in 2017 ($R^2 = 0.72, p < 0.001$). When restricting regression analyses to NPSG stations only, there were no significant correlations between UCYN-A1 nifH gene abundances and any measured abiotic environmental parameters. When restricting regression analyses to each year separately, UCYN-A1 nifH gene abundances correlated with dissolved iron concentration in 2016 ($R^2 = 0.73, p = 0.002$) but inversely correlated with dissolved iron concentration in 2017 ($R^2 = 0.72, p < 0.001$). When restricting regression analyses to NPSG stations only, there were no significant correlations between UCYN-A1 nifH gene abundances and any measured environmental parameter (Supporting Information Table S4).

**Discussion**

**Boundaries for diazotrophy within the North Pacific transition zone**

In the NPTZ, the southward Ekman transport of cold, nitrate-rich subarctic surface waters is generally assumed to favor the growth of large cell size, fast-growing phytoplankton over diazotrophs (e.g., Dutkiewicz et al. 2012). However, increasing reports of active marine diazotrophs in unexpected environments, including cold and high-nitrate waters (Shiozaki et al. 2017), show that the biogeography and environmental controls of marine N2 fixation need to be re-evaluated. Here, we present two springtime meridional surveys of diazotrophic abundance, diversity, and activity through the environmental gradients of the NPTZ. We found a clear boundary for diazotrophs near the subtropical frontal zone that defines the southern-most edge of the NPTZ. South of the front, nifH genes, transcripts, and NFRs were consistently detected, and north of the front, nifH genes were rare or absent and NFRs were low or undetected. Our study expands on previous observations of low diazotrophic abundances in the NPTZ (Church et al. 2008; Shiozaki et al. 2017) and raises questions about the environmental factors that restrict the abundance and activity of diazotrophs in the NPTZ yet retain diazotrophs as members of the rare biosphere.

The high sampling resolution during the 2017 cruise revealed a sharp diazotrophic boundary at ~33.5°N, north of

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**Table 2.** $R^2$ values from simple linear regressions of community NFR vs. nifH gene abundances of diazotrophic groups in surface seawater. Regressions were run using the full data set and also separately for NPSG stations only. During 2016 nine diazotrophic nifH phylotypes were quantified, while in 2017 only the two diazotrophic groups with the highest relative abundances (via nifH gene sequencing) were targeted. Significant correlations ($p < 0.05$) are highlighted in bold text. The groups Trichodesmium and het-3 were below the LOD or LOQ in all samples.

| Cruise | Target organism | $R^2$ value (all stations) | $R^2$ value (NPSG only) |
|--------|----------------|---------------------------|------------------------|
| 2017   | UCYN-A1        | 0.693                     | 0.380                  |
|        | Trichodesmium  | 0.428                     | 0.212                  |
| 2016   | UCYN-A1        | 0.988                     | 0.984                  |
|        | UCYN-B         | 0.173                     | 0.08                   |
|        | UCYN-A2/A3     | 0.239                     | 0.07                   |
|        | UCYN-C         | 0.152                     | 0.04                   |
|        | Trichodesmium  | (Below LOD or LOQ)        | (Below LOD or LOQ)     |
|        | het-1          | 0.100                     | 0.00                   |
|        | het-2          | 0.250                     | 0.09                   |
|        | het-3          | (Below LOD or LOQ)        | (Below LOD or LOQ)     |
|        | Gamma-A        | 0.164                     | 0.08                   |

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which abundances of UCYN-A decreased by orders of magnitude (Fig. 4). This coincided with a sharp increase in Synechococcus abundance. Temperature/salinity curves indicate the transition to a new water mass near this station (Supporting Information Fig. S2); thus, the abrupt shift in abundances of UCYN-A and Synechococcus likely reflects low mixing rates at the physical front between water masses. While our sampling resolution was coarser during the 2016 cruise, the northern boundary for UCYN-A was likewise near the subtropical frontal zone at the southern edge of the NPTZ.

The underlying drivers of the lower diazotroph abundances and NFRs north of this front are unclear. Much of this uncertainty stems from the fact that the dominant diazotroph in our samples was UCYN-A, an uncultivated cyanobacterium that lives in an unusual, poorly understood symbiosis with a prymnesiophyte alga (Zehr et al. 2016). While most aspects of UCYN-A ecology are unresolved, there is evidence that the ecological controls of this organism may diverge from those of other cyanobacterial diazotrophs. For instance, temperature is a known control of marine N2 fixation (Luo et al. 2014), and the temperature at 33.5°N during the 2017 cruise (~18°C) was near the lower thermal limit for Trichodesmium growth (e.g., Breitharth et al. 2007). Temperature has been predicted to restrict N2 fixation in the NPTZ (Wang et al. 2019), and may help explain the absence of certain cyanobacterial diazotrophs from the region. However, members of the same UCYN-A lineage dominant in our study (UCYN-A1) were recently shown to actively fix N2 in the Arctic Ocean at temperatures as low as 4.1°C (Harding et al. 2018). In our study, UCYN-A abundance correlated strongly with sea surface temperature across the full transect, but within the NPTZ, the highest UCYN-A nifH gene abundances were detected at the northernmost station, where temperatures were coldest. Thus, the reduced temperatures in the NPTZ relative to the NPSG do not explain the abrupt shift in UCYN-A between these two ecosystems; instead, the broad temperature tolerance of UCYN-A may enable this organism to remain rare but active in NPTZ waters.

The availability of fixed N is another environmental control for which the response of UCYN-A may diverge from responses of other cyanobacterial diazotrophs. Nitrate availability has been suggested to suppress diazotrophy due to the higher energetic cost of N2 fixation relative to nitrate uptake, direct inhibition, or elevated N : P ratios (Falkowski 1983; Ohki et al. 1991; Knapp 2012). However, short-term deck-board incubation experiments have shown that UCYN-A growth rates may increase following nitrate additions (Turk-Kubo et al. 2018). The effects of macronutrients on the UCYN-A symbiosis are not fully understood, especially in the context of long-term nutrient delivery experienced in situ. In our study, the northern boundary for UCYN-A was not coincident with measurable changes in macronutrient concentrations (Figs. 3, 4) or in N : P ratios (data not shown), and UCYN-A abundances did not correlate with N + N or N : P (Table S4). It is possible that potentially higher nitrate supply rates in the NPTZ may have supported the growth of nondiazotrophic phytoplankton over diazotrophic taxa including UCYN-A (e.g., high N : Fe or N : P supply ratio, Dutkiewicz et al. 2012; Ward et al. 2013). Top-down processes and/or physiological constraints on the prymnesiophyte host may also play an important role in limiting the abundances of UCYN-A in the NPTZ.

Although community NFRs in the NPTZ were lower than those we measured in the NPSG, NFRs were consistently detected across the NPTZ in the 2017 cruise (Fig. 6). This result agrees with the low NFRs predicted in the NPTZ by two separate models in a recent study by Wang et al. (2019) but contrasts with outputs of several global N2 fixation models that predict an absence of diazotrophy in the region (Deutsch et al. 2007; Ward et al. 2013). The lower N2 : C fixation ratios in the NPTZ observed here (Supporting Information Table S3) and previously (Shiozaki et al. 2017) imply that N2 fixation contributes a small fraction of new N to the euphotic zone and is likely of low biogeochemical significance to the region. Nevertheless, our results add to a growing literature documenting marine N2 fixation in temperate and high-latitude environments (e.g., Rees et al. 2009; Blais et al. 2012; Mulholland et al. 2012; Shiozaki et al. 2017, 2017; Harding et al. 2018).

There are several possible explanations for why diazotrophic genes and low NFRs were detected in the NPTZ in 2017 but not in 2016. First, the 2017 cruise was ~1.5 months later in year. The NPTZ exhibits extreme seasonality, so this moderate difference in the timing of the 2017 expedition corresponded to large shifts in macronutrient concentrations, pico-phytoplankton community composition, and 13C primary productivity (Figs. 3, 4). There were also differences in distributions of dissolved iron: during 2016, iron concentrations increased from north to south, but in 2017, the highest iron concentrations were observed in the NPTZ, likely driven by abnormally high inputs of aeolian dust (P. Pinedo, unpublished data). Since the availability of iron is a major constraint of marine N2 fixation, especially in the Pacific Ocean (Weber and Deutsch 2014), the higher iron concentrations in 2017 may have facilitated increased rates of diazotrophic growth and N2 fixation. Finally, differences in methodologies used on the two cruises could also contribute to the observed differences in the NPTZ. During 2016, DNA samples were size-fractionated and smaller filtration volumes were used, which likely decreased the likelihood of detecting rare nifH genes. Additionally, different methods were used to introduce the 15N2 tracer on the two cruises (see Methods), which could have contributed to observed differences in community NFRs. Still, despite the difference in the magnitude of rates and gene abundances, both surveys showed the same overall feature of low or undetectable nifH gene abundances and NFRs in the NPTZ.

It is not clear which diazotrophic taxa drove the observed NFRs in the NPTZ during the 2017 cruise. Diazotrophic species richness was low in the NPTZ (Supporting Information Fig. S4). UCYN-A nifH genes were recovered from stations farther north than those of other cyanobacterial diazotrophs (up to 41.5°N),
consistent with recent findings indicating that UCYN-A broadens the geographic range of diazotrophic cyanobacteria (Moisander et al. 2010; Harding et al. 2018). However, abundances of UCYN-A were below the limit of quantification in most NPTZ samples (<500 $nifH$ copies L$^{-1}$). Assuming the highest UCYN-A cell-specific NFR measured in the NPSG (10.9 fmol N cell$^{-1}$ d$^{-1}$), it would require $1.4 \times 10^{5}$ UCYN-A cells to account for the lowest community NFR measured in the NPTZ (1.35 nmol N L$^{-1}$ d$^{-1}$, Fig. 6). Thus, it is unlikely that this UCYN-A sublineage (UCYN-A1) was responsible for all, or even most, of community N$_2$ fixation in this region. Stations from the NPTZ also contained higher relative abundances of $nifH$ genes from noncyanobacterial diazotrophs, but few of these organisms exist in culture and their biogeochemical significance is not well understood (Moisander et al. 2017). Surprisingly, $nifH$ transcripts were not recovered from any NPTZ stations, and $nifH$ genes were not recovered from the NPTZ station with the highest NFR (Figs. 4, 6). Shiozaki et al. (2017) likewise reported the detection of N$_2$ fixation but not $nifH$ genes (via qPCR) in the NPTZ. Similar enigmatic N$_2$ fixation has been observed in the South Pacific (Turk-Kubo et al. 2014; Gradoville et al. 2017) and suggests that ecologically important groups of diazotrophs are not targeted using the current $nifH$ PCR primers (Comejo-Castillo 2017; Delmont et al. 2018).

Finally, it could be hypothesized that some diazotrophs detected in the NPTZ were advected horizontally from their habitat and may not be active in the NPTZ. A recent study by Cheung et al. (2019) found that diazotrophs are advected through the Kuroshio current, and that in particular, the UCYN-A1 lineage and noncyanobacterial diazotrophs can be advected large distances. While the NPTZ stations sampled in our study do not show temperature/salinity attributes that implicate Kuroshio extension source waters (Supporting Information Fig. S2), the Kuroshio Extension is the origin of the North Pacific Current that flows east through the NPTZ (Yasuda 2003; Hu et al. 2015) and we cannot rule out the possibility that UCYN-A or other diazotrophs were delivered by horizontal advection. Physical transport may likewise explain previous observations of cyanobacterial diazotrophs at high latitudes in the North Atlantic (e.g., Lipschultz and Owens 1996; Díez et al. 2012; Rivero-Calle et al. 2016).

**UCYN-A dynamics within the North Pacific subtropical gyre**

The NPSG is one of the best-studied habitats for marine diazotrophs. While early work in the NPSG focused on _Trichodesmium_ and heterocystous cyanobacterial symbionts of diatoms (Mague et al. 1974; Venrick 1974), the NPSG is now known to harbor the unicellular cyanobacterial groups UCYN-A, UCYN-B (_Crocospheara_) and UCYN-C (_Cyanothecaceae_-like organisms), as well as diverse noncyanobacterial diazotrophs (Zehr et al. 1998; Church et al. 2005a; Farnelid et al. 2011). However, most of our understanding of N$_2$ fixation in the NPSG comes from studies at Sta. ALOHA (Karl and Church 2014), and less is known about the spatial variability of diazotrophic communities and NFRs within the broader gyre (although see Church et al. 2008; Moore et al. 2009; Shiozaki et al. 2010, 2017; Villareal et al. 2012). In our study, UCYN-A dominated the $nifH$ gene and transcript sequences recovered from all surface NPSG samples. While UCYN-A is present year-round and throughout the euphotic zone at Sta. ALOHA (Church et al. 2009), our study adds to mounting evidence suggesting that this organism may dominate diazotrophic communities in the NPSG during springtime (Church et al. 2009; Böttjer et al. 2014; Gradoville et al. 2017).

A challenge of oceanographic surveys is the attribution of a community process, such as N$_2$ fixation, to specific organisms. The strong, positive correlation between UCYN-A $nifH$ gene abundances and community NFRs during both cruises (Figs. 5, 6) and the absence of such strong relationships for other diazotrophic groups (Table 2) suggest that UCYN-A was the primary diazotroph driving the observed community NFRs in the NPSG. A similar relationship between UCYN-A abundance and hydrogen production (a proxy for N$_2$ fixation) has been described in the tropical North Atlantic (Moore et al. 2018), suggesting a major contribution of UCYN-A to community N$_2$ fixation in that region. Additionally, during the 2017 cruise, we directly measured cell-specific NFRs of UCYN-A symbioses in five NPSG stations (Fig. 7). The small differences in cell-specific rates among stations and the absence of a relationship between cell-specific rates and community NFRs suggest that the variability in community NFRs in the NPSG was likely driven by differences in cell abundance rather than by differences in cell-specific activity.

We combined our measurements of UCYN-A cell-specific NFRs with qPCR-derived UCYN-A $nifH$ gene abundances to estimate that the bulk contributions of UCYN-A to community NFRs ranged from 9 ± 2 to 40 ± 18% (Supporting Information - Table S5). This approach has many potential sources of error, including the potential for qPCR $nifH$ gene abundances to underestimate or overestimate cell concentrations due to low DNA extraction efficiency (Bostrom et al. 2004) or polyploidy (Sargent et al. 2016). Furthermore, measuring the $^{15}$N enrichment of UCYN-A symbioses after 24 h does not account for the transfer of recently fixed N to other organisms, and the CARD-FISH protocol has been shown to dilute the heavy isotope signal within single cells (Musat et al. 2014), constituting two other potential sources of underestimation relative to community NFR measurements. Although the bulk contribution estimates should be viewed with caution, these estimates support the notion that UCYN-A contributed to community N$_2$ fixation in the NPSG. Large estimated contributions of UCYN-A to community N$_2$ fixation have also been reported in the North Atlantic Ocean and the Arctic Ocean (Martinez-Perez et al. 2016; Harding et al. 2018), further supporting the emerging global importance of UCYN-A to marine N$_2$ fixation.

The large potential contribution of UCYN-A to community NFRs in the NPSG points to a need for understanding the environmental controls of UCYN-A in this region. During
2017, UCYN-A nifH gene concentrations within the NPSG varied by a factor of ~13, but the mechanisms driving this observed spatial and/or temporal variability are unclear. UCYN-A abundance within the gyre did not correlate with environmental parameters expected to affect intrinsic growth, such as temperature and the concentrations of iron and macronutrients (Supporting Information Table S4). Thus, our findings may indicate that the differences in UCYN-A abundance among NPSG stations were driven primarily by top-down rather than bottom-up processes. There is evidence that top-down processes may play an important role in controlling UCYN-A abundance: UCYN-A nifH gene sequences have been observed in the guts of copepods (Scavotto et al. 2015), and dilution experiments in the NPSG indicate that UCYN-A are preferentially grazed over other diazotrophic taxa (Turk-Kubo et al. 2018). More work is needed in order to understand the environmental controls of UCYN-A and to validate the importance of top-down processes in controlling abundances of this globally abundant marine diazotroph.

Conclusions

We observed clear biogeographical patterns for diazotrophs in the North Pacific. Some diazotrophic groups, including most diazotrophic cyanobacteria, were restricted to the NPSG, while the geographical ranges of noncyanobacterial diazotrophs and of the cyanobacterium UCYN-A extended into the subarctic frontal zone. Our work demonstrates the importance of UCYN-A to N2 fixation in the NPSG, where UCYN-A abundance correlated strongly with community NFR, while cell-specific NFRs by UCYN-A were more uniform. It is less clear which organisms drive N2 fixation in the NPTZ, and whether bottom-up (e.g., nutrient supply ratios) or top-down processes are responsible for the sharp shift in diazotrophic abundances and rates observed between the two regimes. This study and others like it demonstrate the need for a better understanding of the ecological controls of uncultivated marine diazotrophs such as UCYN-A.

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Conflict of Interest
None declared.