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ARTICLE

# New insights into the distributions of nitrogen fixation and diazotrophs revealed by high-resolution sensing and sampling methods

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## Abstract

Nitrogen availability limits marine productivity across large ocean regions. Diazotrophs can supply new nitrogen to the marine environment via nitrogen ( $N_2$ ) fixation, relieving nitrogen limitation. The distributions of diazotrophs and  $N_2$  fixation have been hypothesized to be generally controlled by temperature, phosphorus, and iron availability in the global ocean. However, even in the North Atlantic where most research on diazotrophs and  $N_2$  fixation has taken place, environmental controls remain contentious. Here we measure diazotroph composition, abundance, and activity at high resolution using newly developed underway sampling and sensing techniques. We capture a diazotrophic community shift from *Trichodesmium* to UCYN-A between the oligotrophic, warm (25–29 °C) Sargasso Sea and relatively nutrient-enriched, cold (13–24 °C) subpolar and eastern American coastal waters. Meanwhile,  $N_2$  fixation rates measured in this study are among the highest ever recorded globally and show significant increase with phosphorus availability across the transition from the Gulf Stream into subpolar and coastal waters despite colder temperatures and higher nitrate concentrations. Transcriptional patterns in both *Trichodesmium* and UCYN-A indicate phosphorus stress in the subtropical gyre. Over this iron-replete transect spanning the western North Atlantic, our results suggest that temperature is the major factor controlling the diazotrophic community structure while phosphorous drives  $N_2$  fixation rates. Overall, the occurrence of record-high UCYN-A abundance and peak  $N_2$  fixation rates in the cold coastal region where nitrate concentrations are highest (~200 nM) challenges current paradigms on what drives the distribution of diazotrophs and  $N_2$  fixation.

## Introduction

Nitrogen ( $N_2$ ) fixation, conducted by a group of specialized microorganisms called diazotrophs, provides the largest

external nitrogen input into the ocean [1, 2]. Alleviating nitrogen limitation over a large portion of the global surface oceans,  $N_2$  fixation supports new production and net oceanic carbon uptake [3, 4]. Over geological time scales,  $N_2$  fixation is believed to compensate for nitrogen removal from denitrification and anammox [5]. The balance between these microbial processes influences oceanic productivity, export of carbon to the deep ocean and ultimately

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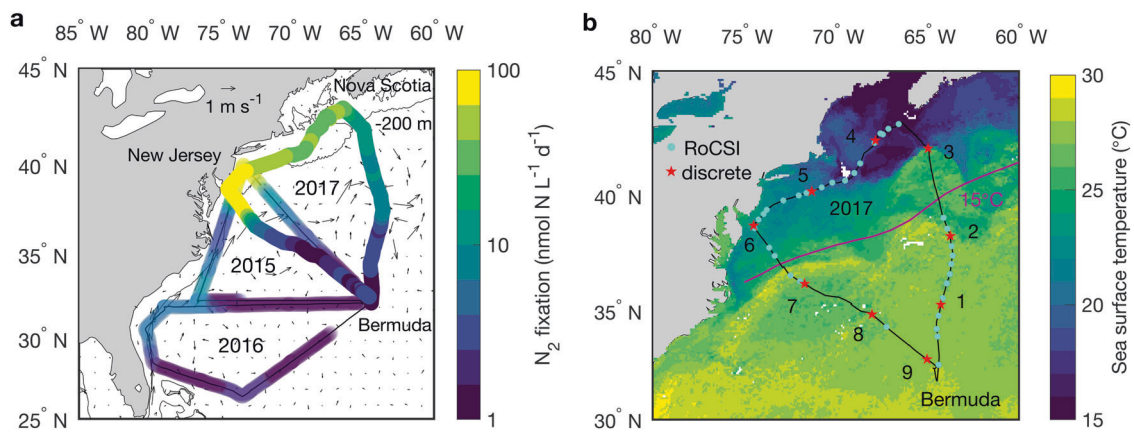
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**Fig. 1**  $N_2$  fixation rates and molecular sampling sites. **a**  $N_2$  fixation rates (colored points) observed in 2017 summer are compared to  $N_2$  fixation rates (transparent colored points) measured in 2015 and 2016 summers (obtained from Tang et al. [23]), superimposed over surface currents (arrows) derived from satellite-observed sea surface height (CMEMS) during the 10-day 2017 cruise. The strong surface currents show the rough position of the Gulf Stream and the solid black line depicts the 200 m bathymetry (ETOPO1). **b** Robotic

Cartridge Sampling Instrument—RoCSI samples (cyan circles) and discrete daily noon samples (number 1–9, red stars) are overlaid over satellite mean sea surface temperatures (MODIS-Aqua) observed during the 10-day 2017 cruise. The subpolar gyre and subtropical gyre are separated by the north wall of the Gulf Stream (purple line) which is defined as the intersection of the 15 °C isotherm with the 200 m isobaths [48].

atmospheric  $CO_2$  concentrations [6, 7]. Therefore, understanding the factors regulating  $N_2$  fixation and diazotrophs is important for evaluating and predicting changes to nitrogen and carbon cycling in the global ocean.

The dominant environmental controls on  $N_2$  fixation rates and diazotrophs in the global ocean remain elusive. Various factors have been proposed as controls, including temperature [8], phosphorus [9], iron [10], nutrient supply ratios [11], zooplankton grazing [12], and a combination of multiple factors [13, 14]. Specifically, warm and oligotrophic subtropical waters enriched in iron while depleted in nitrogen are traditionally recognized as hot spots of  $N_2$  fixation and diazotrophs [11, 15]. In the North Atlantic, phosphorus and iron availability are believed to be the major factors regulating diazotroph distribution and activity [9, 14, 16–18]. However, observations used in these analyses are mostly distributed in the central and eastern North Atlantic, leaving the western and temperate North Atlantic relatively undersampled. In addition, although pioneering studies on  $N_2$  fixation date back to the 1960s in the Sargasso Sea [19], much of the early work focused on large-size *Trichodesmium* colonies captured via plankton nets [20]. This sampling technique may overlook the potential role of small unicellular cyanobacterial (e.g., UCYN-A) and non-cyanobacterial diazotrophs, whose importance has become increasingly recognized [21, 22]. Thus, aside from *Trichodesmium*, the diazotrophic community in the western North Atlantic remains poorly characterized. Since  $N_2$  fixation is the cumulative product of the diazotrophic activity of different microorganisms, a more comprehensive characterization of diazotrophs is required to resolve

the environmental controls on  $N_2$  fixation in the North Atlantic.

To that end, our study builds on the survey of  $N_2$  fixation in Tang et al. [23], revisits the hot spots along the US Eastern Seaboard, and extends observations to higher latitudes (30–43° N) using a method we recently developed to estimate  $N_2$  fixation continuously [24]. We couple these measurements with a newly developed high-frequency autonomous microbial molecular sampling approach to contextualize these rates with diversity and gene expression patterns of  $N_2$ -fixing organisms (Robidart et al., unpublished). This unprecedented combination of high-resolution  $N_2$ -fixation observations and molecular sampling allows us to capture and characterize episodic diazotrophic blooms and evaluate their environmental controls.

## Materials and methods

### Sample collection and analyses

During a 10-day cruise between July 29 and August 7, 2017 traveling north from Bermuda to the Nova Scotia coast, then south to the New Jersey coast and finally back to Bermuda (Fig. 1), surface seawater (~5 m) was continuously pumped via a trace metal clean tow-fish system (GeoFish, manufactured by University of California Santa Cruz) into the laboratory aboard the *R/V Atlantic Explorer* [25]. This seawater was used for nutrient, trace metal, and microbial molecular sample collection and for  $N_2$  fixation incubations. Nutrients (nitrate + nitrite and phosphate)

were sampled in acid-washed 15 ml polypropylene tubes (Falcon, Corning, USA) and immediately preserved in a  $-20^{\circ}\text{C}$  freezer. Nutrients were then analyzed colorimetrically with an automatic nutrient analyzer on land [26, 27]. The detection limits for nitrate + nitrite and phosphate were  $0.01\ \mu\text{M}$  and  $0.014\ \mu\text{M}$ , respectively. The trace metal samples were collected directly in a laminar flow hood within a custom-built clean bubble. Samples were filtered through  $0.2\ \mu\text{m}$  cartridge filters (Sartobran 300, Sartorius, Germany) into 60 ml LDPE bottles cleaned following GEOTRACES protocols [28]. Samples for dissolved manganese (DMn), copper (DCu) and iron (DFe) were acidified on board with HCl (ultrapure, Merck), then stored at ambient temperature and finally analyzed by SF-ICP-MS back in the lab on land [29]. A detailed description of the trace metal sampling and analyses can be found in Tang et al. [23].

### Measurement of $\text{N}_2$ fixation, primary production, and net community production rates

Surface  $\text{N}_2$  fixation rates shown in Fig. 1a were measured continuously underway via Flow-through incubation Acetylene Reduction Assay by Cavity ring-down Spectroscopy (FARACAS) [24]. Briefly, acetylene ( $\text{C}_2\text{H}_2$ ) is dissolved into filtered seawater and is then mixed with surface seawater collected continuously via a tow-fish, reaching a 10%  $\text{C}_2\text{H}_2$  saturation. The mixed seawater is pumped into a 9 L flow-through incubation chamber at  $\sim 100\ \text{ml min}^{-1}$  leading to a 90 min residence time for the flow-through seawater. After incubation, the ethylene ( $\text{C}_2\text{H}_4$ ) from the incubated seawater is extracted by a bubble column reactor using a  $\sim 35\ \text{ml min}^{-1}$   $\text{C}_2\text{H}_4$ -free stripping gas and then measured by cavity ring-down spectroscopy (CRDS). CRDS measures  $\text{C}_2\text{H}_4$  concentration every few seconds at high precision. Bypassing the incubation chamber every few hours,  $\text{C}_2\text{H}_4$  background in the mixture of  $\text{C}_2\text{H}_4$  tracer and seawater is determined. The  $\text{C}_2\text{H}_4$  concentration difference between incubation and bypass can be used to calculate the near real-time  $\text{C}_2\text{H}_4$  production rates. Finally, the  $\text{C}_2\text{H}_4$  production rates are converted to  $\text{N}_2$  fixation rates using a 4:1 ratio. The detection limit of FARACAS is  $\sim 0.19\ \text{nmol N L}^{-1}\ \text{d}^{-1}$ , which is comparable to the discrete  $^{15}\text{N}_2$  addition method. Surface  $\text{N}_2$  fixation and primary production rates at 22 discrete stations were measured in triplicate using the dissolved  $^{15}\text{N}_2$  and  $^{13}\text{C}$  addition methods. The detailed description of the isotope incubation protocols can be found in Berthelot et al. [30].  $\text{N}_2$  fixation rates were considered as detected when the isotopic enrichment was higher than 0.00146 atom% [31]. This led to detection limit ranging  $0.11\text{--}2.23\ \text{nmol L}^{-1}\ \text{d}^{-1}$  (depending on the particulate N collected on filters). Using this criterion,  $\text{N}_2$  fixation was detected at 21 of the 22 stations.

Net community production (NCP) rates were concurrently estimated based on continuous dissolved  $\text{O}_2/\text{Ar}$  measurements by equilibrator inlet mass spectrometry [32]. Since  $\text{O}_2$  and Ar have similar solubility properties while Ar is biologically inert, Ar-normalized  $\text{O}_2$  can infer biological  $\text{O}_2$  production by removing physical influences on  $\text{O}_2$  concentration [33]. NCP represents the difference between primary production and community respiration, which can be used as a proxy for new production and/or export production. The contribution of  $\text{N}_2$  fixation to NCP was assessed by estimating their carbon fixation potentials with conversion factors of  $\text{C:N} = 106:16$  and  $\text{C:O}_2 = 1:1.4$  following Tang et al. [23]. Assuming constant C:N and C: $\text{O}_2$  ratios introduces uncertainties in our estimates. However, while variation in the conversion factors may affect the absolute contribution of  $\text{N}_2$  fixation to NCP, they have smaller effects on the spatial pattern.

### Molecular sampling

Over 200 molecular samples were collected at high frequency using a newly developed autonomous filtration system: Robotic Cartridge Sampling Instrument—RoCSI (Robidart et al., unpublished) (Fig. 1b). Briefly, around 2 L of seawater taken from the tow-fish system was filtered through a  $0.2\ \mu\text{m}$  Sterivex filter (Millipore, MA, USA) approximately every hour. The Sterivex filters were immediately preserved by adding RNAlater solution and stored at room temperature until transferred to a  $-80^{\circ}\text{C}$  freezer within 24 h. In addition to hourly sampling by RoCSI, we collected over 100 discrete molecular samples in triplicate nearly every 6 hours (i.e.  $\sim 4$  times daily) from surface underway line and from 10 CTD stations. Depending on particle concentrations and biomass,  $\sim 1.5$  L to over 4 L of seawater was filtered on each Sterivex filter using a peristaltic pump. The Sterivex filters were flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . These filters were later used for DNA and RNA analyses. Data obtained from 34 RoCSI samples as well as 9 discrete molecular samples collected at around noon local time daily are presented in this study (Fig. 1b).

### DNA and RNA extractions

DNA and RNA were extracted from the Sterivex filters using the AllPrep DNA/RNA Mini kit (Qiagen, MD, USA), including an initial bead-beating step (2 min at 30 Hz in a Qiagen TissueLyser) in RLT + buffer before following the manufacturer's instructions. DNA was digested from RNA samples using a RNase-free DNase set (Qiagen, MD, USA) and the RNA Clean & Concentrator kit (Zymo, CA, USA). DNA and RNA were eluted in  $50\ \mu\text{l}$  EB buffer and  $50\ \mu\text{l}$

RNase-free water respectively. The DNA and RNA extracts were quantified using the Qubit™ DNA/RNA HS Assay Kit (Invitrogen, CA, USA) according to the manufacturer's guidelines. In addition, RNA purity and integrity were checked using RNA chips on an Agilent 2100 Bioanalyser (Agilent, CA, USA).

### ***nifH* PCR amplification, amplicon sequencing, and sequence analysis**

Diazotrophic communities were characterized by sequencing *nifH* genes from nine discrete samples collected at around noon during the cruise (Fig. 1b). DNA extracted from stations 7, 8 and 9 were pooled together due to their low DNA concentrations. *nifH* genes were amplified using a nested PCR protocol [34], using second round PCR primers (*nifH1* and *nifH2*) containing Illumina tag sequences (Supplementary Table 1). *nifH* amplification and PCR conditions are described in Turk et al. [35]. Equimolar concentrations of *nifH* amplicons were pooled and sequenced using the Miseq-Illumina platform at the University of Southampton's Environmental Sequencing Facility. The sequencing data have been deposited in the NCBI Sequence Read Archive under accession number PRJNA554315.

We used QIIME1 to process the amplicon sequencing data [36]. Raw sequences were merged and quality filtered, whereby sequences with a quality score <20, expected number of errors >1, and reads <200-bp long were removed using USEARCH [37]. On average, 37,366 sequences per sample were obtained after quality control (min = 33,500 and max = 43,333). Sequences were further clustered into operational taxonomic units (OTUs) at 97% sequence identity after removing chimeras via UPARSE [38]. Representative sequences from *nifH* OTUs were assigned putative taxonomies using the BLAST resource on NCBI yielding a final dataset of 550 OTUs.

### **Quantification of *nifH* gene copies and *nifH* gene expression**

The abundance and expression of *nifH* genes from selected major diazotroph taxa were quantified using quantitative PCR (qPCR) and reverse transcription qPCR (RT-qPCR), including *Trichodesmium*, UCYN-A, and *Crocospaera* [39]. Complementary DNA (cDNA) was synthesized from purified RNA using the QuantiTect Reverse Transcription Kit (Qiagen, MD, USA) following the manufacturer's guidelines, using equimolar quantities (0.25 µM) of *nifH2* and *nifH3* primers [35] and 10 ng of purified RNA extract. Negative controls (no-RTs) were generated for each sample in parallel. Protocol of qPCR reaction conditions and thermocycling parameters are described in Robidart et al. [40].

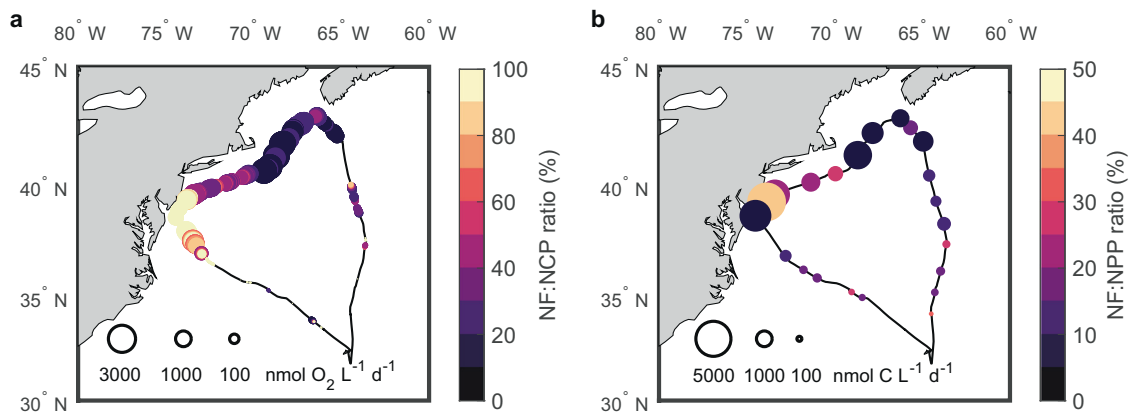
Briefly, the reactions were prepared in 96-well optical reaction plates with optical caps (Applied Biosystems, CA, USA) and run on a Real-time PCR System (Roche Light-Cycler® 96 Instrument, Germany) with the following thermocycling settings: 95 °C for 75 s, 45 cycles of 94 °C for 15 s and 59 °C for 30 s. The sample reactions (30 µl) were run in triplicate or duplicate and contained 15 µl of Accu-Prime™ SuperMix I (Invitrogen, CA, USA), 2.4 µl of 25 mM MgCl<sub>2</sub> solution, 9.86 µl of nuclease-free water (Ambion, CA, USA), 0.12 µl each of the forward and reverse primers (0.4 µM final concentration), 0.5 µl of FAM-labeled TaqMan probe (0.2 µM final concentration) and 2 µl of template DNA. Standard dilution series ranging from 10<sup>0</sup> to 10<sup>7</sup> gene copies were used for each reaction. Negative controls with 2 µl of nuclease-free water (Ambion, CA, USA) instead of the template DNA were run on each plate to check for contamination. No amplification was observed across all negative controls. Samples with no amplification are denoted as below the detection limit (35–133 copies L<sup>-1</sup>) while samples with only one or two amplifications in a triplicate run are denoted as detectable but not quantifiable. The efficiency of the qPCR and RT-qPCR reactions ranged from 98.6% to 107.2%, with an average of 103.7%.

### **RT-qPCR of phosphorus stress marker gene expression in diazotrophs**

To characterize the physiological status of diazotrophs, we designed primers and standards targeting the *pstS* gene for phosphorus stress for both *Trichodesmium* and UCYN-A (Supplementary Table 1). UCYN-A *pstS* genes were collected from GenBank and aligned. Sequences were highly conserved and qPCR primers were designed directly from these sequences. For *Trichodesmium*, a metatranscriptomic sequence database from the Atlantic Ocean was used to collect *pstS* genes. They were aligned with *pstS* genes from GenBank, and qPCR assays were designed based on conserved regions in the gene. Genes were amplified from cruise samples and cloned as described below.

*pstS* amplicons were ligated to the pGEM T-Easy vector and cloned into TOP10 cells (Invitrogen). Plasmids were purified from cultured clones and sequenced to confirm specificity. Sequenced plasmids were used as standards. Standard curves from 10<sup>0</sup>–10<sup>7</sup> copies were run on each plate with cDNA/DNA extracts from the cruise, and standard curve efficiencies were 103.2% (*Trichodesmium*) and 99.2% (UCYN-A). In addition to sequencing from the cruise samples, specificity was determined by comparing *nifH* copy numbers to *pstS* copy numbers (Supplementary Fig. 1), with the expectation that there should be a 1:1 relationship if primer sets are specific ( $n = 7$ ,  $r = 0.99$ ,





**Fig. 2 Contribution of  $N_2$  fixation (NF) to marine production.** Contribution of NF to **a** net community production (NCP) and **b** net primary production (NPP). The circle size represents the magnitude of NCP and NPP.

$q < 0.01$ ). *pstS* gene expression was quantified using RT-qPCR to evaluate potential nutrient limitation in diazotrophs collected near noon across large spatial gradients (stations 1–9). We normalized *pstS* transcription per liter seawater to the diazotroph *nifH* gene abundances per liter, and we reported both transcripts per liter and transcripts per *nifH* copy for *Trichodesmium* and UCYN-A.

## Results and discussion

### Distribution of $N_2$ fixation rates

Large spatial variations in surface  $N_2$  fixation rates were observed in the northwestern North Atlantic, ranging from below the detection limit ( $0.19 \text{ nmol N L}^{-1} \text{ d}^{-1}$ ) to over  $167 \text{ nmol N L}^{-1} \text{ d}^{-1}$  (Fig. 1). FARACAS  $N_2$  fixation (gross rate) measurements agreed strongly with the results of discrete  $^{15}\text{N}_2$  incubations (net rate) over a wide range of rates ( $n = 22$ ,  $r = 0.7$ ,  $p < 0.01$ ; Supplementary Fig. 2). While the warm and oligotrophic Sargasso Sea is traditionally believed to harbor conditions favorable to  $N_2$  fixation, we found relatively low  $N_2$  fixation rates across this region (geometric mean of  $3 \text{ nmol N L}^{-1} \text{ d}^{-1}$ ).  $N_2$  fixation can however still occasionally support a large fraction of local NCP and net primary production (NPP) due to lower NCP and NPP offshore (Fig. 2).

High  $N_2$  fixation rates were primarily located in cold subpolar North Atlantic and coastal waters from Nova Scotia to the Mid-Atlantic Bight (geometric mean of  $43 \text{ nmol N L}^{-1} \text{ d}^{-1}$ ). These waters were more nutrient rich than the adjacent subtropical waters. Intense  $N_2$  fixation in coastal waters has recently been reported by Tang et al. [23]. However, such high  $N_2$  fixation rates as we observed in the subpolar North Atlantic have rarely been detected in other subpolar oceans, deserving further exploration.

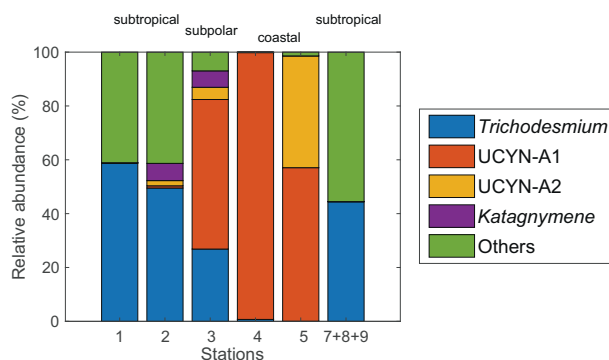
$N_2$  fixation has been reported at measurable rates in the Arctic Ocean [41, 42] and in temperate areas [43]. The elevated rates that we measured off the cold Nova Scotia coast (above  $25 \text{ nmol N L}^{-1} \text{ d}^{-1}$  in  $13^\circ\text{C}$  seawater) further extends the distribution of high  $N_2$  fixation rates (Fig. 1). Our observation of disproportionately large  $N_2$  fixation fluxes in subpolar and coastal waters confirms the high contribution of  $N_2$  fixation in North American continental shelf waters, which Mulholland et al. [44] estimated to be  $0.28 \text{ Tg N yr}^{-1}$  between Cape Hatteras and Nova Scotia. Estimates of N inputs from  $N_2$  fixation in the North Atlantic range between  $4.3$  and  $89.6 \text{ Tg N yr}^{-1}$  [45], and have mostly been derived from tropical and subtropical regions. Our results argue for the inclusion of coastal  $N_2$  fixation when estimating regional and global  $N_2$  fixation fluxes, and for the inclusion of  $N_2$  fixation, rarely considered in coastal studies [46, 47], when evaluating N budgets in coastal waters.

Intensive  $N_2$  fixation activity observed near the New Jersey coast reached the top 1% of  $N_2$  fixation rates ever reported in the literature [49]. The annual recurrence of these diazotrophic blooms (observed in 2015 and 2017 summers shown in Fig. 1a) makes this region one of the most significant  $N_2$  fixation hot spots in the global ocean, alongside the tropical North Atlantic [20], the western tropical South Pacific [50] and the coast of the eastern Arabian Sea [51, 52]. However, we only captured a snapshot of this diazotrophic hotspot in summer as opposed to observing the full pattern of seasonal variability. Recent studies have started to shed light on the temporal patterns of  $N_2$  fixation in the western North Atlantic coastal waters: the peak season of  $N_2$  fixation ranges from spring to fall depending on the location [44]. The mechanisms driving such seasonality remain unclear, warranting further study. Phytoplankton blooms in the Mid-Atlantic Bight are generally thought to be enhanced by nutrients supplied from

physical mixing, upwelling [53], and riverine runoff [54]. Here we demonstrated that  $N_2$  fixation could also be an important source of new nitrogen, contributing substantially to both NCP (>80%) and NPP (>30%) in this area (Fig. 2). We should however interpret the fraction of NCP fueled by  $N_2$  fixation with caution because of differences in the integration time scales of our methods. FARACAS estimates hourly and daily  $N_2$  fixation rates while  $O_2/Ar$ -NCP observations integrate productivity over a few days in this region. However, simultaneous measurements of  $N_2$  fixation and NPP in  $^{15}N_2$ - and  $^{13}C$ -addition incubations, shown in Fig. 2b, avoid the issue of reconciling different measurement time scales and corroborate the role of  $N_2$  fixation in the supporting coastal marine production.  $N_2$  fixation and marine production are thus closely linked, albeit with significant spatial variations in the western North Atlantic (the contribution of  $N_2$  fixation to NPP ranged from 5.6% to 42%). These results also underline the need to further investigate  $N_2$  fixation in poorly sampled and unexplored regions.

### Diazotrophic communities

During the transition from regions of low to high  $N_2$  fixation, the diazotrophic community shifted dramatically from *Trichodesmium* and non-cyanobacterial diazotrophs (e.g.,  $\gamma$ -24774A11) in the subtropical gyre to UCYN-A in the subpolar gyre and coastal areas (Fig. 3). *Trichodesmium* accounted for over 50% of *nifH* gene sequences at stations 1–2. In contrast, UCYN-A constituted a majority portion of *nifH* gene sequences at stations 3–5. One of the unidentified diazotrophs observed in the subtropical gyre matched an organism (accession number: AF016613.2 in NCBI) sampled within the same region reported in Zehr et al. (1998) [55]. Over our study area, low  $N_2$  fixation rates were observed when non-cyanobacterial diazotrophs were abundant. In addition, *Crocospaera* and *Richelia* were not detected in *nifH* amplicon sequencing. Previous studies have also reported low *Crocospaera* abundance in the North Atlantic [56]. Nevertheless, a portion of the diazotrophic community may not be amplifiable with the sequencing primers used in this study, as recent metagenomic deep sequencing has revealed diverse *nifH* phylogenotypes that would not be amplified with existing universal *nifH* primers [22]. Different spatial shifts of diazotrophs distribution have been previously observed in the North Atlantic [17, 57] and in the western Pacific [58, 59]. However, the factors driving such changes in diazotroph communities vary spatially. Therefore, it becomes critical to understand the conditions leading to the shift of diazotrophs and dominance of UCYN-A in coastal waters, as this organism is likely responsible for one of the highest marine  $N_2$  fixation rates ever reported.

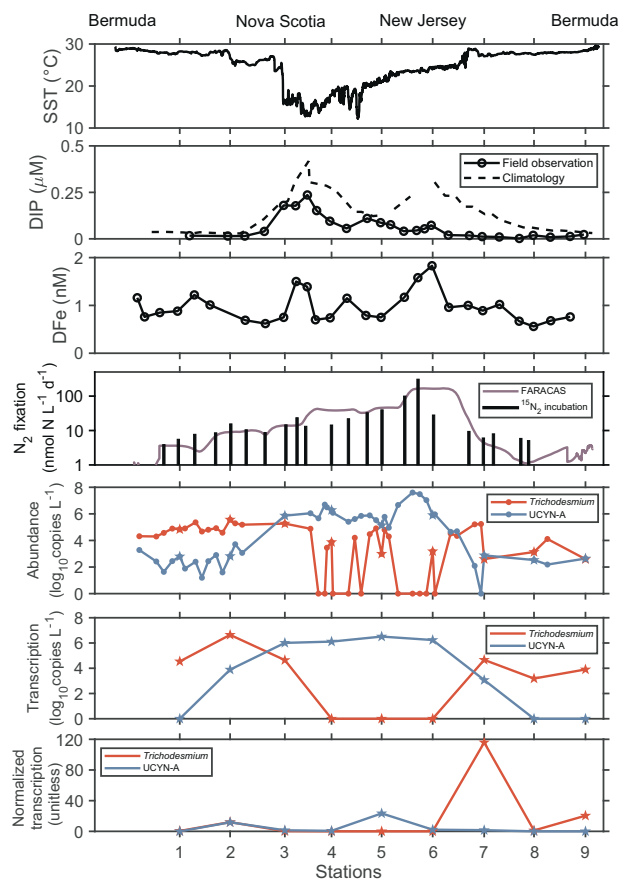


**Fig. 3** Relative abundance of *nifH* OTUs recovered from community DNA in discrete samples collected near noon daily shown in Fig. 1b. “Others” includes non-cyanobacterial diazotrophs, unidentified diazotrophs, and unassigned sequences in NCBI (e.g., AF016613.2; CP009517.1; CP020953.1; CP001649.1; KU248161.1).

### Factors controlling the distribution of $N_2$ fixation

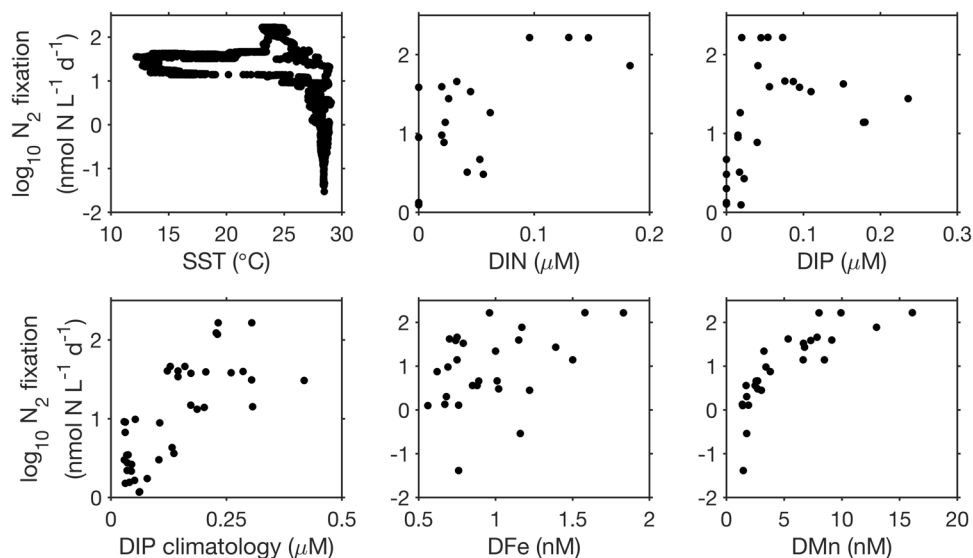
The spatial distribution of  $N_2$  fixation rates paralleled dissolved inorganic phosphorus (DIP) concentrations (Fig. 4 and Supplementary Fig. 3). Median  $N_2$  fixation rates were nearly 8 times higher when DIP concentration exceeded  $0.05 \mu M$  north of the Gulf Stream (Fig. 5). High DIP concentrations in the subpolar gyre and coastal waters may result from multiple sources including mixing and riverine runoff [60, 61]. In contrast, DIP was depleted in the western subtropical North Atlantic [62]. Our results are consistent with modeling work suggesting phosphorus limitation of  $N_2$  fixation in the subtropical North Atlantic [63]. Although the transport of excess phosphorus across the Gulf Stream has been hypothesized to sustain  $N_2$  fixation in the subtropical North Atlantic [64], we observed relatively low  $N_2$  fixation rates that are comparable to previous estimates [49] in the subtropical North Atlantic.

Iron is another potential factor limiting  $N_2$  fixation [16]. We measured relatively high DFe concentrations ( $0.56$ – $1.83 \text{ nM}$ ) over our research area (Supplementary Fig. 3), similar to previous observations [65]. These DFe concentrations were generally higher than the iron half-saturation constant for the growth of *Trichodesmium* [66]. While  $N_2$  fixation was highest in regions with elevated DFe (Fig. 5), a stoichiometric excess of DFe ( $Fe^* = DFe - R_{Fe} * DIP$ ; where  $R_{Fe} = 0.47 \text{ mmol DFe} : 1 \text{ mol DIP}$ ) [67] suggested that iron was not limiting within our study area (Supplementary Fig. 3), an implication in line with recent nutrient addition experiments in this region [68]. However, diazotrophs generally have a higher iron requirement compared to other phytoplankton, e.g., *Trichodesmium*’s iron use efficiency is fourfold lower than phytoplankton growing on ammonium [69]. The excess DFe at some coastal stations could disappear (and in fact become negative, indicative of iron limitation) if we increase the DFe:DIP ratio (i.e.  $R_{Fe}$ ) by approximately



**Fig. 4 Environmental properties,  $N_2$  fixation rates, diazotroph abundances, and *nifH* transcription patterns along the transect.** Environmental properties include sea surface temperature (SST), and concentrations of dissolved inorganic phosphorus (DIP) and dissolved Fe (DFe). Station numbers correspond to discrete sampling sites shown in Fig. 1b. Molecular samples collected by the discrete method and by RoCSI are depicted using filled stars and circles, respectively. *nifH* abundances and transcription data are presented in Supplementary Tables 2 and 3. *nifH* abundances and transcription below the detection limits are shown as 0 on the y-axis.

**Fig. 5 Spearman's rank correlation analyses between surface daily  $N_2$  fixation rates and environmental properties.**  $N_2$  fixation rates vs **a** SST ( $r = -0.75$ ,  $p < 0.01$ ), **b** surface DIN ( $r = 0.54$ ,  $p = 0.02$ ), **c** surface DIP ( $r = 0.60$ ,  $p < 0.01$ ), **d** surface DIP climatology in August ( $r = 0.72$ ,  $p < 0.01$ ), **e** surface DFe ( $r = 0.35$ ,  $p = 0.07$ ), **f** surface DMn ( $r = 0.92$ ,  $p < 0.01$ ).

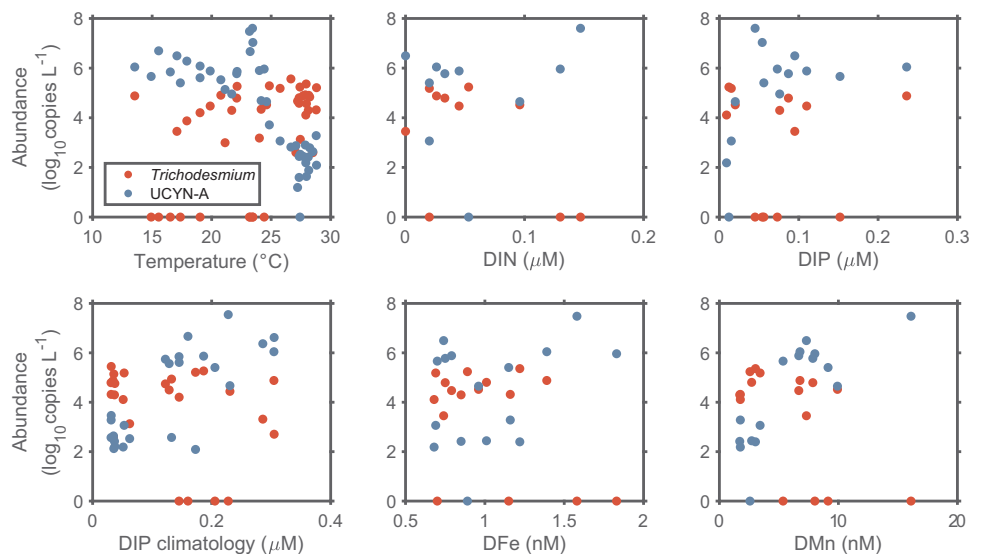


tenfold. Furthermore, iron bioavailability to microorganisms is linked to its organic complexation, rather than the total concentration. Most dissolved iron is present in strong complexes in this region [70]. However, the availability of this strongly complexed iron to diazotrophs is not well understood. Overall, we propose that a fraction of the excess phosphorus supply to the surface waters north of and near the Gulf Stream is assimilated by diazotrophs, supporting intense  $N_2$  fixation where iron is available. In contrast, the subtropical gyre harbors less  $N_2$  fixation due to phosphorus depletion (Fig. 4).

Other good predictors of  $N_2$  fixation in this study include some trace metals e.g. DCu and DMn, and chlorophyll-a concentrations ([Chl]) (Fig. 5 and Supplementary Fig. 4). Although high concentration of copper may be toxic to diazotrophs and phytoplankton [71], a positive correlation was found between  $N_2$  fixation rates and DCu. DCu concentrations along the transect ranged from 0.68 to 4.2 nM near the coast, typical of this region [72, 73]. However, the presence of very strong copper-complexing agents in seawater can reduce the available copper concentration to levels low enough to limit phytoplankton growth. For example, diatoms have been demonstrated to be co-limited by copper, iron, and light availability in the Northeast Pacific [74]. In addition, copper may limit denitrification activity of denitrifying bacteria [75]. Higher DCu concentrations could support denitrification in the coastal sediment and anoxic waters, removing bioavailable nitrogen and creating conditions favorable to  $N_2$  fixation. Manganese has been shown to be essential for some terrestrial  $N_2$ -fixing bacteria [76, 77], but the physiological requirement for manganese in marine diazotrophs is not well understood. Manganese shares some sources with DIP in the ocean, including lithogenic dust deposition, sediments, and rivers [78, 79]. A relationship



**Fig. 6** Relations between quantitative diazotroph *nifH* gene abundances and various environmental properties. *nifH* abundances below detection limits are shown as 0 on the y-axis.



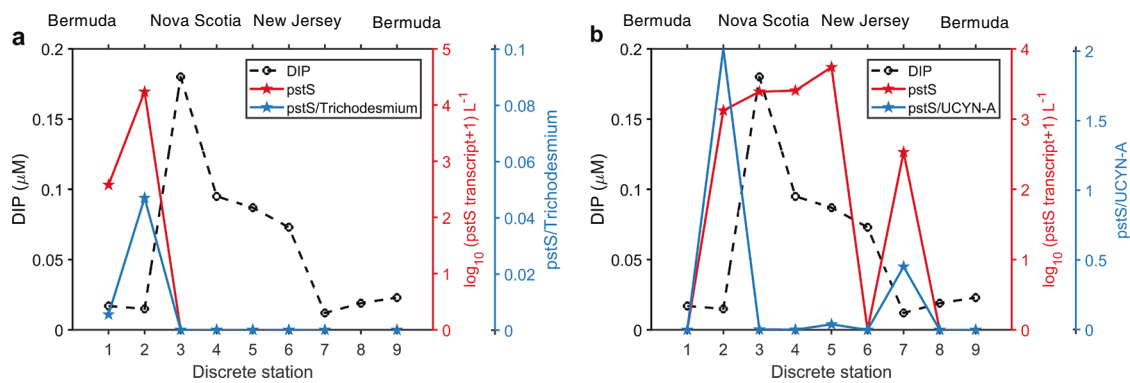
between DCu, DMn, and  $N_2$  fixation activity may simply reflect supplies of DIP and Fe or co-occurrence with other unrecognized factors beneficial to  $N_2$  fixation in our study area (Supplementary Fig. 5). Finally,  $N_2$  fixation rates were highly positively correlated to [Chl] (Supplementary Fig. 4). This result further emphasizes the presence of  $N_2$  fixation in highly productive regions [23, 80] in addition to in oligotrophic areas. However, it is unclear whether  $N_2$  fixation drives increases in [Chl] and marine production or whether  $N_2$  fixation is enhanced after depletion of nitrogen relative to phosphorus and release of organic matter by phytoplankton blooms.

### Factors controlling the distribution of diazotrophs

Absolute *nifH* gene abundances quantified by qPCR indicate that *Trichodesmium* was more abundant in the subtropical gyre while UCYN-A dominated in the subpolar and coastal oceans (Fig. 4), paralleling spatial patterns of their relative abundances shown in Fig. 3. Temperature may be the dominant factor driving such community shifts, with phosphorus availability acting as a secondary influence (Fig. 6). *Trichodesmium* reached maximum abundances ( $3.7 \times 10^5$  *nifH* copies  $L^{-1}$ ) at stations 2 and 7 near or within the Gulf Stream, where seawater was warm (28 °C) and DIP was low. Their *nifH* gene transcription was also high here compared to other regions (Fig. 4). This pattern may be driven by *Trichodesmium*'s preference for higher temperatures with an optimal growth temperature at 27 °C [81] and their ability to exploit diverse sources of dissolved organic phosphorus [82]. Our results support findings in earlier studies [83], suggesting that the Gulf Stream serves as a transport highway for *Trichodesmium*. This strong current could carry *Trichodesmium* from the tropical Atlantic and

along the Eastern American coast to temperate oceans (Supplementary Fig. 6), which may explain why *Trichodesmium* has been captured at high latitudes [84], e.g., near the British Isles [85]. Thus, the Gulf Stream may transport diazotrophs and nitrogen incorporated via  $N_2$  fixation in addition to its physical transport of nutrients [86]. Transport of *Trichodesmium* is also observed in the Kuroshio Current in the North Pacific [87], indicating the potentially widespread role of strong western boundary currents in transporting diazotrophs. Although *Trichodesmium* was also present in coastal and cold waters, their *nifH* gene transcription was undetectable (Fig. 4). This may indicate  $N_2$  fixation may not be the main N source for *Trichodesmium* in these regions. The activities and preferred nutrient strategies of *Trichodesmium* in the cold higher latitudes warrant further study.

In contrast, UCYN-A was more abundant in subpolar and coastal areas where temperature was low and phosphorus was more available despite the presence of nitrate. UCYN-A abundance estimated by *nifH* qPCR agrees with the results from quantitative 16S rRNA amplicon sequencing (Supplementary Fig. 7). UCYN-A abundance reached  $4 \times 10^7$  copies  $L^{-1}$  near the New Jersey coast (Fig. 4), among the highest abundances ever reported in the literature [49]. High transcription of the *nifH* gene of UCYN-A in coastal waters implies an active role in  $N_2$  fixation. Therefore, the highest  $N_2$  fixation rate across our transect was likely driven by this UCYN-A bloom. The hypothesized host of UCYN-A, *Braarudosphaera bigelowii*, also thrived in this area (Supplementary Fig. 8), with statistical co-occurrence patterns suggesting that it plays a key role in the regional microbial community (Wang et al., unpublished). The UCYN-A/*B. bigelowii* association has recently been reported in the waters off the coasts of



**Fig. 7 Evidence suggesting phosphorus limitation in diazotrophs.** Phosphorus stress patterns in **a** *Trichodesmium* and **b** UCYN-A at nine discrete stations shown in Fig. 1b. *pstS* transcription data are presented in Supplementary Table 2.

California [88] and Brazil [89], suggesting a widespread distribution.

UCYN-A was more abundant in waters with higher DIP concentrations ( $>0.05 \mu\text{M}$ ) (Fig. 6). This is consistent with Robidart et al. [40] at DIP concentrations below  $0.2 \mu\text{M}$  in the North Pacific but contradicts the findings of Stenegren et al. [58] who showed a negative correlation between UCYN-A abundance and DIP concentration globally. This discrepancy highlights our limited understanding of the yet-uncultivated UCYN-A group, motivating further physiological observations. The highest UCYN-A abundance observed during our cruise occurred in  $\sim 24^\circ\text{C}$  seawater, in line with its predicted optimal temperature [8] and observed niches in the eastern Atlantic [56]. However, our observations revealed high abundances ( $>10^6$  copies  $\text{L}^{-1}$ ) and expression ( $>10^5$  copies  $\text{L}^{-1}$ ) in colder environments with temperatures as low as  $13^\circ\text{C}$  near Nova Scotia (Figs. 4 and 6). This is also consistent with a recent report of UCYN-A actively fixing  $\text{N}_2$  in the cold waters of the Arctic Ocean [90]. These unexpected findings expand the temperature-based distribution of active diazotrophs. Some models may consequently be underestimating the  $\text{N}_2$  fixation budget by setting the threshold for  $\text{N}_2$  fixation based on the temperature ecophysiology of *Trichodesmium*, overlooking the role of other diazotrophs in temperate and polar regions. Similarly, the influence of nitrate on  $\text{N}_2$  fixation in models may need to be revised. Peak  $\text{N}_2$  fixation and UCYN-A abundance were found in coastal waters with nitrate and nitrite concentrations close to  $200 \text{ nM}$  (Figs. 5 and 6), reaching or exceeding half-saturation values of nitrate uptake in many marine phytoplankton cultures [91] and in natural phytoplankton assemblages from oceanic waters [92]. Several mechanisms have been hypothesized to explain  $\text{N}_2$  fixation in the presence of the reactive nitrogen [93–96] but the control of reactive nitrogen on UCYN-A and its hypothesized hosts deserves further investigation. Finally, iron enrichment may provide another advantage for UCYN-

A to flourish in coastal waters while the effect from co-occurrent manganese is poorly understood (Fig. 6).

Overall, the shift in diazotrophic community structure between subtropical, subpolar and coastal waters and its relation to corresponding changes of environmental controls was further confirmed and visualized by a redundancy analysis (Supplementary Fig. 9): temperature predominantly determined diazotroph composition, with phosphorus and iron playing regulatory roles.

### Expression patterns of an inorganic phosphorus stress marker gene in diazotrophs

We further evaluated the nutrient stress experienced by the dominant diazotrophs by examining the transcription of the inorganic phosphorus stress biomarker *pstS* [97]. *pstS* codes for a phosphate-binding subunit of the transporter PstS and has been used to show *Trichodesmium*'s phosphorus stress in the North Atlantic subtropical gyre [98]. *pstS* expression in *Trichodesmium* was only detected in the subtropical gyre with the highest transcription observed at station 2 (Fig. 7a). In contrast, UCYN-A exhibited high *pstS* transcription in subpolar and coastal seawaters where DIP concentration was higher (Fig. 7b). However, *pstS* transcription normalized to *nifH* gene abundance was significantly lower in these subpolar and coastal regions compared to the subtropical gyre, indicating that phosphorus limitation may be stronger in the subtropical regions where DIP was depleted. Organic phosphorus concentrations were not measured on this cruise, but may also play a role. Both *Trichodesmium* and UCYN-A may have highest inorganic phosphorus stress in the subtropical waters, but *Trichodesmium* are able to assimilate organic forms of phosphorus to mitigate phosphorus stress in the North Atlantic subtropical gyre [98]. In addition to assessing the phosphorus stress, future work should target marker gene expression indicative of iron limitation.

## Conclusion

By applying high-resolution biogeochemical and biological sampling techniques, we captured the large-scale distribution of  $N_2$  fixation and diazotrophs from the subtropical North Atlantic to the US East Coast, with contrasting patterns between *Trichodesmium* and UCYN-A, the dominant diazotrophs in this study. The overlooked high  $N_2$  fixation fluxes in the subpolar gyres and in coastal oceans could be significant contributors to global marine  $N_2$  fixation. Substantial variations in diazotroph abundance over short distances were observed thanks to our high-frequency sampling techniques, further demonstrating the patchy spatial distribution of diazotrophs. Further, record abundances of UCYN-A quantified here are largely attributed to the high-resolution sampling scheme detecting bloom conditions, which would have been less likely to be captured by the traditional discrete CTD sampling.

Strong gradients in physical and chemical factors enabled the clear attribution of ecological drivers for  $N_2$  fixation and diazotrophs. Dramatic changes in  $N_2$  fixation rates (from undetectable to the highest 1% of rates measured globally) and shifts in diazotrophic community structure were best explained by phosphorus availability and temperature gradients, respectively. Taken together, our results imply that the environmental controls on  $N_2$  fixation rates and diazotroph composition may be different, with the best predictors of diazotroph abundances varying across species. For example, UCYN-A's niches are distinct from other diazotrophs: high  $N_2$  fixation rates and UCYN-A abundance were unexpectedly observed in cold and nitrogen-present regions, challenging traditional assumptions and models about favorable conditions to diazotrophy. Therefore, modeling of  $N_2$  fixation ultimately requires observation and simulation of more diverse diazotrophic groups in addition to well-recognized *Trichodesmium*. Furthermore, deeper investigation of factors unobserved in this study, which may play important roles, such as top-down controls upon  $N_2$  fixation [12], would represent valuable avenues for future research. Nevertheless, our approaches for studying the ecophysiology of diverse diazotrophs and for conducting large-scale, high-resolution surveys over broad ocean regimes offer considerable potential for improving our understanding of  $N_2$  fixation and our ability to simulate  $N_2$  fixation in a changing climate.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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