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Evidence of limited N_2 fixation in the Southern Ocean

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Biological nitrogen fixation is an important source of new nitrogen, influencing ocean fertility and carbon uptake. While recently documented in Arctic waters, its role in the Southern Ocean remains uncertain. We measured nitrogen fixation along the Western Antarctic Peninsula and at Palmer Station over two austral summer months. Rates from $^{15}N_2$ assay were below conservative detection limits but detectable under less stringent detection thresholds. Continuous acetylene reduction assay provided further support. *nifH* gene sequencing identified Gammaproteobacteria as the dominating identified diazotrophs, while Epsilonproteobacteria contributed disproportionally to *nifH* expression when putative nitrogen fixation was highest. Combined with environmental observations, we hypothesize that vertical water mixing resuspended sediments into the water column and contributed to the limited nitrogen fixation. Given the sporadic and low rates, further research is needed to determine whether nitrogen fixation plays a minor role or represents an overlooked process with biogeochemical significance in the Southern Ocean.

Nitrogen is a fundamental element of life, present in biomolecules such as proteins and nucleic acids. In many regions of the world oceans such as oligotrophic regions, nitrogen availability limits photosynthesis and the biological uptake and sequestration of carbon dioxide (CO_2)[1]. Biological dinitrogen (N_2) fixation to ammonium is a major source of new nitrogen in the ocean[2] and can support up to half of new production in oligotrophic waters[1]. However, estimates of global N_2 fixation harbor substantial uncertainties, ranging from less than 100 to over 200 Tg N year^{-1}[2–4]. By comparison, global ocean denitrification estimates are ~400 Tg N year^{-1}[5]. Other sources of nitrogen, such as riverine input and atmospheric deposition, are not large enough to balance the nitrogen budget in the ocean[2]. The implication is that either the oceanic nitrogen inventory is decreasing (with implications for ocean fertility), or that nitrogen sources (sinks) are under-(over-) estimated. In order to address a potential missing source of nitrogen, researchers are actively looking for other biomes where N_2 fixation may be important. Until recently, N_2 fixation was believed to be conducted predominantly by cyanobacteria such as *Trichodesmium* living in warm tropical and subtropical waters[6]. However, recent studies have not only expanded the range of habitats over which N_2 fixation may be important, including coastal[7] and polar oceans[8], but also the microbial taxa who may be responsible for it[9–12]. In polar waters, most observations of N_2 fixation have been collected in the Arctic with a dominance of diazotrophs more eurythermal than *Trichodesmium* such as UCYN-A and cluster III *nifH* phylotypes, and with rates ranging from below detection limit to 17.2 nmol N L^{-1} d^{-1}[8,13,14]. In contrast, few observations are available in Antarctic waters. Traditional views would dictate that the cold and nitrogen rich waters of the Southern Ocean should not be conducive to N_2 fixation. However, low N_2 fixation rates have been reported in the high-latitude waters of the South Pacific and South Indian Oceans[15,16]. Recently, Shiozaki et al.[17] published evidence of N_2 fixation in eastern Antarctic waters with a particularly high rate (44.4 nmol N L^{-1} d^{-1}) at the ice-edge[17]. This high rate has recently been questioned by White et al. as it was calculated from an outlier and the observed UCYN-A abundance was not sufficient to support such a high rate[18]. More recently, N_2 fixation was reported during late summers in the coastal waters of Chile Bay at the Western Antarctic Peninsula (WAP)[19].

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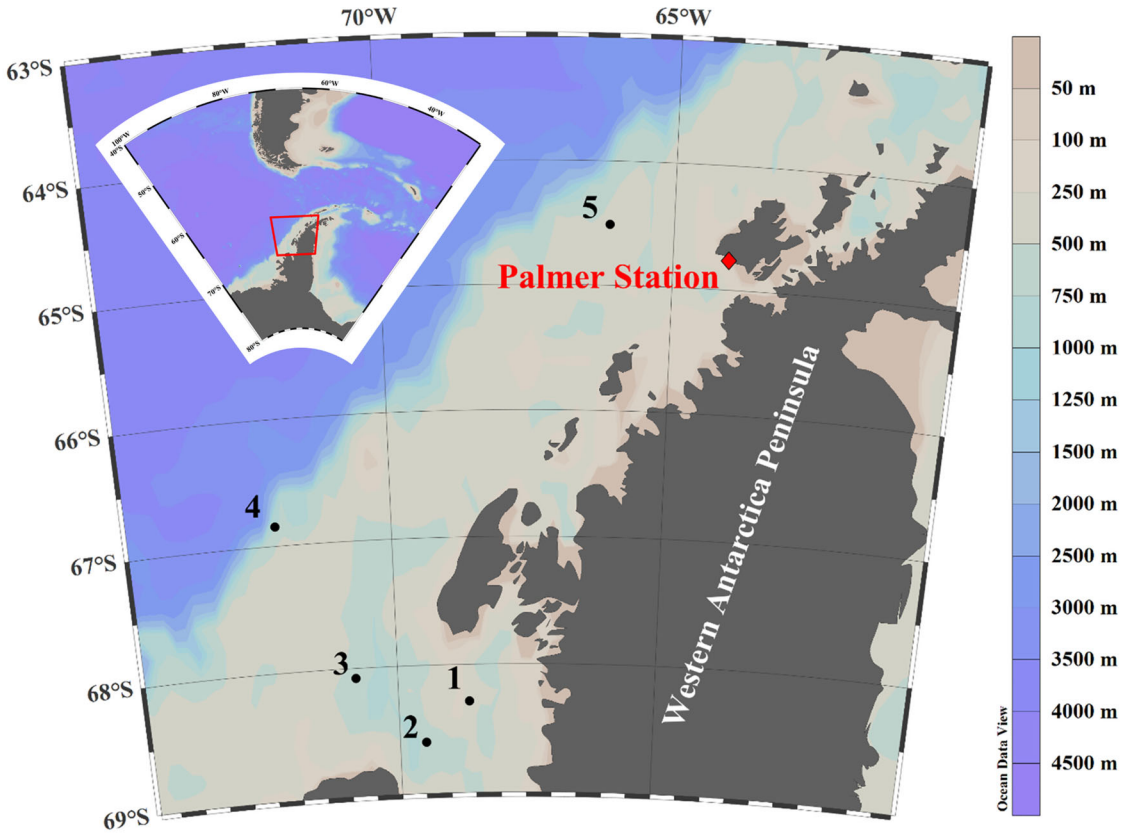

Fig. 1 | Map of the sampling region at the Western Antarctic Peninsula. Numbers 1-5 indicate discrete sampling stations from January to February in 2018. The red diamond shows Palmer Station, where time-series observations were conducted from January to March in 2019.

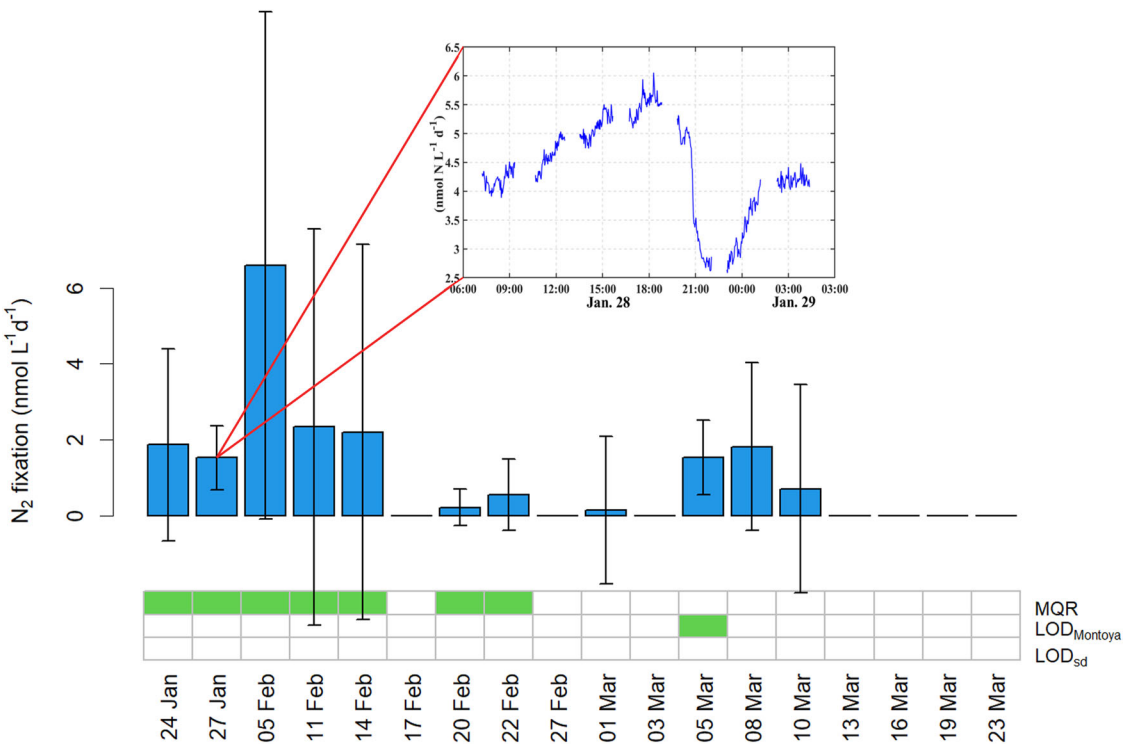

Fig. 2 | Nitrogen fixation rates measured at Palmer Station. Nitrogen fixation rates (nmol N L^{-1} d^{-1}) measured by $^{15}N_2$ incubation (blue bars) from January to March and by FARACAS (blue line) from Jan. 27th to 28th in 2019. The error bars show standard deviation of the triplicate incubations (except for duplicates on Feb. 11th). Rates above the MQR, $LOD_{Montoya}$, or LOD_{sd} are depicted with green marks.

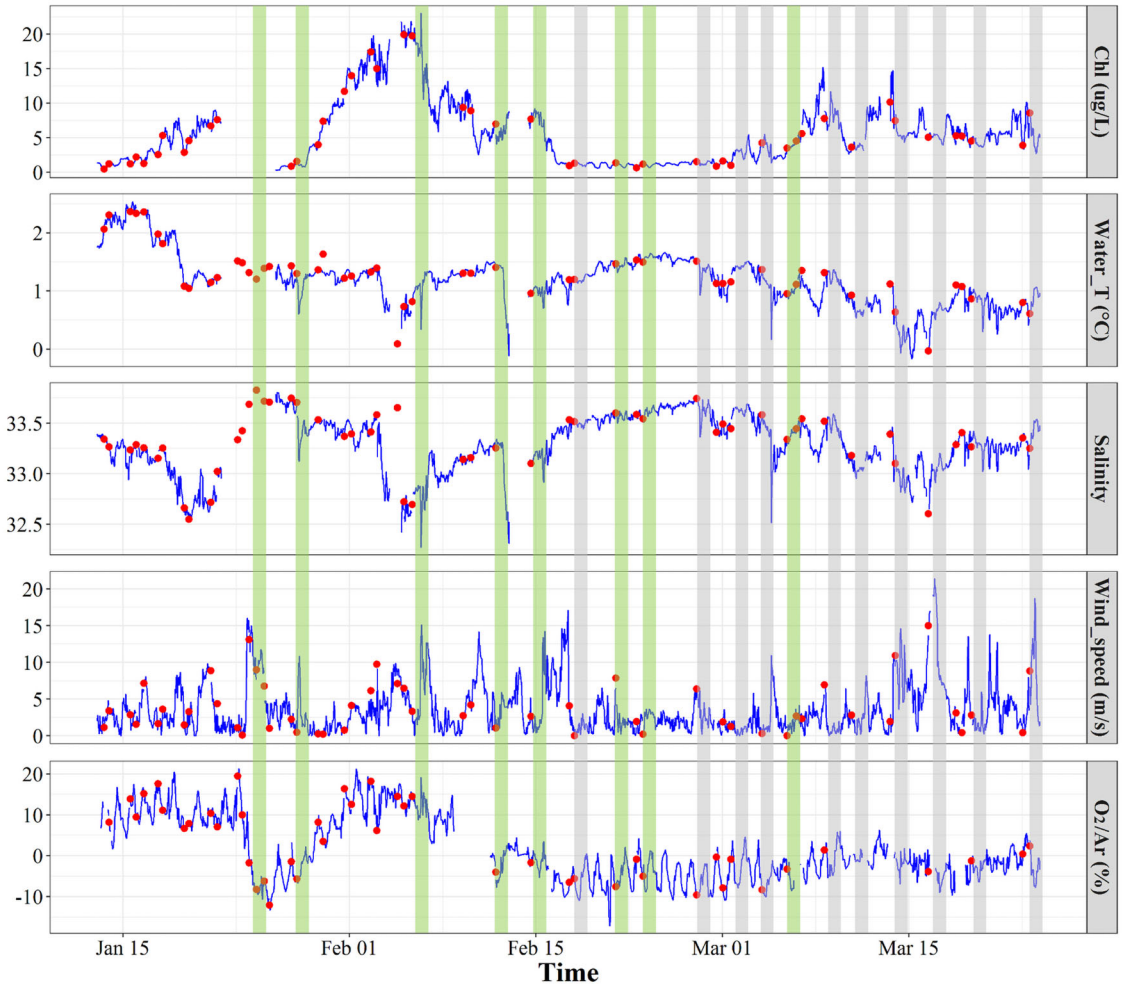

Fig. 3 | Times series of environmental parameters from Jan. to Mar. at Palmer Station in 2019. 1-h averaged chlorophyll, water temperature, salinity, wind speed and O_2/Ar ratio are shown by blue lines in each panel. Each red dot represents a 10-min average at the sampling time point for discrete DNA/RNA samples. The shaded area indicates the time periods when $^{15}N_2$ incubation experiments were performed and the signals were below (gray shaded) or above the MQR or the $LOD_{Montoya}$ (green shaded).

In this study, we provide evidence of potential but limited N_2 fixation rates in Antarctic waters. We focus our study on the Western Antarctic Peninsula (Fig. 1), a region which has experienced warming, sea ice retreat, and changes in phytoplankton community structure over the last decades[20–22]. We conducted a survey of N_2 fixation along the WAP shelf from January to February in 2018, and time-series observations at Palmer Station during the growing season from January to March in 2019. The rates measured by $^{15}N_2$ incubation method were below the most stringent detection limit at all offshore stations in 2018 and from February to March at Palmer Station in 2019. However, some measured N_2 fixation rates were above less stringent detection limits suggesting potential N_2 fixation. This was corroborated by estimates with our high-frequency instrument FARACAS, a flow-through method that measures N_2 fixation continuously based on acetylene reduction[23]. While Shiozaki et al.[17] found a dominance of UCYN-A in *nifH* gene expression[17], our *nifH* gene amplicon sequencing results showed that Proteobacteria were likely the dominant diazotroph in our study region. Combined with the analysis of environmental properties, we hypothesize that the diazotrophs were associated with the sediments. In our study, the potential N_2 fixation fluxes were sporadic and low. Additional work is needed to determine if N_2 fixation in Antarctic waters is an interesting biological process with little biogeochemical implications, or if our limited observations encountered an important new biome of N_2 fixation.

Results and Discussion

Measuring low N_2 fixation rates

Due to relatively low fluxes compared to the available pool, measuring N_2 fixation in the ocean using $^{15}N_2$ assays has long been a methodological challenge and a matter of discussion, with methodological bias toward potential over-estimation[24] or under-estimation[25–27]. More recently, Gradoville et al.[28] pointed out the need to qualify systematically the detection limits when using $^{15}N_2$ assays in particular when reporting low rates to avoid interpreting false positives[28]. They proposed two ways to qualify the detection threshold. The first one is to propagate all the experimental uncertainties associated with each parameter used for the determination of the rate, defined as to the minimal quantifiable rate (MQR)[29]. The second one is to define a minimum ^{15}N enrichment between the start and end of the incubations of 4‰ (0.00146 atom%) according to Montoya et al.[30], referred here as the limit of detection ($LOD_{Montoya}$). In addition, we defined an alternative limit of detection (LOD_{sd}) using three times the standard deviation of the initial ^{15}N abundance in our samples as a threshold. In our experiments, while the isotopic composition of the particulate organic N (PON) was measured in triplicate after the incubations, the initial value was measured in monoplicate only. In order to define the LOD_{sd}, we modeled the standard deviation of the initial ^{15}N abundance in PON using the observed trend over time at Palmer station (see the "Method" section for more details).

N_2 fixation was detected at seven, one, and none of the time points out of 18 at Palmer Station depending on whether we use the MQR, $LOD_{Montoya}$,

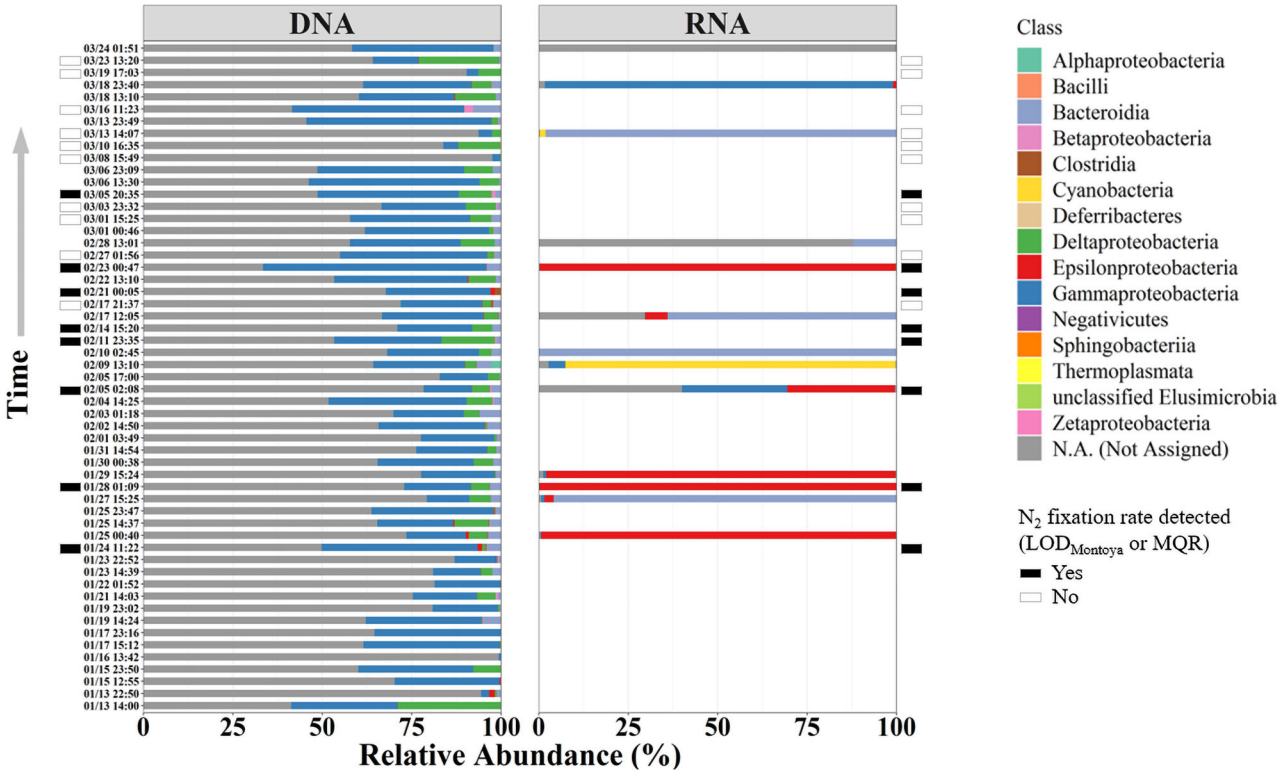

Fig. 4 | Time series of diazotroph community composition at class/phylum level, identified from *nifH* sequencing. The left and right panels show DNA and RNA, respectively. Only samples with more than 1000 reads are included. The black and white squares indicate samples for which N_2 fixation rates were above or below detection thresholds, respectively.

and LOD_{sd} as thresholds, respectively (Fig. 2). Interestingly, the only sample above the $LOD_{Montoya}$ is not above the MQR. This discrepancy is due to the fact that only the ^{15}N enrichment difference between the beginning and the end of the incubation in the particulate matter is used to calculate LOD while all the parameters used in the calculation of the N_2 fixation rate are included to calculate MQR. Nevertheless, since the N_2 fixation rates presented here were always close to the different detection limits used (Supplementary Data 1 and 2), we interpret them as potential N_2 fixation rates.

Time series of nitrogen fixation measurements and other environmental factors

$^{15}N_2$ fixation rates were above the MQR between Jan. 24th and Feb. 22nd (with the exception of Feb. 17th) with rates ranging 0.21–6.59 nmol L^{-1} d^{-1} when detected and then remained below the MQR for the rest of the studied period. N_2 fixation rates were only above the $LOD_{Montoya}$ on Mar. 5th at a rate of 1.53 nmol L^{-1} d^{-1}. These measured N_2 fixation rates were comparable to most of the maximum N_2 fixation rates reported by Shiozaki et al.[17], except for one of their extremely high value (44.4 nmol N L^{-1} d^{-1}) at Station E near the sea ice edge[17], although our study was conducted during austral summer when there was no sea ice formation in the northern WAP. This rate was also comparable to the rates in surface waters under light conditions between 2013 and 2017 reported by Alcamán-Arias et al.[19] at Chile Bay, but considerably lower than those in 2018 and 2019[19]. On a more global scale, between Jan. 24th and Feb 14th N_2 fixation rates were higher than the 7th decile of the compiled rates reported in Shao et al.[31]. However, when normalized to the high biomass observed in the study region (N_2 fixation rate/particulate organic N), our rates (<0.001 d^{-1}) are much lower than what is measured in subtropical waters (typically ranging 0.01–0.1 d^{-1})[31], suggesting a limited impact of N_2 fixation in this Antarctic ecosystem. To capture temporal patterns of N_2 fixation at higher resolutions than $^{15}N_2$ incubations, measurements by FARACAS were carried out, which also showed detectable N_2 fixation from Jan. 27th to 28th (Fig. 2), ranging from 1.2 to 5.8 nmol N L^{-1} d^{-1}. These rates were comparable to $^{15}N_2$ incubation measurements. The stir bar in FARACAS incubator was found to be stuck before 9 pm on Jan. 28th and could have caused overestimation of N_2 fixation rates between 4 am and 9 pm because of ethylene buildup in the incubator. The discrepancy between the N_2 fixation rates measured by two different methods could also be caused by uncertainties in the conversion ratio between acetylene reduction and N_2 fixation and release of newly fixed N into dissolved phase not captured by the $^{15}N_2$ incubation. The increasing N_2 fixation trends at night as observed after 9 pm on 28th is consistent with the higher rates under dark conditions at the WAP[19], potentially contributed by the heterotrophic diazotrophs (as described in the next section).

The days before Feb. 2nd and 14th, when N_2 fixation rates were at their highest, were characterized by a drastic change in environmental conditions. Strong winds on Jan 22nd (>15 m/s) caused considerable water mixing with a substantial drop in biological oxygen saturation (as estimated with O_2/Ar) at the ocean surface from around +10% to below −10% (Fig. 3). The water temperature did not change substantially, but salinity increased from 33.4 to 33.8, the highest value observed over the study period (Fig. 3) and was consistent with upwelling of bottom waters or Upper Circumpolar Deep Water (salinity 34.62–34.68)[32]. Vertical profiles of water density at station B 1 km from Palmer Station also showed elevated values in the surface layer during this time (Supplementary Fig. 1), indicating the enhanced vertical water mixing associated with the strong winds. Nutrients (N and P, Supplementary Fig. 1) also increased during this water mixing event, and triggered a diatom bloom in early February, as shown by the increase in chlorophyll, O_2/Ar ratios (Fig. 3), and particulate organic N (Supplementary Data 1). The color of the filtered biomass, when N_2 fixation was measurable, was also visually distinct from other samples (Supplementary Fig. 2), indicating potentially different plankton communities or particle sources. Based on these environmental conditions, and our *nifH* analyses showing likely sediment-associated diazotrophs (see "Discussion" below), we

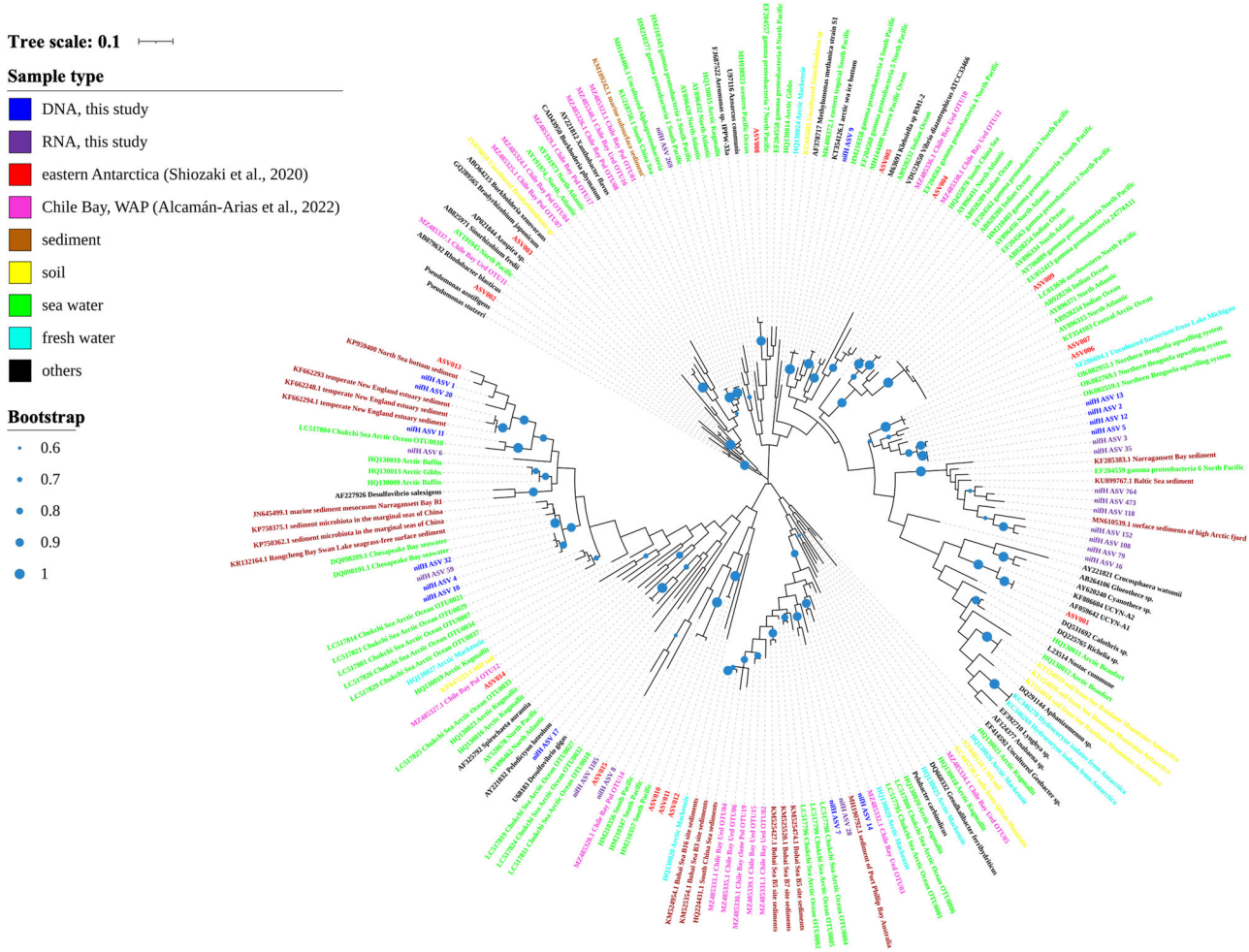

Fig. 5 | Maximum likelihood phylogenetic analysis of potential diazotroph communities based on *nifH*. The analysis includes a total of 206 *nifH* sequences, color coded by source: blue and purple for the top 15 *nifH* amplicon sequencing variants (ASVs) in DNA and RNA samples in this study, respectively, with overlap in *nifH* ASV 3; red labels denote top ASVs identified in eastern Antarctica waters by Shiozaki et al.[17]; pink labels for sequences identified from Chile Bay by Alcamán-Arias et al.[19]. Additional *nifH* sequences from other environmental samples are represented in various colors. Bootstrap values were determined from 1000 iterations (blue circles).

hypothesize that the strong winds resuspended sediments in the shallow water column (bottom depth within 10 m).

The importance of sedimentary N_2 fixation is increasingly evident[33,34], especially in coastal oceans[35–38]. Mixing could inoculate the water column with sedimentary diazotrophs[39] and/or alleviate water column diazotrophic iron limitation through resuspension[40,41]. Limited organic matter availability (with lower chlorophyll and negative O_2/Ar ratios), or higher N/P ratios (Supplementary Fig. 1) may explain the undetectable N_2 fixation on other windy days later during the growing season. Further studies are needed to ascertain the factors controlling diazotrophy in the region.

To expand the spatial coverage of N_2 fixation observations along the WAP, we conducted $^{15}N_2$ incubations at 5 different stations along the WAP during the 2018 PAL-LTER cruise from Jan 22nd to Feb 3rd (Fig. 1). All N_2 fixation rates were below the detection limits ($LOD_{montoya}$, LOD_{sd}, and MQR) except for station 150.00 where the measured N_2 fixation rate of 1.9 nmol L^{-1} d^{-1} which was associated with a high standard deviation (±8.3 nmol L^{-1} d^{-1}) exceeded the $LOD_{montoya}$ of only 0.3 nmol L^{-1} d^{-1}. As opposed to the time-series sampling site at Palmer Station, where bottom depth is 10–20 m, these 5 regional cruise stations were farther offshore, with the water depth ranging from 250 to 460 m. The absence of detectable N_2 fixation rates in surface waters at these offshore deeper stations also aligns with our hypothesis that sedimentary diazotrophs are the main contributors to measurable N_2 fixation rates in the study region.

Diazotroph community composition in Antarctica

DNA and RNA were extracted from a total of 55 filters collected from Jan. 13th to Mar. 24th in 2019. *nifH* genes were amplified from all 55 DNA samples. The sequences show the presence of putative diazotrophs in the WAP coastal waters (Fig. 4). A maximum likelihood phylogenetic tree was built for the top 15 *nifH* ASVs among DNA and RNA samples from this study (named as "*nifH* ASV 1", etc.) together with the top 15 ASVs from eastern Antarctica (named as "ASV001" to "ASV015" in Shiozaki et al.)[17], 18 OTUs from Chile Bay[19] and other representative *nifH* sequences from environmental samples or diazotroph isolates (Fig. 5).

While there are some similarities in diazotrophic communities between different Antarctic waters (e.g., clustering of *nifH* ASV 1 and ASV013, *nifH* ASV 14 and Ued OTU03, ASV004 and Ued OTU12), most of the top *nifH* ASVs from our study are distinct from those in eastern Antarctic or Chile Bay waters (Fig. 5). The top sequences from our study are also different from Arctic samples based on the phylogenetic tree (Fig. 5). For example, UCYN-A was described as a major contributor to N_2 fixation in eastern Antarctica[17] and the Arctic waters[14,42]. In contrast, no *nifH* ASVs from our study sites at the WAP are close to UCYN-A (ASV001) on the phylogenetic tree (Fig. 5). The absence of UCYN-A was also reported in the Chile Bay waters[19], indicating distinct diazotroph communities between eastern and western Antarctic waters. In our study, Proteobacteria were the most well-represented diazotrophic phylum, with Gammaproteobacteria as

the dominant class (Fig. 4). 22 out of the 55 RNA samples showed positive *nifH* RT-PCR amplifications. Shown on Fig. 4 are the 13 RNA samples with more than 1000 reads per library after removal of the non-*nifH* gene sequences. Epsilonproteobacteria and Bacteroidia dominated the RNA samples. The discrepancy between DNA and RNA *nifH* compositions indicates that some rare diazotrophs may contribute disproportionally to N_2 fixation rates.

Potential for sedimentary diazotrophy

Some Epsilonproteobacteria can fix N_2 and have been shown to be potentially important in high-latitude coastal sediments[43]. We hypothesize that Epsilonproteobacteria were likely key diazotrophs at the WAP. When measurable N_2 fixation rates and substantial *nifH* RNA reads were detected (Fig. 4), the most abundant RNA reads were identified as Epsilonproteobacteria, with *nifH* ASV 35 being the most abundant ASV on Jan. 28 and Feb. 2 and *nifH* ASV 108 and *nifH* ASV 152 accounting for >70% of the reads on Feb. 23. Strains under genus *Arcobacter* were among the top blast hits for both *nifH* ASV 108 and *nifH* ASV 152. *Arcobacter* has been reported in diverse habitats including estuarine sediments[44,45]. The top blast hit was *Candidatus Sulfurimonas* under genus *Sulfurimonas* (score = 468, *e*-value = 2e−127) for *nifH* ASV 35. Genus *Sulfurimonas* is under the order Campylobacterales, which has been reported in high-latitude ocean sediments[46,47]. Similarly, species of genus *Sulfurimonas* can be dominant in pelagic and sedimentary redoxclines[48], again agreed with the hypothesis that *nifH* ASV 35 resulted from resuspended sediments. Furthermore, *nifH* ASV 35, *nifH* ASV 108 and *nifH* ASV 152, which were the dominantly expressed RNA ASVs during measurable N_2 fixation in our study, clustered together with sequences isolated from sedimentary samples (Fig. 5). *nifH* ASV 1 and *nifH* ASV 20 cluster with ASV013 on the phylogenetic tree, together with four other sedimentary samples, further supporting a sedimentary origin. Notably, Chile Bay diazotrophs also clustered together with sedimentary samples but differed from the top *nifH* ASVs in our RNA samples on the phylogenetic tree (Fig. 5).

A bloom of the genus *Arcobactor* and *Sulfurimonas* was reported after oxygenation of sediments collected from the coastal Baltic Sea[43], a similar eutrophic high latitude coastal environment to the WAP, which was traditionally believed unfavorable for N_2 fixation. A recent study also showed that N_2 fixation occurred under simulated sediment resuspensions with coastal Baltic Sea sediments, dominated by sulfur-reducing bacteria[49]. Reported to be prominent in diazotrophic communities from temperate estuary sediments[39], sulfur-reducing Deltaproteobacteria were also present at our study site. Sediment resuspension at the WAP likely led to a re-oxygenation similar to one observed in the Baltic Sea study[43]. The authors hypothesized that re-oxygenation may have led to favorable microaerophilic conditions for these genera, and high heterogeneity in sediments created micro niches which permitted anaerobic N_2 fixation[43]. N_2 fixation may also have been stimulated by subdued inhibition by ammonium when resuspended and diluted into the water column[50].

Considering that a large portion of the *nifH* sequences were not identified based on the currently available reference database (Fig. 4), an alternative to the sediment source is that we may have encountered new heterotrophic N_2 fixers. Recent studies have shown that heterotrophic N_2 fixers may be more important in the ocean than previously thought[9–12]. Our study demonstrates that they may also be important in Antarctic waters. For example, many of our *nifH* ASVs also showed substantial levels of sequence identity (i.e., over 85%) to *nifH* genes found in genomes of prevalent heterotrophic bacterial diazotrophs recently reconstructed from surface ocean metagenomes[10].

In our study, characterization of the community was also performed on days when N_2 fixation was below the detection limit. On some of these days, the *nifH* gene was successfully amplified (RT-PCR) from RNA samples (Fig. 4) (e.g., Mar. 24th). This could result from low absolute abundance of active diazotrophs or below detection limit N_2 fixation, or a change in diazotroph community between the start of the incubations and molecular sampling. For example, an RNA sample collected in the afternoon of Jan. 27th, right before the second $^{15}N_2$ incubation, displayed notably different active taxa based on *nifH* gene expression, dominated by *nifH* ASV 8 under class Bacteroidia (93.7%). The top blast hit of *nifH* ASV 8 is an uncultured bacterium from the OMZ water column (score = 348, *e*-value = 2e−91). This substantial change in *nifH* gene expression within 10 h could be caused by diel variability or changes in water masses, perhaps associated with tidal activity.

With the results indicating that resuspended sediments may have contributed to measurable N_2 fixation in coastal Antarctic waters, our study challenges the traditional view that polar, coastal, and nutrient-rich oceans are inhospitable to diazotrophs and adds to recent studies expanding their potential niches[7,17,19,51–55]. However, our observations showed N_2 fixation to be low (or absent when using more stringent thresholds) and sporadic. The major diazotroph communities were distinct from other studies in Antarctic waters[17,19], showing the Southern Ocean to be a potentially diverse N_2 fixer habitat. This time-series dataset also complements our understanding of temporal variabilities of N_2 fixation in Antarctica waters. It remains to be determined how widespread diazotrophy is in Antarctic waters and how it is influenced by sediment resuspension. Furthermore, given that vertical mixing is common in this region, sediment resuspension may also influence elemental cycling within the water column[56]. In light of the disproportionate influence of climate change in polar regions (e.g., warming, stratification, and community changes), further studies are needed to examine the importance of N_2 fixation in this newly discovered biome.

Methods

High-resolution continuous measurement of N_2 fixation rates (FARACAS)

High frequency measurements of aquatic N_2 fixation were conducted at Palmer Station, Antarctica, from January to March in 2019 using the FARACAS system[23]. Due to technical issues, the FARACAS data were only usable at the start of the deployment during January 27–28th. Unfiltered seawater was continuously pumped into the laboratory from 6 m depth in Arthur Harbor, west of Palmer Station. C_2H_2 gas was prepared in Tedlar bag by reacting high-purity calcium carbide (CaC_2, Alfa Aesar) with ultrapure water, and then dissolved in 0.2 μm filtered seawater to a saturation of 70% (v/v%). The 70% C_2H_2 tracer water was then mixed with unfiltered seawater at a ratio of 1:6, reaching a final saturation of 10%. This water mixture was pumped into a modified 9 L glass incubator, where flow-through incubation was performed. Ambient temperature was maintained by a water-jacket outside of incubator and light was simulated using an LED-wrap based on the solar altitude. The incubator was thoroughly acid washed every two days. C_2H_4 production rates were measured during the incubation using a Picarro CRDS (cavity ring-down laser absorption spectroscopy) C_2H_4 analyzer (model G1106, Santa Clara, CA), and converted to N_2 fixation rates using a factor of 4:1.

$^{15}N_2$ incubation

Concurrently, discrete $^{15}N_2$ addition incubations were conducted from late January to March 2019 at Palmer Station, following the protocols outlined in Mohr et al.[25]. Briefly, $^{15}N_2$ enriched water was made by dissolving 10 mL $^{15}N_2$ tracer (Cambridge Isotope Laboratories, 98%+) into 1 L 0.2 μm filtered seawater after degassing under vacuum for more than 1 h. Surface seawater was collected into 4 L transparent polycarbonate bottles directly from the shore adjacent to the inlet of unfiltered water source for the FARACAS system at Palmer Station or into 2.5 L transparent polycarbonate bottles from Niskin bottles during the 2018 LTER cruise. In case of unfavorable weather conditions at Palmer Station, water was collected from the tap in the Palmer Station pump house. $^{15}N_2$ enriched water was injected to the bottom of each triplicate bottles (5% v:v) at Palmer Station or directly injected as $^{15}N_2$ during the 2018 LTER cruise, after which the triplicate bottles were incubated for 24 h in an outdoor transparent tank, with continuously circulating surface seawater controlling the temperature. Dissolved $^{15}N_2$ samples were collected in gas tight Exetainer vials (Labco) from initial $^{15}N_2$ enriched water as well as from each bottle after incubation. The samples were poisoned with $HgCl_2$ and

stored until analysis using Membrane Inlet Mass Spectrometer (Bay Instruments). Particulate matter was recovered by filtering the water through pre-combusted 25 mm GF/F filters (Whatman) at the end of each incubation. Non-incubated particulate matter was also sampled at each time point (in monoplicate) to reflect the natural $^{15}N/^{14}N$ ratio in the environment. Filters were stored at −80 °C and dried before N isotope analysis using an elemental analyzer coupled with a mass spectrometer. N_2 fixation rates were calculated according to Montoya et al.[30]. At Palmer Station, the natural (i.e., non-incubated) ^{15}N abundance of particulate matter measured at each time point in monoplicate showed a scattered pattern but a linear increasing trend over the time course of the survey (Sieve Bootstrap Based Test, "notrend_test" function in "funtimes" R package, p-value < 0.0001). We assume that this trend reflects an ecological reality and the scattered nature of the samples is mostly due to analytical uncertainty. In order to account for this, we modeled the linear increasing trend ($r = 0.75$, p-value = 0.0003, $n = 18$, Supplementary Fig. 3) and used the fitted values to define the initial ^{15}N abundance in particulate matter for each time point. During the 2018 LTER cruise, no pattern in the ^{15}N abundance of non-incubated was observed and the average of all values was used for all stations. To determine the LOD_{sd}, the standard deviation of the initial ^{15}N abundance in the PON was calculated using the rolling standard deviation method with a width of 3, to account for the trend, leading to a value of 0.00063 atom%. $LOD_{Montoya}$ and MQR were calculated according to Gradoville et al.[28].

DNA and RNA sample collection

DNA and RNA samples were collected twice a day around mid-day and mid-night from January 13th to March 24th in 2019. In order to avoid potential contamination within the pipes between Palmer Station pump house and the wet lab, unfiltered seawater was collected into acid-washed LDPE Cubitainers (Thermo Scientific) from the tap in the pump house. A peristaltic pump was used to filter seawater onto 0.22 µm polyethersulfone membrane filters (Millipore) or 0.22 µm Sterivex filters (Millipore). The filtration was limited to no more than 30 min to minimize RNA degradation, achieving a filtration volume ranging between 600–4000 mL. The filters were flash frozen immediately using liquid nitrogen and then stored at −80 °C until further analysis.

DNA and RNA extraction, nested PCR and *nifH* gene sequencing

DNA and RNA were extracted from 0.22 µm membrane or 0.22 µm Sterivex filters using Allprep DNA/RNA mini kit (Qiagen) following the manufacture's instruction with an additional 2-min bead-beating step at 30 Hz using 0.2 g of 0.1 mm Zr beads. DNA and RNA were eluted using 50 uL EB buffer or RNase-free water respectively. RNA samples were further cleaned using RNase-Free DNase set (Qiagen) and the RNA Clean & Concentrator kit (Zymo) to remove DNA, and finally eluted in 15 µL RNase free water. DNA and RNA concentrations were measured on a Qubit fluorometer using Qubit DNA/RNA HS Assay Kit. In order to synthesize cDNA from RNA samples for *nifH* gene amplification, reverse transcription was conducted using QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's guidelines. *nifH* genes were then amplified using a nested PCR protocol (nifH3 and nifH4 primers during the 1st round, nifH1 and nifH2 primers with Illumina adapter during the 2nd round) as described by Turk et al.[57]. The thermal cyle of nested PCR was 95 °C for 5 min followed by 35 cycle of 1 min at 95 °C, 55 °C, 72 °C, respectively, and then 5 min at 72 °C. Finally, customized dual indexed barcodes (6 bp) with heterogenous spacers[58] were added through a ligation process using KAPA HiFi ReadyMix[59]. The thermal cycle of ligation PCR was 95 °C for 3 min followed by 8 cycle of 30 s at 95 °C, 55 °C, 72 °C, respectively, and then 5 min at 72 °C. Samples were pooled in equal molar concentrations before sending for Illumina sequencing at Duke Center for Genomic and Computational Biology in one MiSeq 300PE run.

Sequence data processing

Raw sequencing data was demultiplexed using QIIME 1 (version 1.9.1). The reads were then merged, and barcodes, primers and adapters were trimmed using BBDuk (version 1.1.2). ASVs were inferred using DADA2 pipeline after quality filtering and dereplication[60]. Chimeras were then removed, and taxonomy was assigned using the function "assignTaxonomy" with reference *nifH* ARB database[61]. All ASVs were blasted against Refseq database using blastx in DIAMOND[62] and the ASVs that do not encode nitrogenase were filtered out as non-*nifH* sequences. Some of the featured ASVs were further blasted on National Center for Biotechnology Information (NCBI) database for more information. In total, 25403 ASVs were identified from 55 DNA samples and 22 RNA samples. The 55 DNA samples had an average of 101846 reads and the 13 RNA samples (>1000 reads) had an average of 41147 reads. All *nifH* gene sequencing data has been deposited in NCBI.

Phylogenetic analyses were conducted in MEGA7 (version 7.0.26)[63]. 206 sequences were first aligned using ClustalW and then constructed the phylogenetic tree by Maximum Likelihood method based on the Tamura-Nei model[64]. Bootstrap values were calculated based on 1000 times of replications.

Environmental parameters

Temperature, salinity, chlorophyll, and wind speed data were downloaded from the Antarctic Meteorological Research Center (AMRC) FTP site (ftp://amrc.ssec.wisc.edu/pub/palmer/) and averaged over a 10-min time span at the sampling time or an 1-hour time span during the time series. Temperature was measured at the Palmer Station pump house using a Seabird SBE 38 thermometer. Conductivity was measured using Seabird SBE45 thermosalinograph in the Palmer Station aquarium lab. On the days when these temperature and conductivity data were not available, data were interpolated based on the Palmer Station tide gauge sensor data and salinity was calculated[65] in order to obtain a wider time coverage. Chlorophyll was measured using a WetLab fluorometer (FLRT 3759) in the aquarium lab. Wind speed was measured at the weather station in the backyard of Palmer Station. O_2/Ar ratios were measured in the wet lab using an equilibrator inlet mass spectrometer (EIMS)[66] and averaged to the same 10-min or 1-h resolution as other environmental parameters.

Data availability

Source data used to create all the figures are available on Figshare (https://doi.org/10.6084/m9.figshare.28386431.v2). The sequence datasets generated for this study have been deposited at DDBJ/ENA/GenBank under the accession KIWL00000000. The version described in this paper is the first version, KIWL01000000.

Code availability

No custom code or mathematical algorithm used to be deemed central to the conclusions.

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Author contributions

N.C. designed the study. S.G. performed measurements and collected samples at Palmer Station, with contributions from W.T. and H.W.D. H.B. conducted measurements at offshore stations. S.G. and H.B. analyzed nitrogen fixation data. S.G. performed molecular sequencing with contributions from J.R. and Y.L. S.G. analyzed environmental data. S.G., Y.L., and A.M.E. conducted sequencing data analyses. S.G. and N.C. wrote the manuscript with contributions from all co-authors.

Competing interests

The authors declare no competing interests.

Additional information

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