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The response of diazotrophs to nutrient amendment in the South China Sea and western North Pacific

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Abstract. The availability of iron (Fe) and phosphorus (P) have been shown to be key factors regulating rates of nitrogen fixation in the western Subtropical Pacific. However, their relative importance at finer spatial scales between the northern South China Sea (NSCS) and the western boundary of the North Pacific is poorly constrained. Furthermore, nutrient limitation of specific diazotroph types has not yet been assessed. Here we investigated these unknowns by carrying out measurements of (i) finer scale spatial variabilities in N_2 fixation rates and diazotroph abundances throughout these regions, and (ii) conducting eight additional Fe and phosphate addition bioassay experiments where both changes in N_2 fixation rates and the abundances of specific diazotrophs were measured. Overall, nitrogen fixation rates were lower in the NSCS than around the Luzon Strait and the western North Pacific, which we hypothesize was due to lower Fe-to-fixed nitrogen supply ratios that decrease their competitive ability with non-diazotrophic phytoplankton. The nutrient addition bioassay experiments demonstrated that nitrogen fixation rates in the central northern South China Sea (NSCS) were co-limited by Fe and P, whereas in the western boundary of the North Pacific they were P-limited. Changes in the abundances of *nifH* in response to nutrient addition varied in how well they correlated with changes in nitrogen fixation rates, and the largest responses were always dominated by either *Trichodesmium* or UCYN-B. In general, nutrient addition had a relatively restricted impact on diazotroph community structure apart from on UCYN-B, which showed increased contribution to the diazotroph community

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following P addition at sites where N_2 fixation rates were P-limited. We further hypothesize the importance of absolute Fe supply rates in regulating spatial variability in diazotroph community structure across the study area.

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1 Introduction

Nitrogen fixation by diazotrophic bacteria converts abundant dinitrogen (N_2) gas into ammonia, providing nearly half of the ocean's bioavailable nitrogen (N) (Gruber and Galloway, 2008), which goes on to support >30% of carbon export from surface to deep waters in the N-limited ocean (Böttjer et al., 2016; Wang et al., 2019). A diverse community of diazotrophs has been described across the oligotrophic ocean that includes *Trichodesmium*, unicellular cyanobacteria (UCYN-A and *Crocosphaera*, also referred to as UCYN-B), the heterocystous symbiont *Richelia* associated with diatoms (DDAs, diatom-diazotroph associations), and noncyanobacterial diazotrophs (NCDs, heterotrophic or photoheterotrophic bacteria) (Zehr and Capone, 2020). However, there is still a lack of knowledge on what controls diazotrophic distribution, activity and community structure in the current ocean.

Iron (Fe) and phosphorus (P) are believed to be key factors controlling the biogeographic distribution of marine N_2 fixation (Sohm et al., 2011; Zehr and Capone, 2020; Wen et al., 2022). Fe is particularly important for N_2 fixers as a cofactor for the FeS-rich nitrogenanse enzyme (Berman-Frank et al., 2001), whereas P is also required for genetic information storage, cellular structure and energy generation. A number of nutrient-addition bioassay experiments conducted in the field have shown that N_2 fixation in the oligotrophic oceans can be limited by Fe or P, or co-limited by both nutrients at the same

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time (Mills et al., 2004; Needoba et al., 2007; Grabowski et al., 2008; Watkins-Brandt et al., 2011; Langlois et al., 2012; Dekaezemacker et al., 2013; Krupke et al., 2015; Tanita et al., 2021; Wen et al., 2022; Turk-Kubo et al., 2012). However, few studies have quantified how the supply of Fe and/or P impacts the abundance of individual diazotrophic phylotypes and their community structure (Langlois et al., 2012; Moisander et al., 2012; Turk-Kubo et al., 2012). Experiments conducted so far that investigated this were located in the South Pacific and North Atlantic, and found diverse responses among diazotrophic phylotypes to the addition of Fe and/or P. Furthermore, the responses of total diazotroph abundances assessed from *nifH* gene quantifications were not qualitatively match the responses of bulk N_2 fixation rates (Langlois et al., 2012; Moisander et al., 2012; Turk-Kubo et al., 2012). Resolution of the specific types of diazotrophs responding to nutrient supply, in addition to overall N_2 fixation rates, are potentially crucial for understanding their biogeography, which in turn could be important for biogeochemical function. For example, the presence of large *Trichodesmium* filaments is expected to have a different fate in the microbial food web and contribute differently to the sinking flux of carbon than that of small unicellular species (Bonnet et al., 2016).

The northern South China Sea (NSCS) and the neighboring western boundary of the North Pacific are interacting water bodies, with the major western boundary Kuroshio Current intruding into the NSCS across the Luzon Strait, generating frontal zones with

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unique physical and biogeochemical characteristics (Du et al., 2013; Guo et al., 2017; Huang et al., 2019; Li et al., 2021; Lu et al., 2019; Xu et al., 2018). Common to the full regime, however, is surface waters that are warm, stratified and N-depleted, but subject to elevated dust input from the Gobi Desert (Duce et al., 1991; Jickells et al., 2005). These conditions potentially provide an ideal habitat for diazotrophs (Chen et al., 2003; Wu et al., 2003). Investigations in these regions have shown high variability in diazotroph abundances and N_2 fixation rates (Chen et al., 2003; Chen et al., 2014; Chen et al., 2008; Lu et al., 2019; Wu et al., 2018), which overall increased from the NSCS basin to the western boundary of the North Pacific (Wen et al., 2022). Along this gradient in N_2 fixation, the dominant diazotroph types switched from *Trichodesmium* in the NSCS to UCYN-B in the western boundary of the North Pacific (Wen et al., 2022). Several studies have hypothesized that these gradients of diazotroph abundances and N_2 fixation rates were regulated by nutrient availability (specifically, Fe, P and N; Wu et al., 2003; Chen et al., 2003; Chen et al., 2008; Shiozaki et al., 2014a; Shiozaki et al., 2015a). More recent observational and experimental evidence supported the hypothesis that Fe:N supply ratios are the main drivers of the abundance of diazotrophs and N_2 fixation rates across the western North Pacific (Wen et al., 2022). With an increasing supply ratio of Fe:N from the North Equatorial Current (NEC) to the Philippines Sea, Wen et al. (2022) found that diazotroph abundances and N_2 fixation rates increased, and bioassay experiments demonstrated evidence for N_2 fixation rates switching from Fe to P limitation or to

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nutrient-replete conditions. In the NSCS, Wen et al. (2022) found N_2 fixation rates fell in between NEC and Kuroshio values and bioassay experiments demonstrated rates were co-limited by Fe and P, which they hypothesized was due to intermediate Fe:N supply ratios (Wen et al., 2022).

Although this previous study has outlined the broad spatial pattern of nutrient regulation of marine N_2 fixation throughout the western Subtropical Pacific (Wen et al., 2022), important questions remain. Two specific examples are: (i) the relatively lower spatial resolution of the experiments in Wen et al. (2022) and other studies (Chen et al., 2019; Shiozaki et al., 2014b) remain insufficient to delineate Fe and P controls at finer spatial scales between the neighboring NSCS and the western boundary of the North Pacific; and (ii) In addition to controls on N_2 fixation rates, broad-scale differences in the types of diazotrophs dominating the N_2 fixer community were not concretely associated with environmental drivers in experimental tests for nutrient limitation, because changes in type-specific diazotroph abundances following nutrient addition were not measured (Chen et al., 2019; Shiozaki et al., 2014b; Wen et al., 2022). Therefore, in the present study we extend the findings of Wen et al. (2022) and others by carrying out additional, higher-spatial resolution observations of volumetric N_2 fixation rates and measurements of the abundances of key diazotrophic phylotypes from the NSCS basin to the western boundary of the North Pacific (including the upstream Kuroshio) between 2016 and 2018

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(Fig. 1). These new observations were supplemented by a further additional eight, high volume (10 L) nutrient amendment bioassay experiments throughout the transect to directly test the response of both (i) N_2 fixation rates, and (ii) *nifH* gene abundances to supply of potentially limiting nutrients (Fe, P, and Fe+P).

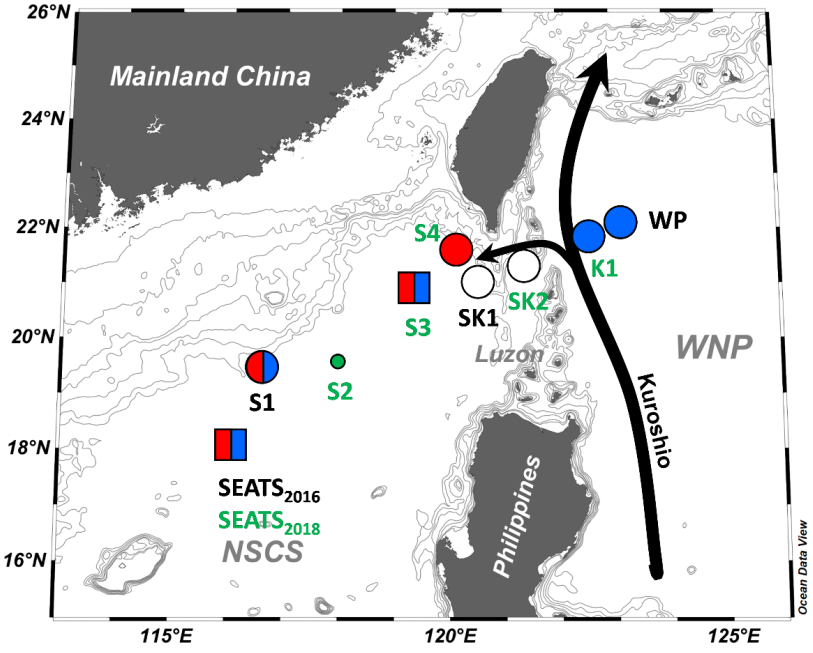

Figure 1. Sampling and nutrient amendment experiment locations in the northern South China Sea and the western boundary of the North Pacific. One station ($SEATS_{2016}$) was sampled in 2016, three (S1, SK1, WP) were in 2017, and six (stations with green labels) were in 2018. Nutrient amendment experiments were conducted at 8 of 10 stations. Symbols summarize the nutrient limitation of N_2 fixation rates found at each site: red, Fe limitation; blue, P limitation; split red/blue, Fe-P co-limitation; white, nutrient replete. Co-limitation type is indicated by symbol type (square, independent co-limitation; circle, simultaneous co-limitation). WNP, the western North Pacific. Black arrows indicate Kuroshio Current and its branch. Gray lines indicate 50, 100, 300, 500, 1000, 1500 and 2000 m bathymetric depth contours.

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2 Method

2.1 Sample collection

Investigations and bioassay experiments were conducted on three cruises to the NSCS (stations SETAS and S1 to S4), the Luzon Strait (stations SK1 and SK2), the upstream Kuroshio (station K1), and the western boundary of the North Pacific (station WP) (Fig. 1), between May 2016 and June 2018 onboard the R/V *Dongfanghong* 2 and R/V *Tan Kah Kee*. At each station (except station SK2 where no hydrological data are available), temperature and salinity were recorded by a Seabird 911 CTD. Water samples were collected using Niskin-X bottles at five or six depths (except SK2, only surface waters were sampled) throughout the upper 150 m for the determination of N_2 fixation and primary production rates. Seawaters from each depth were also sampled for the analysis of *nifH* gene abundance. Samples for nutrient analysis were also collected. Seawater for the bioassay experiments (at 8 of 10 stations) was collected using a trace-metal-clean towed sampling device located around 2-5 m depth with suction provided by a Teflon bellows pump. Seawaters were sampled in a dedicated trace-metal-clean laminar flow hood maintained over-pressurized by HEPA-filtered air. During the cruise in 2018 (stations with green labels in Fig. 1), surface waters were sampled under trace-metal-clean condition for the determination of total particulate Fe concentration.

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2.2 N_2 fixation and primary production rate measurements

N_2 fixation rates were determined by the $^{15}N_2$ gas dissolution method (Mohr et al., 2010), combined with a primary production assay using $NaH^{13}CO_3$ (99 atom% ^{13}C, Cambridge Isotope Laboratories). Briefly, 0.22 μm-filtered surface seawater was degassed using a Sterapore membrane unit (20M1500A: Mitsubishi Rayon Co., Ltd., Tokyo, Japan) as described in Shiozaki et al. (2015b). After that, 20 mL 98.9 atom% pure $^{15}N_2$ gas (Cambridge Isotope Laboratories) was injected into a gas-tight plastic bag containing 2 L of the degassed seawater and allowed to fully equilibrate before use. The N_2 fixation and primary production incubations were conducted in duplicate 4.3 L Nalgene polycarbonate bottles. Samples were spiked with 100 mL $^{15}N_2$ enriched filtered seawater from the same site and incubated on-deck for 24 h. The final $^{15}N_2$ enriched seawater concentration in the incubation bottles was not measured directly during this study. We thus employed a $^{15}N_2$ atom% of 1.40 ± 0.08 atom% (n = 17) measured in a following cruise in 2020 (Wen et al., 2022), during which the N_2 fixation incubations were conducted using the same approach, reagents, and equipment as for the study described here. For primary production measurements, $NaH^{13}CO_3$ solution was added at a concentration of 100 μM. After that, the bottles were covered with a neutral-density screen to adjust the light to the levels at sampling depths, and then were incubated for 24 h in an on-deck incubator continuously flushed with surface seawater. Incubated samples were filtered onto pre-combusted (450 °C, 4 h) GF/F filters, and the particulate organic matter from each depth

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were also collected to determine background POC/PON concentrations and their natural $^{13}C/^{15}N$ abundances.

All filter samples were acid fumed to remove the inorganic carbon and then analyzed using an elemental analyzer coupled to a mass spectrometer (EA-IRMS, Thermo Fisher Flash HT 2000-Delta V plus). The N_2 fixation and primary production rates were then calculated according to Montoya et al. (1996) and Hama et al. (1983), respectively. The detection limits of N_2 fixation rates were then calculated according to Montoya et al. (1996), taking 4‰ as the minimum acceptable change in the $\delta^{15}N$ of particulate nitrogen. All parameters involved in N_2 fixation rate calculation are shown in Supplementary Materials. To represent the inventories, the upper 150 m depth-integrated N_2 fixation rate and primary production were calculated by the trapezoidal integration method.

2.3 *nifH* gene abundance

At each depth, 4.3 L seawater samples for DNA extraction were filtered onto 0.22 μm pore-sized membrane filters (Supor200, Pall Gelman, NY, USA) and then frozen in liquid N_2. To extract the DNA, membranes were cut into pieces under sterile conditions, and then extracted using the QIAamp® DNA Mini Kit (Qiagen) following the manufacturer's protocol. The quantitative polymerase chain reaction (qPCR) analysis was targeted on the *nifH* phylotypes of *Trichodesmium* spp., unicellular cyanobacterial UCYN-A1, UCYN-

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A2, and UCYN-B, *Richelia* spp. (het-1), and a gamma-proteobacterium (γ-24774A11), using previously designed primers and probe sets (Supplementary Table S1; Church et al., 2005a; Church et al., 2005b; Moisander et al., 2008; Thompson et al., 2014). A recent study suggested that the primers for UCYN-A2 also target UCYN-A3 and thus cannot be used to differentiate between these two phylotypes (Farnelid et al., 2016). Therefore, we used the convention UCYN-A2/A3 when referring to these two groups. The *nifH* standards were obtained by cloning the environmental sequences from previous samples collected from the SCS. qPCR analysis was carried out as described previously (Church et al., 2005a) with slight modifications. Triplicate qPCR reactions were run for each environmental DNA sample and for each standard on a CFX96 Real-Time System (Bio-Rad Laboratories). Standards corresponding to between 10^1 and 10^7 copies per well were amplified in the same 96-well plate. The amplification efficiencies of PCR were always between 90-105%, with R^2 values > 0.99. The quantification limit of the qPCR reactions was 10 *nifH* gene copies per reaction, and 1 µL from 100 or 150 µL template DNA was applied to qPCR assay, which was equivalent to approximately ~230-350 gene copies per L of seawater sample filtered (4.3 L).

2.4 Bioassay experiments

Acid-cleaned Nalgene polycarbonate carboys (10 L) were filled with near surface seawater from the towed fish system. Trace metal clean techniques were strictly applied

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in experimental setup and manipulations. All materials coming in contact with the incubation water were acid-washed in a Class-100 cleanroom before use. Nutrient amendments at all sites were Fe, P, and Fe+P. The amended Fe and P (chelexed and filter-sterilized) concentrations were 2 nM and 100 nM, respectively. Control bottles incubated with no nutrient treatment were included in all experiments. All treatments were conducted with 2 or 3 replicates and incubated for 3 days in a screened on-deck incubator continuously flushed with surface seawater. After pre-incubation, subsamples were collected for the determination of N_2 fixation rate and *nifH* gene abundance. $^{15}N_2$ enriched seawater was prepared as described above, except that all the materials coming in contact with the seawater were acid-cleaned before use.

2.5 Macronutrient and chlorophyll *a* analyses

Samples for macronutrient analyses were collected in 125-mL acid-washed high-density polyethylene (HDPE) bottles (Nalgene), and analyzed onboard using a Four-channel Continuous Flow Technicon AA3 Auto-Analyzer (Bran-Lube GmbH). The detection limits for NO_3^-+NO_2^- and PO_4^{3-} were 0.1 μmol L^{-1} and 0.08 μmol L^{-1}, respectively. The nitracline was defined as the depth at which NO_X concentration equaled 0.1 μmol L^{-1} (Le Borgne et al., 2002). Samples for chlorophyll *a* analysis were collected on nominal 0.7 μm pore-size GF/F filters (Whatman) and chlorophyll *a* concentration was determined using a Trilogy fluorometer (Turner-Designs, USA).

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2.6 Particulate Fe concentration

Total particulate Fe (PFe_{total}) and intracellular Fe (PFe_{intra}) were sampled under laminar flow hood. Briefly, 4-9 L of surface waters were filtered onto acid-cleaned 0.22-μm polycarbonate membrane filters. For PFe_{intra} samples, in order to remove metal-bound to the cell surface, cells were exposed twice to an oxalate-EDTA solution for 5 minutes and rinsed nine times with Chelex-cleaned 0.56 mol L^{-1} NaCl solution (Li et al., 2020). PFe_{total} and PFe_{intra} concentrations were then determined by ICP-MS (ICP-MS 7700X, Agilent).

2.7 Statistical analysis

Significance of differences among nutrient treatments of bioassay experiments (for N_2 fixation rate) were tested by ANOVA followed by Fisher PSLD test, using R-4.1.2. Pairwise correlation between N_2 fixation rates, diazotroph groups and environmental factors was analyzed using Pearson correlation. A significance level of $p < 0.05$ was applied, except as noted where significance was even greater.

3 Results

3.1 Spatial variations of N_2 fixation rates and diazotroph composition

Our survey revealed substantial spatial variability in N_2 fixation rates and *nifH* gene

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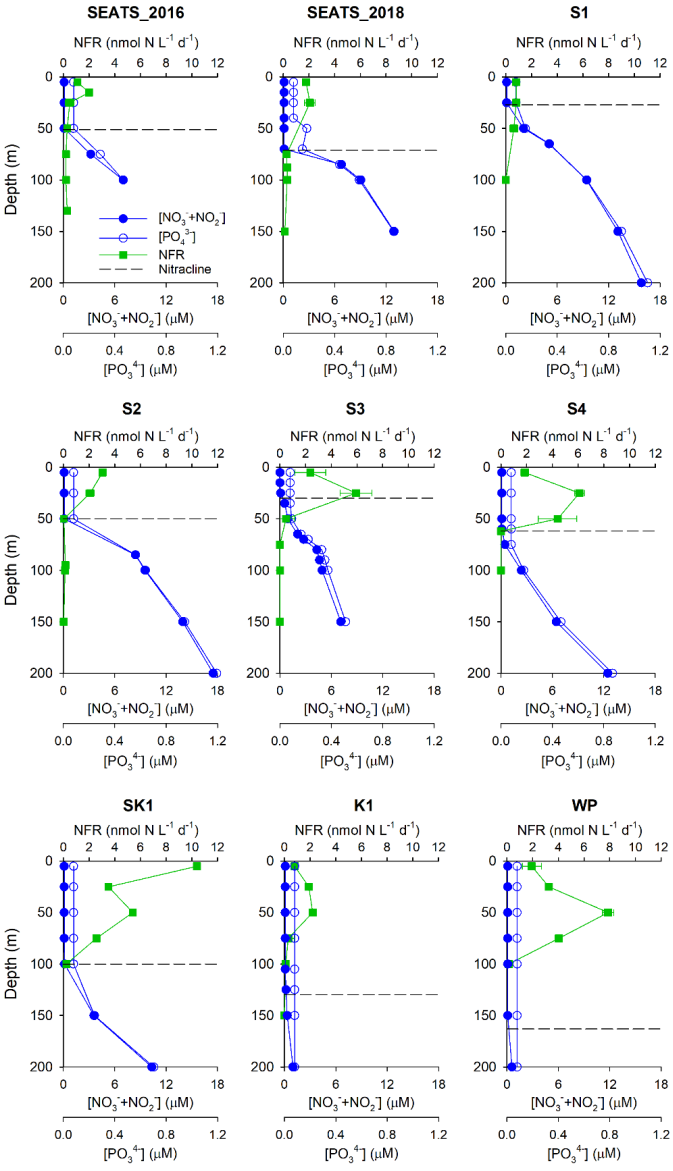

Figure 2. Vertical profiles of N_2 fixation rates. Green squares, N_2 fixation rate (NFR, nmol N L^{-1} d^{-1}); blue solid circles, NO_3^-+NO_2^- concentrations (μM); blue open circles, PO_4^{3-} concentrations (μM). The dashed line indicates the nitracline depth. Note that no profile data were available at station SK2.

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abundances across the study area (Figs. 2 and 3). Vertically, high N_2 fixation rates were found in the upper 50 m (ranged from bellow detection limit to 10.4 ± 0.01 nmol N L^{-1} d^{-1}), rates dropped rapidly at greater depths (Fig. 2), and surface rates were positively correlated with depth-integrated rates (Pearson $r = 0.68$, $p = 0.043$, Supplementary Table S2). Horizontally, depth-integrated N_2 fixation rates were generally low at the central NSCS basin stations (SEATS, S1 and S2, on average 86 ± 33 μmol N m^{-2} d^{-1}), elevated at stations close to the western edge of the Luzon Strait (S3 and S4, on average 214 ± 47 μmol N m^{-2} d^{-1}), and were highest at the Luzon Strait station (SK1, 437 μmol N m^{-2} d^{-1}) and the western North Pacific boundary station (WP, 403 μmol N m^{-2} d^{-1}) (Figs. 1, 3 and Table 1).

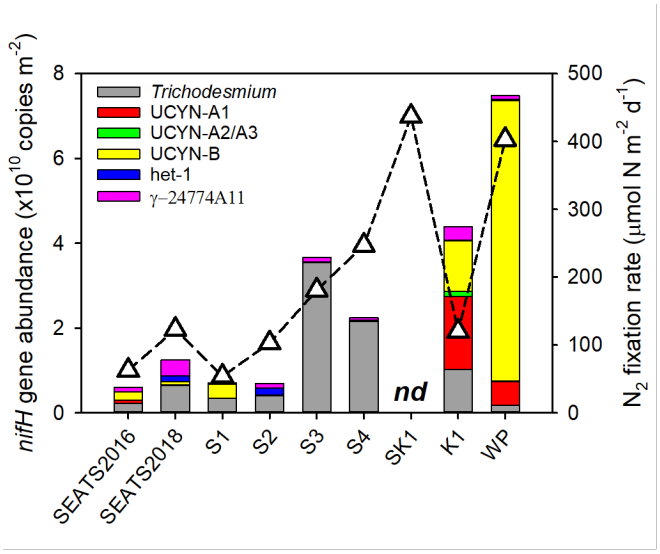

Figure 3. Depth-integrated (upper 150 m) *nifH* gene abundances (bars) and N_2 fixation rates (triangles). Note that depth-integrated N_2 fixation rates and *nifH* gene abundances were not available at station SK2. nd, not determined.

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Table 1. Environmental conditions, N_2 fixation, and primary production rates. Sea surface temperature (SST) and salinity (SSS), nitracline depth (D_{Nitr}), surface N_2 fixation rate (SNF), upper 150 m depth-integrated N_2 fixation rate (INF) and primary production (IPP) at each station. nd, not determined.

Station	SST (°C)	SSS	Chl *a* (μg/L)	D_{Nitr} (m)	SNF (nmol N L^{-1} d^{-1})	INF (μmol N m^{-2} d^{-1})	IPP (mmol C m^{-2} d^{-1})
$SEATS_{2016}$	30.3	33.46	0.26	51	1.1	63	44
$SEATS_{2018}$	30.3	33.46	0.11	71	1.8	123	24
S1	29.5	33.73	0.24	27	0.8	54	43
S2	29.4	33.75	0.10	50	3.0	103	24
S3	28.7	33.53	0.15	30	2.4	181	98
S4	29.5	33.74	0.17	62	1.8	247	59
SK1	30.5	33.62	0.22	100	10.4	437	11
SK2	nd	nd	0.11	nd	2.0	nd	nd
K1	29.1	34.45	0.11	130	0.8	120	19
WP	30.9	34.47	0.11	163	1.9	403	9

A significant positive correlation was found between the depth-integrated *nifH* gene abundance and N_2 fixation rate (Pearson $r = 0.72$, $p = 0.046$, Supplementary Table S2), demonstrating that the abundances of these major diazotroph phylotypes well explained the major variability in measured rates. However, considerable spatial variation was found in the specific diazotrophs supporting N_2 fixation (Fig. 3). *Trichodesmium*

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dominated the diazotroph assemblage throughout the water column of the NSCS (52-96% of the total *nifH* gene abundance, excluding station $SEATE_{2016}$). In contrast, at the Kuroshio station K1, unicellular diazotrophic cyanobacteria (UCYN-A and UCYN-B) were the most abundant phylotypes, and at station WP, UCYN-B alone was dominant (Fig. 3 and Supplementary Table S3).

3.2 Diazotroph response to Fe and P supply

To directly test which nutrients were limiting overall N_2 fixation rates and the abundance of individual diazotrophs, we conducted eight, ~3-day nutrient addition bioassay experiments (Figs. 4 and 5). The responses of N_2 fixation rate to different combinations of Fe and P supply demonstrated a coherent geographic switch across the study area (Figs. 1, 4 and 5). At stations towards to the NSCS basin ($SEATS_{2016}$, S1 and S3), N_2 fixation rates were co-limited by Fe and P. Two forms of this co-limitation were identified: (i) only simultaneous Fe and P addition stimulated N_2 fixation rates ('simultaneous co-limitation', station S1, Fig. 4); (ii) independent addition of either Fe or P alone, or supply of Fe and P in combination, enhanced N_2 fixation rates ('independent co-limitation', stations $SEATS_{2016}$ and S3, Fig 4). Further to the northeast, in contrast, N_2 fixation rates were only stimulated by nutrient combinations containing Fe at S4 and by combinations containing P at K1 and WP, suggesting single limitation by Fe or P, respectively, at these sites (Fig. 4). Although Fe addition also appeared to stimulate N_2

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fixation rates at station K1, P was generally the major limiting nutrient at this station taking into account the responses of both N_2 fixation rates and *nifH* gene abundance (see below) (Figs. 4 and 5). At stations (SK1 and SK2) in the Luzon Strait, mean N_2 fixation rates were highest in treatments containing P, but responses were not significantly greater than the untreated controls, suggesting that both Fe and P availability were not limiting N_2 fixation rates (Fig. 4).

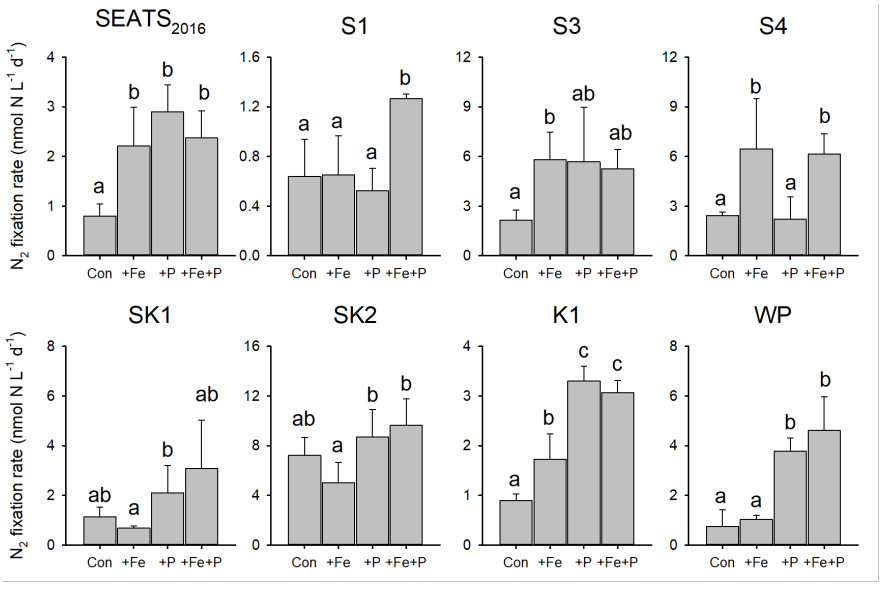

Figure 4. Response of N_2 fixation to nutrient amendment. Error bars represent the standard deviation of biological replicates (n = 2 or 3). Different letters above error bars indicate statistically significant differences ($p < 0.05$) between treatments (ANOVA followed by Fisher PLSD test).

Further detail as to the drivers of the N_2 fixation responses to Fe and P additions was

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provided by the species-level analysis of diazotroph *nifH* from the treatment bottles. In general, responses of total *nifH* gene abundance to Fe and P amendments were qualitatively consistent with N_2 fixation rates at most sites, that is, the nutrient(s) limiting N_2 fixation rates also limited the diazotroph abundance (Figs. 4 and 5). The exceptions were at stations S3 and S4, where variability in *nifH* abundances was observed in response to nutrient treatment (station S3) or overall trends differed between *nifH* abundances and N_2 fixation rates (station S4; enhanced *nifH* abundance in response to +P, whereas rates only responded to +Fe). Quantitatively, the responses of N_2 fixation

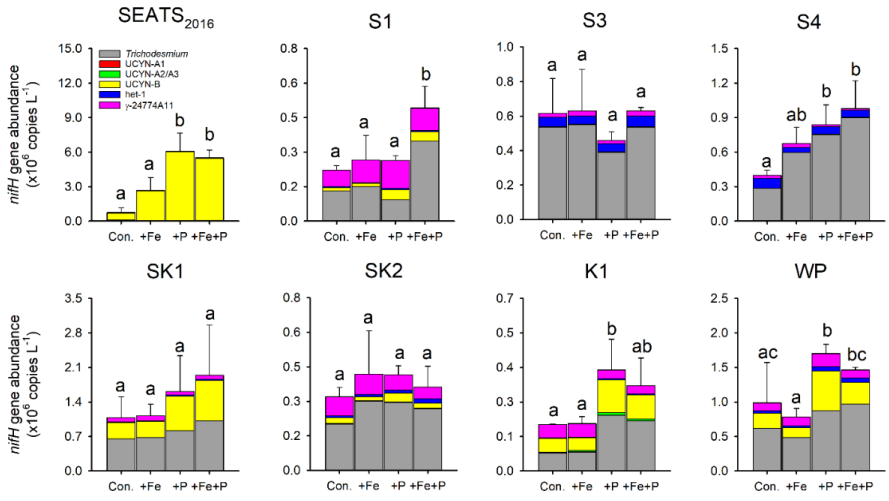

Figure 5. Response of diazotroph phylotypes to nutrient amendment. Bar heights represent the mean total *nifH* concentration and error bars the standard deviation of biological replicates (n = 2 or 3). Different letters above error bars indicate a statistically significant difference ($p < 0.05$) between treatments (ANOVA followed by Fisher PLSD test).

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rates and *nifH* biomass to nutrient addition were not well correlated (total *nifH* abundance increase rate versus N_2 fixation increase rate following nutrient supply, $R^2 = 0.07$, $p = 0.21$; Supplementary Fig. S1), despite initial background *nifH* abundances and N_2 fixation rates being well correlated (Pearson $r = 0.72$, $p = 0.046$, Supplementary Table S1). This suggested a decoupling of the rates of change in biomass and N_2 fixation rates following nutrient addition over the relative short incubation timescales (~3 days).

Overall, the diazotroph community structure was not greatly changed after nutrient amendments (Fig. 5). *Trichodesmium* and UCYN-B were the two most dominant species in all experimental waters that contributed to the enhanced total *nifH* gene abundance after nutrient additions (Figs. 3, 5 and Supplementary Fig. S1). Despite showing independent co-limitation in response to Fe and P supply at station $SEATS_{2016}$ (Fig. 4), as reflected by equally responding N_2 fixation rates, UCYN-B, the dominant diazotroph in non-amended control waters, increased 2-fold more following P addition in comparison to Fe addition (Fig. 5 and Supplementary Fig. S2). Furthermore, no significant changes in *nifH* were observed at station S3, where N_2 fixation rates were also independently Fe-P co-limited. More consistent between the N_2 fixation rates and *nifH* biomass changes were the *nifH* responses at station S1, with overall *nifH* concentrations only responding to Fe+P additions, matching the N_2 fixation response. This was mostly driven by co-limitation of *Trichodesmium*, whereas UCYN-B responded only to P supply (Fig. 5 and

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Supplementary Fig. S2).

In contrast to the Fe limitation of N_2 fixation rates found at station S4, *nifH* abundances showed the most significant responses to the combined supply of Fe and P. However, at sites where N_2 fixation rates were P-limited (K1 and WP) overall *nifH* concentrations also responded most to P addition, with contributions from both *Trichodesmium* and UCYN-B (Fig. 5). In addition, het-1 also increased significantly with +P combinations at stations K1 and WP (Supplementary Fig. S2). By contrast, γ-24774A11, which also accounted for a substantial fraction of the diazotroph community (up to 31%), did not show clear enhancement to nutrient additions (Supplementary Fig. S2), suggesting that it was not Fe- and/or P-limited.

4 Discussion

In the present study, rates and *nifH* gene abundances were much higher in the northeast region of our study area than in the NSCS basin (Fig. 3). Rates at stations SK1 and WP were comparable to those recently reported in this region (~450 μmol N m^{-2} d^{-1}) measured using the same $^{15}N_2$ gas dissolution method (Lu et al., 2019; Wen et al., 2022). Although relatively low rates were measured at the Kuroshio Current station (K1) compared with previous observations (e.g., Wen et al., 2022), high *nifH* gene abundance was nevertheless observed at this site (Fig. 3 and Supplementary Table S3). Therefore,

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our observations provide increasing evidence for this western (sub)tropical North Pacific boundary region containing important "hot spots" of N_2 fixation (Shiozaki et al., 2010; Shiozaki et al., 2015a; Wen et al., 2022). However, the elevated total *nifH* concentration in the western boundary of the North Pacific during our study was largely attributed to an increased abundance of unicellular diazotrophs (UCYN-A and B, Fig. 3), but not *Trichodesmium* as previously reported (Chen et al., 2003; Chen et al., 2014; Chen et al., 2008; Shiozaki et al., 2014a). Instead, we found that *Trichodesmium* was most abundant at stations (S3 and S4) close to the western edge of the Luzon Strait (Fig. 3 and Supplementary Table S3), where Kuroshio intrusion water has been hypothesized to introduce *Trichodesmium* into a favorable biogeographic regime (Lu et al., 2019). Either this region is spatially and/or temporally heterogeneous with respect to the presence of unicellular versus *Trichodesmium* diazotrophs, or the environmental changes have led to a shift in diazotroph community structure (Gruber, 2011; Hutchins and Fu, 2017).

Depth-integrated N_2 fixation rate and *nifH* gene abundance were not correlated with sea surface temperature (SST), but a significant positive correlation was found between nitracline depth and total *nifH* gene abundance (Pearson $r = 0.74$, $p = 0.037$, Supplementary Table S2). This was suggestive of subsurface N supply into the euphotic zone, which is inversely related to nitracline depth, potentially being important in regulating diazotroph abundance in our study area, with lower N supply leading to

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enhanced diazotroph abundances (Chen et al., 2003; Shiozaki et al., 2014b). The presence of diazotrophs in the ocean will be a function of how well they can compete with non-diazotrophic phytoplankton for limiting resources (e.g., Fe and P) under grazing pressure (Dutkiewicz et al., 2014; Landolfi et al., 2021; Ward et al., 2013). Accordingly, because of the growth characteristics of diazotrophs in comparison to non-diazotrophs, in particular their lack of requirement for pre-fixed N, but higher requirement for Fe and P, the relative supply rates of N, Fe and P are highly important in dictating where diazotrophs can succeed (Ward et al., 2013). Aligning with earlier global model predictions (Ward et al., 2013), and investigations in the (sub)tropical Atlantic (Schlosser et al., 2014), Wen et al. (2022) recently found that the Fe:N supply ratio (including subsurface and aerosol N and Fe supplies) was a robust predictor of diazotroph standing stock across the broader western North Pacific, including our study region.

Although the current study lacked the data to calculate nutrient supply rates into the euphotic zone (matching Fe concentration profiles, euphotic depths), the correlation found between *nifH* and nitracline depth suggested the potential for the same driver (i.e., Fe:N supply rates) to be operating over this smaller spatial scale. In line with Wen et al. (2022), we further hypothesize that the expected significant N supply rate to surface waters of the NSCS (due to a shallower nitracline, alongside riverine and aerosol inputs) reduces, but does not eliminate the competitive ability of diazotrophs, as Fe supply rates

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to this region are likely also high (Duce et al., 1991; Jickells et al., 2005; Zhang et al., 2019), thereby maintaining Fe:N supply ratios at levels supporting diazotrophs (Ward et al., 2013; Wen et al., 2022). At these Fe:N supply levels, we observed that N_2 fixation rates were either (i) 'simultaneously co-limited' by Fe and P (identified at station S1), which represents a state where two, non-substitutable nutrients (in this case, Fe and P) have been drawn down to equally limiting levels (Sperfeld et al., 2016), or (ii) 'independently co-limited' (stations $SEATS_{2016}$ and S3), which represents a state where the resources are substitutable at biogeochemical (Saito et al., 2008), or community levels (Arrigo, 2005).

The measured contributions of individual diazotrophs to total *nifH* concentration in response to nutrient supply suggested that simultaneous Fe-P co-limitation of N_2 fixation rates at station S1 was via regulation of *Trichodesmium*, which only responded to Fe+P addition (Fig. 5). The *nifH* responses also suggested that independent Fe-P co-limitation of N_2 fixation rates at sites $SEATS_{2016}$ and S3 was not operating at the community level (i.e., one diazotroph type limited by Fe and the other by P) (Arrigo, 2005), as different diazotroph community structure responses to either Fe or P addition were not observed (Fig. 5). We suggest three possible causes for this observation: (i) co-limitation was at the biochemical rather than community level (i.e., either Fe or P could enhance the rates of processes ultimately driving elevated N_2 fixation) (Saito et al., 2008); (ii) a more subtle

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community co-limitation was occurring at the level of ecotypes not resolved by the *nifH* qPCR analyses; or (iii) community co-limitation of N_2 fixation rates for the measured groups was occurring, but, unlike the simultaneous co-limitation scenario at station S1, experimental durations were too short for this to be reflected in diazotroph biomass changes. Surprisingly, stations with independent co-limitation of N_2 fixation rates by Fe and P ($SEATS_{2016}$ and S3) were not additive (i.e., increases in N_2 fixation rates in Fe+P treatments were not larger than Fe and P alone) (Sperfeld et al., 2016). Although the available data do not allow us to provide a concrete reason for this, it could reflect serial limitation of N_2 fixation by another resource (e.g., a different nutrient or light).

In contrast to the more central NSCS, in the western boundary of the North Pacific, elevated Fe:N supply ratios are expected as a result of deepening nitraclines (Fig. 2 and Table 1) and continued aerosol Fe inputs (Wen et al., 2022). Additional Fe inputs other than aerosol deposition may have also contributed to further enhanced Fe:N supply in the Luzon Strait. At station SK2, much higher surface particulate Fe concentrations (both intracellular and total forms) were observed (Supplementary Table S4), implying additional Fe inputs, potentially sourced from the adjacent islands and the surrounding shallow sub-surface bathymetry (Shiozaki et al., 2014a; Shiozaki et al., 2015a). In turn we hypothesize that elevated Fe:N supply rates enhance N_2 fixation rates at these sites (Fig. 3), which leads to P drawdown and subsequent P limitation of the enhanced

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diazotroph stock (Figs. 4 and 5; Hashihama et al., 2009; Ward et al., 2013; Wen et al., 2022).

In addition to the Fe:N supply ratio regulating the total *nifH* gene abundance and activity (Wen et al., 2022), we also further hypothesize that overall Fe supply rates might be an important factor in determining the diazotroph community structure in our study area (Church et al., 2008; Langlois et al., 2008; Shiozaki et al., 2017). Specifically, the depth-integrated diazotroph compositions switched from being co-dominated by *Trichodesmium* and other diazotrophs in the central NSCS (SEATS, S1 and S2), *Trichodesmium*-dominated in the more northern NSCS (S3 and S4), and finally dominated by UCYN-B in the western boundary of the North Pacific (Fig. 3 and Supplementary Table S3). Elevated Fe supply in the NSCS, particularly around the islands and shallow bathymetry of the Luzon Strait, might create a more favorable condition for *Trichodesmium* (Fig. 3 and Supplementary Table S3), consistent with elevated Fe demands of this species (Kupper et al., 2008; Sohm et al., 2011), as well as its ability to use particulate Fe forms (Rubin et al., 2011), and in line with the elevated contribution of this species found in other regions with enhanced Fe supply (e.g., the tropical North Atlantic and western South Pacific; Bonnet et al. 2018; Sañudo-Wilhelmy et al., 2001; Sohm et al., 2011; Stenegren et al., 2018). Conversely, unicellular species may be more competitive than *Trichodesmium* in regions with lower Fe supply rates (Fig. 3). In addition to having a higher surface to

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volume ratio that favors Fe uptake (Hudson and Morel 1990; Jacq et al., 2014), UCYN-B species such as *Crocosphaera* have been reported to employ a repertoire of Fe-conservation strategies, e.g., daily synthesis and breakdown of metalloproteins to recycle Fe between the photosynthetic and N_2 fixation metalloenzymes and increased expression of flavodoxin at night even under Fe-replete conditions (Saito et al., 2011). These potentially explain why UCYN-B was less Fe-limited in the NSCS basin (stations $SEATS_{2016}$ and S1; Fig. 5 and Supplementary Fig. S2) and dominates the diazotroph community on the western Pacific side of the Luzon Strait (Fig. 3; Chen et al., 2019; Wen et al., 2022).

5 Conclusions

Observations and experiments conducted in the NSCS and the western boundary of the North Pacific demonstrated that in the more central NSCS, Fe and P were co-limiting the lower overall observed N_2 fixation rates, whereas P was limiting the higher rates on the western Pacific side of the Luzon Strait. This matched the expectation of higher Fe:N supply ratios in the western Pacific generating a more favorable niche for diazotrophs, leading to a drawdown of P. *Trichodesmium* and UCYN-B were the most dominant diazotroph types in the incubation waters and both dominated the responses of the total *nifH* gene after nutrient amendments. In general, nutrient addition had a relatively restricted impact on diazotroph community structure apart from on UCYN-B, which

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showed increased contribution in the diazotroph community following P addition at sites where N_2 fixation rates were P-limited. We hypothesize that overall switches in diazotroph community structure from *Trichodesmium*-dominated in the NSCS to single-celled UCYNA/B was related to declines in overall Fe supply rates and the different physiological strategies of these diazotrophs to obtain and use Fe. Future research that more accurately constrains nutrient supply rates to these different regions would be beneficial for further resolving this hypothesis.

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Data availability. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data associated with the paper are available from the corresponding authors upon request.

Author contributions. D.S., H.H., and Z.W. designed the research. Z.W., R.D., W.W., W.L., X.H., W.L., and L.W. performed the experiments. Z.W., D.S., H.H., T.J.B., X.L., and Z.C. analyzed the data. Z.W., T.J.B., H.H., and D.S. wrote the manuscript. All authors discussed the results and commented and edited the manuscript.

Competing interests. The authors declare that they have no conflict of interest.

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