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

Zuozhu Wen[1,2], Thomas J. Browning[2], Rongbo Dai[1], Wenwei Wu[1], Weiying Li[1,a], Xiaohua Hu[1], Wenfang Lin[1], Lifang Wang[1], Xin Liu[1], Zhimian Cao[1], Haizheng Hong[1], and Dalin Shi[1]

[1]State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, Fujian, PR China

[2]Marine Biogeochemistry Division, GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany

Correspondence to: Dalin Shi (dshi@xmu.edu.cn) and Haizheng Hong (honghz@xmu.edu.cn)

a. Present address: Key Laboratory of Marine Ecosystem Dynamics, Second Institute of Oceanography, Ministry of Natural Resources, Hangzhou, Zhejiang, PR China

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 *nifH* gene 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 *nifH* gene abundances of specific diazotrophs were measured. Overall, nitrogen fixation rates and *nifH* gene abundances were lower in the NSCS than around the Luzon Strait and the western North Pacific. The nutrient addition bioassay experiments demonstrated that N_2 fixation rates in the central 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 N_2 fixation rates, and in 6 out of 8 experiments the largest responses in *nifH* gene abundances were dominated by either *Trichodesmium* or UCYN-B. In general, nutrient addition had a relatively restricted impact on the composition of the six phylotypes that we surveyed apart from on UCYN-B. This unicellular cyanobacterium group showed increased contribution to the total *nifH* gene abundance following P addition at sites where N_2 fixation rates were P-

limited. Our study provides comprehensive evidence of nutrient controls on N_2 fixation biogeography in the margin of the western North Pacific. Future research that more accurately constrains nutrient supply rates to this region would be beneficial for resolving what controls diazotroph community structure.

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

time (Mills et al., 2004; Needoba et al., 2007; Grabowski et al., 2008; Watkins-Brandt et al., 2011; Langlois et al., 2012; Turk-Kubo et al., 2012; Dekaezemacker et al., 2013; Krupke et al., 2015; Tanita et al., 2021; Wen et al., 2022). 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 did 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, is 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

unique physical and biogeochemical characteristics (Du et al., 2013; Guo et al., 2017; Xu et al., 2018; Huang et al., 2019; Lu et al., 2019; Li et al., 2021). 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., 2008; Chen et al., 2014;Wu et al., 2018; Lu et al., 2019), 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

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 Kuroshio intrusion generates a frontal zone with a unique diazotrophy regime in the NSCS (Lu et al., 2019), and thus the relatively lower spatial resolution of the experiments in Wen et al. (2022) and other studies (Shiozaki et al., 2014b; Chen et al., 2019) remains 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 (Shiozaki et al., 2014b; Chen et al., 2019; 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 *nifH* gene 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 (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).

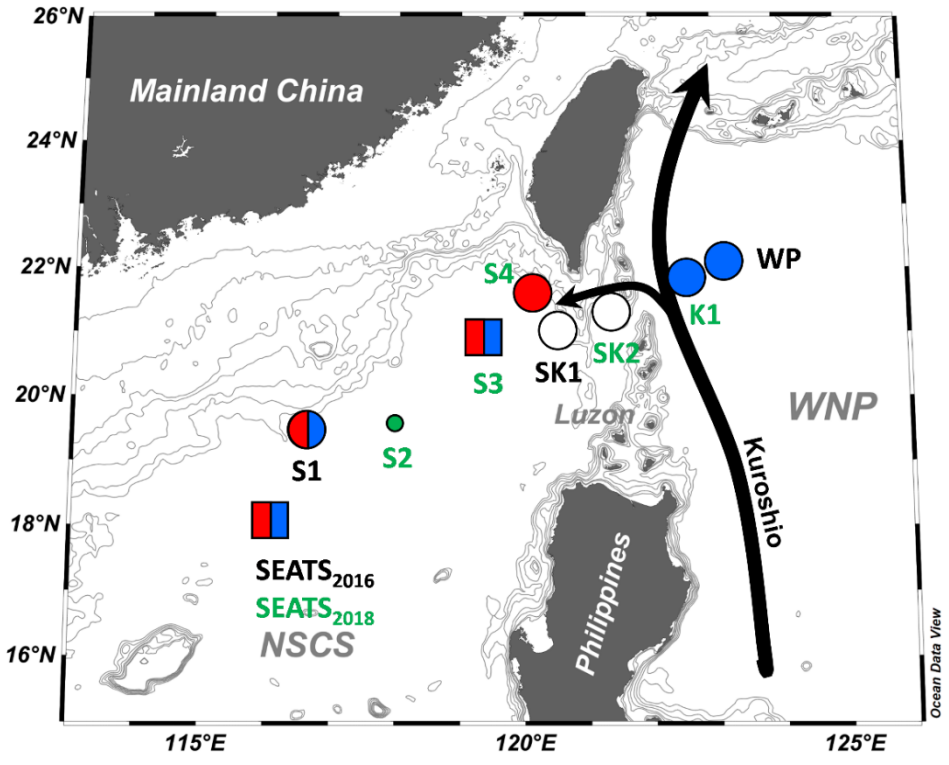


Figure 1. Sampling and nutrient amendment experiment locations in the northern South China Sea and the western boundary of the North Pacific. One station ($SEATS_{2016}$) was sampled in 2016, three (S1, SK1, WP) were in 2017, and six (stations with green labels) were in 2018. Nutrient amendment experiments 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.

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 conditions for the determination of total particulate Fe concentration.

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 (Tedlar®PVF, Dalian Delin Gas Packing Co., Ltd) 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% (ranges from 1.28 to 1.56 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 to a final amended 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 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

198 then extracted using the QIAamp® DNA Mini Kit (Qiagen) following the manufacturer's
199 protocol. The quantitative polymerase chain reaction (qPCR) analysis was targeted on the
200 *nifH* phylotypes of *Trichodesmium* spp., unicellular cyanobacterial UCYN-A1, UCYN-
201 A2, and UCYN-B, *Richelia* spp. (het-1), and a gamma-proteobacterium (γ-24774A11),
202 using previously designed primers and probe sets (Supplementary Table S1; Church et al.,
203 2005a; Church et al., 2005b; Moisander et al., 2008; Thompson et al., 2014). A recent
204 study suggested that the primers for UCYN-A2 also target UCYN-A3 and thus cannot be
205 used to differentiate between these two phylotypes (Farnelid et al., 2016). Therefore, we
206 used the convention UCYN-A2/A3 when referring to these two groups. The *nifH*
207 standards were obtained by cloning the environmental sequences from previous samples
208 collected from the SCS. qPCR analysis was carried out as described previously (Church
209 et al., 2005a) with slight modifications. Triplicate qPCR reactions were run for each
210 environmental DNA sample and for each standard on a CFX96 Real-Time System (Bio-
211 Rad Laboratories). Standards corresponding to between 10^1 and 10^7 copies per well were
212 amplified in the same 96-well plate. The amplification efficiencies of PCR were always
213 between 90-105%, with R^2 values > 0.99. The quantification limit of the qPCR reactions
214 was 10 *nifH* gene copies per reaction, and 1 μL from 100 or 150 μL template DNA was
215 applied to qPCR assay, which was equivalent to approximately ~230-350 gene copies per
216 L of seawater sample filtered (4.3 L).
217

A previous study has reported that *nifH* gene polyploidy exists in *Trichodesmium* (Sargent et al., 2016), which may impact the estimates of diazotroph compositions. However, given that the degree of polyploidy can vary significantly (ranging from 1 to 1405; Sargent et al., 2016; White et al., 2018), with a potential dependence on the growth conditions, nutrient status, developmental stage, and cell cycle (see references in Karlusich et al., 2021), we did not attempt to account/correct for this in calculations of proportions of the different diazotrophs.

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 in experimental setup and manipulations. All materials including the degas unit and Tedlar®PVF bags that came 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. Surface dissolved Fe and P concentrations previously reported in the NSCS were 0.17-1.01 nM and 5-20 nM respectively (Wu et al., 2003; Zhang et al., 2019). In order to obtain a measurable response within the relatively short 72-hour experimental period, 2 nM Fe and 100 nM P (chelexed and filter-sterilized) were added to each of the treatment bottles. Control bottles incubated with no nutrient treatment were included in all experiments. Treatments for 7 out of 8 experiments were conducted with 3 replicates.

However, there were three cases when one of the triplicate samples was lost due to filtration errors (one +Fe+P carboy at station S1, one NFR/PP sample of +Fe+P at station WP, and one NFR/PP sample of +P sample at station S3). In addition, for the bioassay experiment at station $SEATS_{2016}$, sufficient water was only available to conduct the experiment with 2 replicates for control and +Fe+P treatments, while +Fe and +P retained 3 replicates. All carboys were then incubated for 3 days in a screened on-deck incubator (a ~400-L clear on-deck incubator with inflow and outflow) continuously flushed with surface seawater. After 72 hours pre-incubation, subsamples were collected for the determination of Chl *a* concentration, 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). Surface seawaters were additionally sampled for the measurement of low-level nutrient concentrations in the 2018 cruise and NO_3^-+NO_2^- was determined

following Zhang (2000) whilst PO_4^{3-} was analyzed following Ma et al., (2008). Samples for chlorophyll *a* analysis were collected on nominal 0.7 μm pore-size GF/F filters (Whatman), extracted in 90% acetone, and analyzed fluorometrically on a Turner Designs fluorometer (Welschmeyer 1994).

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. It should be noted that

278 those statistical results that were produced from only two replicates are not strictly

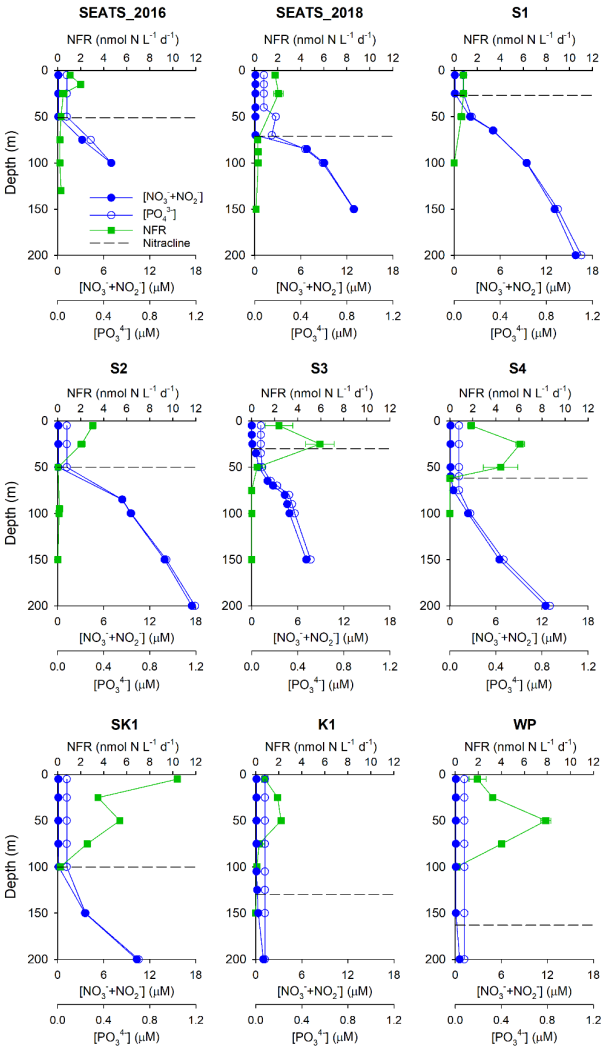
279 statistically valid, but for completeness the posthoc test results are nevertheless still

280 included here.

281

282 # 3 Results

283 ## 3.1 Spatial variations of N_2 fixation rates and diazotroph composition



284

285

286 **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.

Our survey revealed substantial spatial variability in N_2 fixation rates and *nifH* gene abundances across the study area (Figs. 2 and 3). Vertically, high N_2 fixation rates were found in the upper 50 m (ranged from below 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).

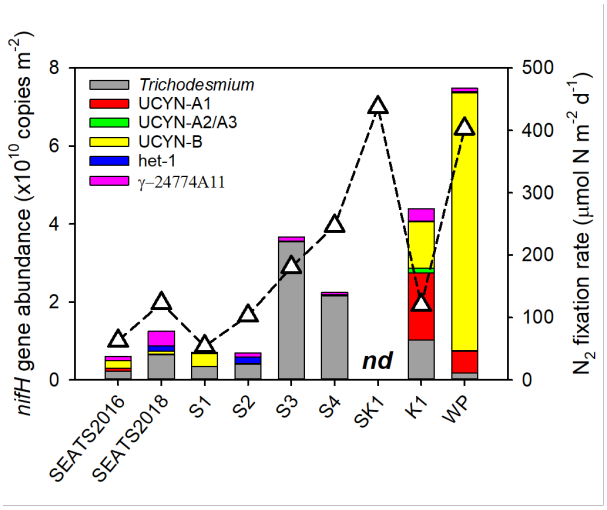


Figure 3. Depth-integrated (upper 150 m) *nifH* gene abundances (bars) and N_2 fixation rates (triangles). Note that depth-integrated N_2 fixation rates and *nifH* gene abundances were not available at station SK2. nd, not determined.

Table 1. Environmental conditions, N_2 fixation, and primary production rates. Sea surface temperature (SST) and salinity (SSS), chlorophyll *a* concentration, surface dissolved inorganic nitrogen (SDIN) and phosphorus (SDIP), 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)	SDIN (nM)	SDIP (nM)	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	nd	nd	51	1.1	63	44
$SEATS_{2018}$	30.3	33.46	0.11	9.5	16.8	71	1.8	123	24
S1	29.5	33.73	0.24	nd	nd	27	0.8	54	43
S2	29.4	33.75	0.10	9.6	13.0	50	3.0	103	24
S3	28.7	33.53	0.15	11.1	16.2	30	2.4	181	98
S4	29.5	33.74	0.17	5.1	11.7	62	1.8	247	59
SK1	30.5	33.62	0.22	nd	nd	100	10.4	437	11
SK2	nd	nd	0.11	nd	nd	nd	2.0	nd	nd
K1	29.1	34.45	0.11	5.9	16.8	130	0.8	120	19
WP	30.9	34.47	0.11	nd	nd	163	1.9	403	9

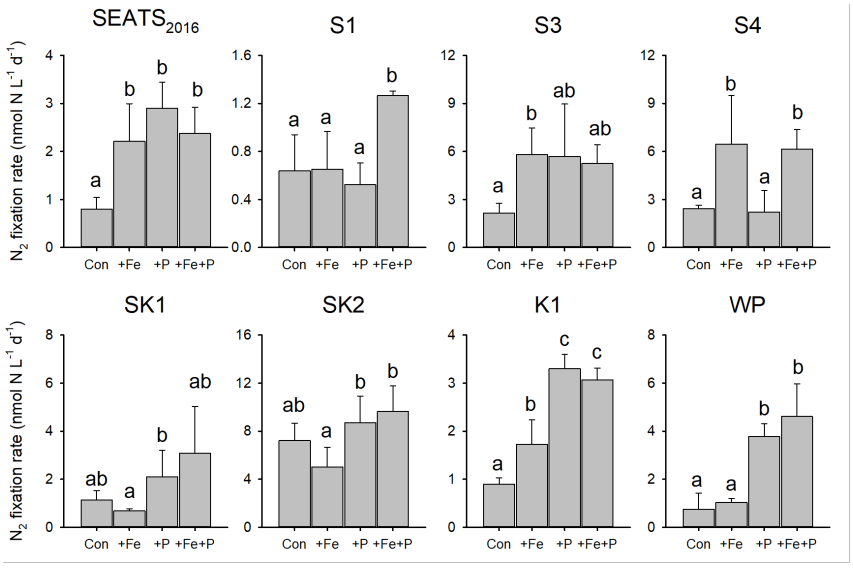
A significant positive correlation was found between the depth-integrated *nifH* gene abundance and N_2 fixation rate (Pearson $r = 0.72$, $p = 0.046$, Supplementary Table S2), demonstrating that the *nifH* gene abundances of these major diazotroph phylotypes that we surveyed well explained the major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

fixation (Fig. 3). *Trichodesmium* dominated the total *nifH* gene abundance 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 *nifH* gene phylotypes, and at station WP, UCYN-B alone was dominant (Fig. 3 and Supplementary Table S3). It should be noted that *nifH* gene polyploidy exists in *Trichodesmium* (Sargent et al., 2016), which may have an important impact on the estimates of both in situ and nutrient-treated diazotroph compositions (see Method).

3.2 Diazotroph response to Fe and P supply

To directly test which nutrients were limiting overall N_2 fixation rates and the *nifH* gene abundances 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', station $SEATS_{2016}$, Fig 4). For the experiment conducted at station S3, in

339 which N_2 fixation was also recognized to be independently co-limited, the rates in all
340 nutrient-amended groups increased by 1.44-1.70 times compared to control values,
341 although statistical significances were not observed in +P and +Fe+P treatments (Fig. 4).
342 Further to the northeast, in contrast, N_2 fixation rates were only stimulated by nutrient
343 combinations containing Fe at S4 and by combinations containing P at K1 and WP,
344 suggesting single limitation by Fe or P, respectively, at these sites (Fig. 4). Although Fe
345 addition also appeared to stimulate N_2 fixation rates at station K1, P was generally the
346 major limiting nutrient at this station taking into account the responses of both N_2 fixation
347 rates and *nifH* gene abundance (see below) (Figs. 4 and 5). At stations SK1 and SK2 in
348 the Luzon Strait, mean N_2 fixation rates and *nifH* gene abundances were not significantly
349 affected by nutrient amendments, suggesting that both Fe and P availability were not
350 limiting N_2 fixation rates (Figs. 4 and 5).
351



352

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). Note that statistical results were produced from only two replicates for control and +Fe+P at station $SEATS_{2016}$, +Fe+P at stations S1 and WP, and +P at station S3, and should thus be treated with caution.

Further detail as to the drivers of the N_2 fixation responses to Fe and P additions was 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 *nifH* 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 rates and *nifH* biomass to nutrient addition were not well correlated (total *nifH* abundance increase rate, calculated as Ln(*nifH*_treatment/*nifH*_control)/incubation time, versus the 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).

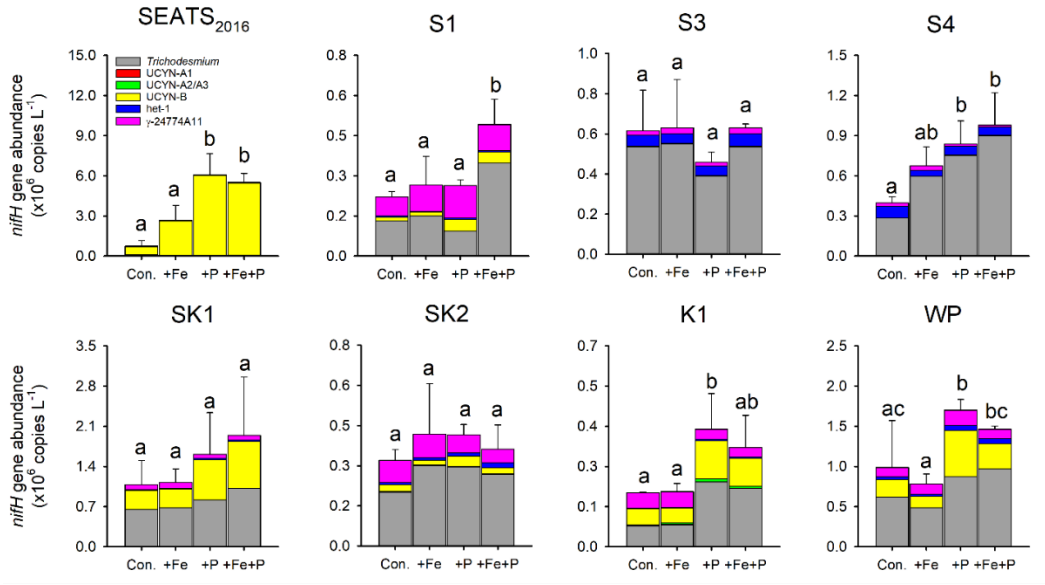


Figure 5. Response of diazotroph phylotypes to nutrient amendment. Bar heights represent the mean total *nifH* concentration and error bars the standard deviation of biological replicates (n = 2 or 3). Different letters above error bars indicate a statistically significant difference ($p < 0.05$) between treatments (ANOVA followed by Fisher PLSD test). Note that statistical results were produced from only two replicates for control and +Fe+P at station $SEATS_{2016}$, control and +Fe+P at station S1, and +P and +Fe+P at station SK2, and should thus be treated with caution.

Overall, the composition of the six diazotroph phylotypes that we surveyed were not greatly changed after nutrient amendments (Fig. 5). *Trichodesmium* and UCYN-B were the two most dominant *nifH* phylotypes 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

394 rates, UCYN-B, the dominant *nifH* phylotype in non-amended control waters, increased
395 2-fold more following P addition in comparison to Fe addition (Fig. 5 and Supplementary
396 Fig. S2). Furthermore, no significant changes in *nifH* were observed at station S3, where
397 N_2 fixation rates were also independently Fe-P co-limited. More consistent between the
398 N_2 fixation rates and *nifH* biomass changes were the *nifH* responses at station S1, with
399 overall *nifH* concentrations only responding to Fe+P additions, matching the N_2 fixation
400 response. This was mostly driven by co-limitation of *Trichodesmium*, whereas UCYN-B
401 responded only to P supply (Fig. 5 and Supplementary Fig. S2).
402
403 In contrast to the Fe limitation of N_2 fixation rates found at station S4, *nifH* abundances
404 showed the most significant responses to the combined supply of Fe and P. However, at
405 sites where N_2 fixation rates were P-limited (K1 and WP) overall *nifH* concentrations also
406 responded most to P addition, with contributions from both *Trichodesmium* and UCYN-B
407 (Fig. 5). In addition, het-1 also increased significantly with +P combinations at stations
408 K1 and WP (Supplementary Fig. S2). By contrast, γ-24774A11, which also accounted for
409 a substantial fraction of the total *nifH* gene abundance (up to 31%), did not show clear
410 enhancement to nutrient additions (Supplementary Fig. S2), suggesting that it was not Fe-
411 and/or P-limited. Interestingly, UCYN-A disappeared in the nutrient amendment
412 experiments, although they were abundant in the initial water at stations K1 and WP
413 (Figs. 3 and 5), possibly due to an unconstrained bottle effect (Göran et al., 2003).

4 Discussion

In the present study, surface dissolved inorganic nitrogen (DIN) and phosphorus (DIP) concentrations in our study area were depleted (5.1-11.1 nM DIN, 11.7-16.8 nM DIP, Table 1). Furthermore, bioassay incubations shown no significant responses of Chl *a* concentration to the amendments of Fe and/or P (Supplementary Fig. S3), together implying the overall phytoplankton community across entire area was N-limited and the upper waters were favorable for N_2 fixation. However, the 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, 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 *nifH* abundance of unicellular diazotrophs (UCYN-A and B, Fig. 3), but not *Trichodesmium* as previously reported (Chen et al.,

2003; Chen et al., 2008; Chen et al., 2014; Shiozaki et al., 2014a). Instead, we found that *Trichodesmium nifH* gene 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 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 (Ward et al., 2013; Dutkiewicz et al., 2014; Landolfi et al., 2021). 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.

By carrying out bioassay incubations, we observed that N_2 fixation rates in the NSCS basin 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 biochemical (Saito et al., 2008), or community levels (Arrigo, 2005). Previous studies reported relatively low surface Fe concentrations in the NSCS basin (0.2-0.3 nM; Wu et al., 2003; Wen et al., 2022), although Fe supply rates to this region are likely elevated via riverine, sedimental and aerosol inputs (Duce et al., 1991; Jickells et al., 2005; Zhang et al., 2019). Wu et al (2003) hypothesized that the low Fe concentrations were due to a lack of Fe-binding organic ligands, which was subsequently restricting the growth of diazotrophs. We suggest that this may also be

attributed to the rapid consumption of Fe (as well as P) by the faster-growing non-diazotrophs under elevated N supply (due to shallower nitraclines, alongside riverine and aerosol inputs). Thus we hypothesize that with the lower Fe:N supply ratio, diazotrophs in this region were outcompeted by non-diazotrophic phytoplankton and co-limited by both Fe and P (Fig. 4). These results add to increasing evidence for the potentially widespread Fe-P colimitation of N_2 fixation under elevated Fe supply (Mills et al., 2004; Snow et al., 2015; Cerdan-Garcia et al., 2022).

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 qPCR-based 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). For instance, in addition to serving as a cofactor in nitrogenase, Fe is also a cofactor in alkaline phosphatases (Rodriguez et al., 2014; Yong et al., 2014). Thus, the addition of Fe

494 may allow for enhanced utilization of dissolved organic P (DOP) under depleted DIP
495 (Browning et al., 2017); (ii) other diazotrophs, which were not analyzed by the qPCR
496 assay, may be responsible for the enhanced N_2 fixation rates after nutrient additions; or
497 (iii) community co-limitation of N_2 fixation rates for the measured groups was occurring,
498 but, unlike the simultaneous co-limitation scenario at station S1, experimental durations
499 were too short for this to be reflected in diazotroph biomass changes. Surprisingly,
500 stations with independent co-limitation of N_2 fixation rates by Fe and P ($SEATS_{2016}$ and
501 S3) were not additive (i.e., increases in N_2 fixation rates in Fe+P treatments were not
502 larger than Fe and P alone; Sperfeld et al., 2016). Although the available data do not
503 allow us to provide a concrete reason for this, the absence of this additive response may
504 reflect one or a combination of (i) addition of Fe or P leading to the depletion of another
505 secondary limitation nutrient (e.g., Ni), (ii) overall light levels setting an upper limit of N_2
506 fixation rates, which prevented further enhancements after nutrient additions, or (iii)
507 grazer regulation of diazotroph biomass accumulation.
508
509 In contrast to the more central NSCS, the deeper nitraclines in the western boundary of
510 the North Pacific appeared more favorable for N_2 fixation (Fig. 3 and Table 1). P
511 limitation of N_2 fixation at these sites implied that Fe supply (e.g., via aerosols)
512 stimulated diazotroph growth (Fig. 3; Wen et al., 2022) and subsequently drawdown P to
513 limiting levels (Table 1, Figs 4 and 5, Hashihama et al., 2009; Ward et al., 2013; Wen et

al., 2022). Additional Fe inputs other than aerosol deposition are also potentially important in supporting the elevated N_2 fixation in the Luzon Strait. At station SK2, much higher surface particulate Fe concentrations (both intracellular and total forms) were observed (Supplementary Table S4), implying supplementary Fe inputs potentially sourced from the adjacent islands and the surrounding shallow sub-surface bathymetry (Shiozaki et al., 2014a; Shiozaki et al., 2015a).

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 of the six phylotypes 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 (Kustka et al., 2003; 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

534 with the elevated contribution of this species found in other regions with enhanced Fe
535 supply (e.g., the tropical North Atlantic and western South Pacific; Sañudo-Wilhelmy et
536 al., 2001; Sohm et al., 2011; Bonnet et al. 2018; Stenegren et al., 2018). In fact, Fe
537 stimulation of *Trichodesmium nifH* abundance was observed in the experiment conducted
538 at station S4 (Supplementary Fig. S2). At station S3, however, this was only observed for
539 N_2 fixation rates but not *Trichodesmium nifH* abundance (Supplementary Fig. S2). We
540 suggest this could reflect a variable decoupling of N_2 fixation rates and diazotroph
541 abundance, depending on other environmental and/or ecological conditions. Conversely,
542 unicellular species may be more competitive than *Trichodesmium* in regions with lower
543 Fe supply rates (Fig. 3). In addition to having a higher surface to volume ratio that favors
544 Fe uptake (Hudson and Morel 1990; Jacq et al., 2014), UCYN-B species such as
545 *Crocosphaera* have been reported to employ a repertoire of Fe-conservation strategies,
546 e.g., daily synthesis and breakdown of metalloproteins to recycle Fe between the
547 photosynthetic and N_2 fixation metalloenzymes and increased expression of flavodoxin at
548 night even under Fe-replete conditions (Saito et al., 2011). These potentially explain why
549 UCYN-B was less Fe-limited in the NSCS basin (stations $SEATS_{2016}$ and S1; Fig. 5 and
550 Supplementary Fig. S2) and dominates the diazotroph community on the western Pacific
551 side of the Luzon Strait (Fig. 3; Chen et al., 2019; Wen et al., 2022). Future work with
552 paired measurements of Fe supply rates to surface waters and diazotroph community
553 structure throughout the region would allow for more robust testing of this hypothesis.

554

555 # 5 Conclusions

556 Observations and experiments conducted in the NSCS and the western boundary of the
557 North Pacific demonstrated that in the more central NSCS, Fe and P were co-limiting the
558 lower overall observed N_2 fixation rates, whereas P was limiting the higher rates on the
559 western Pacific side of the Luzon Strait. This matched the expectation of higher Fe:N
560 supply ratios in the western Pacific generating a more favorable niche for diazotrophs,
561 leading to a drawdown of P. *Trichodesmium* and UCYN-B were the most dominant *nifH*
562 phylotypes in the incubation waters and both dominated the responses of the total *nifH*
563 gene after nutrient amendments. In general, nutrient addition had a relatively restricted
564 impact on qPCR-based diazotroph community structure apart from on UCYN-B, which
565 showed increased contribution in the diazotroph community following P addition at sites
566 where N_2 fixation rates were P-limited. We hypothesize that overall switches in
567 diazotroph community structure from *Trichodesmium*-dominated in the NSCS to single-
568 celled UCYNA/B was related to declines in overall Fe supply rates and the different
569 physiological strategies of these diazotrophs to obtain and use Fe. Future research that
570 more accurately constrains nutrient supply rates to these different regions would be
571 beneficial for further resolving this hypothesis.

572 *Data availability.* All data needed to evaluate the conclusions in the paper are present in
573 the paper and/or the Supplementary Materials. Additional data associated with the paper
574 are available from the corresponding authors upon request.
575
576 *Author contributions.* D.S., H.H., and Z.W. designed the research. Z.W., R.D., W.W.,
577 W.L., X.H., W.L., and L.W. performed the experiments. Z.W., D.S., H.H., T.J.B., X.L.,
578 and Z.C. analyzed the data. Z.W., T.J.B., H.H., and D.S. wrote the manuscript. All authors
579 discussed the results and commented and edited the manuscript.
580
581 *Competing interests.* The authors declare that they have no conflict of interest.
582
583 *Acknowledgements.* The authors acknowledge the captains and crew of the R/V
584 *Dongfanghong* 2 and R/V *Tan Kah Kee* for the help during the cruises. This work was
585 supported by the National Science Foundation of China (41890802, 42076149,
586 41925026, 42106041, and 41721005), the “111” Project (BP0719030), and the
587 XPLORER Prize from the Tencent Foundation to D. Shi.

References

Arrigo, K. R.: Marine microorganisms and global nutrient cycles, Nature, 437, 349-355, 10.1038/nature04158, 2005.

Berman-Frank, I., Cullen, J. T., Shaked, Y., Sherrell, R. M., and Falkowski, P.: Iron availability, cellular iron quotas, and nitrogen fixation in *Trichodesmium*, Limnol. Oceanogr., 46, 1249-1260, 10.4319/lo.2001.46.6.1249, 2001.

Bonnet, S., Baklouti, M., Gimenez, A., Berthelot, H., and Berman-Frank, I.: Biogeochemical and biological impacts of diazotroph blooms in a low-nutrient, low-chlorophyll ecosystem: synthesis from the VAHINE mesocosm experiment (New Caledonia), Biogeosciences, 13, 4461-4479, 10.5194/bg-13-4461-2016, 2016.

Bonnet, S., Caffin, M., Berthelot, H., Grosso, O., Benavides, M., Helias-Nunige, S., Guieu, C., Stenegren, M., and Foster, R. A.: In-depth characterization of diazotroph activity across the Western Tropical South Pacific hot spot of N_2 fixation (OUTPACE cruise), Biogeosciences, 15, 4215-4232, 10.5194/bg-15-4215-2018, 2018.

Boström, K. H., Simu, K., Hagström, Å., and Riemann, L.: Optimization of DNA extraction for quantitative marine bacterioplankton community analysis, Limnol. Oceanogr.-Methods, 2, 365-373, 10.4319/lom.2004.2.365, 2004.

Böttjer, D., Dore, J. E., Karl, D. M., Letelier, R. M., Mahaffey, C., Wilson, S. T., Zehr, J. P., and Church, M. J.: Temporal variability of nitrogen fixation and particulate

nitrogen export at Station ALOHA, Limnol. Oceanogr., 62, 200-216, 10.1002/lno.10386, 2016.

Browning, T. J., Achterberg, E. P., Yong, J. C., Rapp, I., Utermann, C., Engel, A., and Moore, C. M.: Iron limitation of microbial phosphorus acquisition in the tropical North Atlantic, Limnol. Oceanogr. Lett., 8, 15465, 10.1038/ncomms15465, 2017.

Cerdan-Garcia, E., Baylay, A., Polyviou, D., Woodward, E. M. S., Wrightson, L., Mahaffey, C., Lohan, M. C., Moore, C. M., Bibby, T. S., and Robidart, J. C.: Transcriptional responses of *Trichodesmium* to natural inverse gradients of Fe and P availability, Isme J, 16, 1055-1064, 10.1038/s41396-021-01151-1, 2022.

Chen, M., Lu, Y., Jiao, N., Tian, J., Kao, S. J., and Zhang, Y.: Biogeographic drivers of diazotrophs in the western Pacific Ocean, Limnol. Oceanogr., 64, 1403-1421, 10.1002/lno.11123, 2019.

Chen, Y. L. L., Chen, H. Y., and Lin, Y. H.: Distribution and downward flux of *Trichodesmium* in the South China Sea as influenced by the transport from the Kuroshio Current, Mar. Ecol. Prog. Ser., 259, 47-57, 10.3354/meps259047, 2003.

Chen, Y. L. L., Chen, H. Y., Tuo, S. H., and Ohki, K.: Seasonal dynamics of new production from *Trichodesmium* N_2 fixation and nitrate uptake in the upstream Kuroshio and South China Sea basin, Limnol. Oceanogr., 53, 1705-1721, 10.4319/lo.2008.53.5.1705, 2008.

Chen, Y. L. L., Chen, H. Y., Lin, Y. H., Yong, T. C., Taniuchi, Y., and Tuo, S. H.: The

relative contributions of unicellular and filamentous diazotrophs to N_2 fixation in the South China Sea and the upstream Kuroshio, Deep Sea Research Part I, 85, 56-71, 10.1016/j.dsr.2013.11.006, 2014.

Church, M. J., Björkman, K., and Karl, D.: Regional distributions of nitrogen-fixing bacteria in the Pacific Ocean, Limnol. Oceanogr., 53, 63-77, 2008.

Church, M. J., Jenkins, B. D., Karl, D. M., and Zehr, J. P.: Vertical distributions of nitrogen fixing phylotypes at Stn ALOHA in the oligotrophic North Pacific Ocean, Aquat. Microb. Ecol., 38, 3-14, 10.3354/ame038003 2005a.

Church, M. J., Short, C. M., Jenkins, B. D., Karl, D. M., and Zehr, J. P.: Temporal patterns of nitrogenase gene (*nifH*) expression in the oligotrophic North Pacific Ocean, Appl. Environ. Microbiol., 71, 5362-5370, 10.1128/AEM.71.9.5362-5370.2005, 2005b.

Dekaezemacker, J., Bonnet, S., Grosso, O., Moutin, T., Bressac, M., and Capone, D. G.: Evidence of active dinitrogen fixation in surface waters of the eastern tropical South Pacific during El Niño and La Niña events and evaluation of its potential nutrient controls, Global Biogeochem. Cycles, 27, 768-779, 10.1002/gbc.20063, 2013.

Du, C., Liu, Z., Dai, M., Kao, S. J., Cao, Z., Zhang, Y., Huang, T., Wang, L., and Li, Y.: Impact of the Kuroshio intrusion on the nutrient inventory in the upper northern South China Sea: Insights from an isopycnal mixing model, Biogeosciences, 10,

6419-6432, 10.5194/bg-10-6419-2013, 2013.

Duce, R. A., Liss, P. S., Merrill, J. T., Atlas, E. L., Buat-Menard, P., Hicks, B. B., Miller, J. M., Prospero, J. M., Arimoto, R., Church, T. M., Ellis, W., Galloway, J. N., Hansen, L., Jickells, T. D., Knap, A. H., Reinhardt, K. H., Schneider, B., Soudine, A., Tokos, J. J., Tsunogai, S., Wollast, R., and Zhou, M.: The atmospheric input of trace species to the world ocean, Global Biogeochem. Cycles, 5, 193-259, 10.1029/91gb01778, 1991.

Dutkiewicz, S., Ward, B. A., Scott, J., and Follows, M. J.: Understanding predicted shifts in diazotroph biogeography using resource competition theory, Biogeosciences, 11, 5445-5461, 10.5194/bg-11-5445-2014, 2014.

Farnelid, H., Turk-Kubo, K., Muñoz-Marín, M. C., and Zehr, J. P.: New insights into the ecology of the globally significant uncultured nitrogen-fixing symbiont UCYN-A, Aquat. Microb. Ecol., 77, 125-138, 10.3354/ame01794, 2016.

Göran, E. and Cooper, S. D.: Scale effects and extrapolation in ecological experiments, Adv. Ecol. Res., 33, 161-213, 10.1016/S0065-2504(03)33011-9, 2003.

Grabowski, M. N. W., Church, M. J., and Karl, D. M.: Nitrogen fixation rates and controls at Stn ALOHA, Aquat. Microb. Ecol., 52, 175-183, 10.3354/ame01209, 2008.

Gruber, N.: Warming up, turning sour, losing breath: ocean biogeochemistry under global change, Philos T R Soc A, 369, 1980-1996, 10.1098/rsta.2011.0003, 2011.

668 Gruber, N. and Galloway, J. N.: An Earth-system perspective of the global nitrogen cycle,
669 Nature, 451, 293-296, 10.1038/nature06592, 2008.
670 Guo, L., Xiu, P., Chai, F., Xue, H. J., Wang, D. X., and Sun, J.: Enhanced chlorophyll
671 concentrations induced by Kuroshio intrusion fronts in the northern South China
672 Sea, Geophys. Res. Lett., 44, 11565-11572, 10.1002/2017GL075336, 2017.
673 Hama, T., Miyazaki, T., Ogawa, Y., Iwakuma, T., Takahashi, M., Otsuki, A., and
674 Ichimura, S.: Measurement of photosynthetic production of a marine phytoplankton
675 population using a stable ^{13}C isotope., Mar. Biol., 73, 31-36, 10.1007/BF00396282,
676 1983.
677 Hashihama, F., Furuya, K., Kitajima, S., Takeda, S., Takemura, T., and Kanda, J.: Macro-
678 scale exhaustion of surface phosphate by dinitrogen fixation in the western North
679 Pacific, Geophys. Res. Lett., 36, L03610, 10.1029/2008gl036866, 2009.
680 Huang, Y., Laws, E. A., Chen, B., and Huang, B.: Stimulation of heterotrophic and
681 autotrophic metabolism in the mixing zone of the Kuroshio Current and northern
682 South China Sea: Implications for export production, J Geophys Res-
683 Biogeosciences, 124, 2645-2661, 10.1029/2018jg004833, 2019.
684 Hudson, R. J. M. and Morel, F. M. M.: Iron transport in marine-phytoplankton - kinetics
685 of cellular and medium coordination reactions, Limnol. Oceanogr., 35, 1002-1020,
686 10.4319/lo.1990.35.5.1002, 1990.
687 Hutchins, D. A. and Fu, F.: Microorganisms and ocean global change, Nat. Microbiol., 2,

17058, 10.1038/nmicrobiol.2017.58, 2017.

Jacq, V., Ridame, C., L'Helguen, S., Kaczmar, F., and Saliot, A.: Response of the unicellular diazotrophic cyanobacterium *Crocosphaera watsonii* to iron limitation, PLoS One, 9, e86749, 10.1371/journal.pone.0086749, 2014.

Jickells, T. D., An, Z. S., Andersen, K. K., Baker, A. R., Bergametti, G., Brooks, N., Cao, J. J., Boyd, P. W., Duce, R. A., Hunter, K. A., Kawahata, H., Kubilay, N., laRoche, J., Liss, P. S., Mahowald, N., Prospero, J. M., Ridgwell, A. J., Tegen, I., and Torres, R.: Global iron connections between desert dust, ocean biogeochemistry, and climate, Science, 308, 67-71, 10.1126/science.1105959, 2005.

Karlusich, J. J. P., Pelletier, E., Lombard, F., Carsique, M., Dvorak, E., Colin, S., Picheral, M., Cornejo-Castillo, F. M., Acinas, S. G., Pepperkok, R., Karsenti, E., de Vargas, C., Wincker, P., Bowler, C., Foster, R. A.: Global distribution patterns of marine nitrogen-fixers by imaging and molecular methods, Nat. Commun., 12, 10.1038/s41467-021-24299-y, 2021.

Krupke, A., Mohr, W., LaRoche, J., Fuchs, B. M., Amann, R. I., and Kuypers, M. M.: The effect of nutrients on carbon and nitrogen fixation by the UCYN-A-haptophyte symbiosis, Isme J, 9, 1635-1647, 10.1038/ismej.2014.253, 2015.

Kustka, A., Sañudo-Wilhelmy, S., Carpenter, E. J., Capone, D. G., and Raven, J. A.: A revised estimate of the iron use efficiency of nitrogen fixation, with special reference to the marine cyanobacterium *Trichodesmium* spp. (cyanophyta), J.

708 Phycol., 39, 12-25, 10.1046/j.1529-8817.2003.01156.x, 2003.
709 Kupper, H., Setlik, I., Seibert, S., Prasil, O., Setlikova, E., Strittmatter, M., Levitan, O.,
710 Lohscheider, J., Adamska, I., and Berman-Frank, I.: Iron limitation in the marine
711 cyanobacterium *Trichodesmium* reveals new insights into regulation of
712 photosynthesis and nitrogen fixation, New Phytol., 179, 784-798, 10.1111/j.1469-
713 8137.2008.02497.x, 2008.
714 Landolfi, A., Prowe, A. E. F., Pahlow, M., Somes, C. J., Chien, C. T., Schartau, M.,
715 Koeve, W., and Oschlies, A.: Can top-down controls expand the ecological niche of
716 marine N_2 fixers?, Front Microbiol, 12, 690200, 10.3389/fmicb.2021.690200, 2021.
717 Langlois, R. J., Hummer, D., and LaRoche, J.: Abundances and distributions of the
718 dominant *nifH* phylotypes in the Northern Atlantic Ocean, Appl. Environ.
719 Microbiol., 74, 1922-1931, 10.1128/AEM.01720-07, 2008.
720 Langlois, R. J., Mills, M. M., Ridame, C., Croot, P., and LaRoche, J.: Diazotrophic
721 bacteria respond to Saharan dust additions, Mar. Ecol. Prog. Ser., 470, 1-14,
722 10.3354/meps10109, 2012.
723 Le Borgne, R., Barber, R. T., Delcroix, T., Inoue, H. Y., Mackey, D. J., and Rodier, M.:
724 Pacific warm pool and divergence: Temporal and zonal variations on the equator
725 and their effects on the biological pump, Deep Sea Research Part II, 49, 2471-2512,
726 10.1016/S0967-0645(02)00045-0, 2002.
727 Li, W., Sunda, W. G., Lin, W., Hong, H., and Shi, D.: The effect of cell size on cellular Zn

and Cd and Zn-Cd-CO_2 colimitation of growth rate in marine diatoms, Limnol. Oceanogr., 65, 2896-2911, 10.1002/lno.11561, 2020.

Li, X., Wu, K., Gu, S., Jiang, P., Li, H., Liu, Z., and Dai, M.: Enhanced biodegradation of dissolved organic carbon in the western boundary Kuroshio Current when intruded to the marginal South China Sea, J Geophys Res-Oceans, 126, e2021JC017585, 10.1029/2021jc017585, 2021.

Lu, Y., Wen, Z., Shi, D., Lin, W., Bonnet, S., Dai, M., and Kao, S. J.: Biogeography of N_2 fixation influenced by the western boundary current intrusion in the South China Sea, J Geophys Res-Oceans, 124, 6983-6996, 10.1029/2018jc014781, 2019.

Ma, J., Yuan, D. X., Liang, Y., and Dai, M. H.: A modified analytical method for the shipboard determination of nanomolar concentrations of orthophosphate in seawater, J Oceanogr, 64, 443-449, 2008.

Mohr, W., Großkopf, T., Wallace, D. W., and LaRoche, J.: Methodological underestimation of oceanic nitrogen fixation rates, PLoS One, 5, e12583, 10.1371/journal.pone.0012583.g001, 2010.

Moisander, P. H., Beinart, R. A., Voss, M., and Zehr, J. P.: Diversity and abundance of diazotrophic microorganisms in the South China Sea during intermonsoon, Isme J, 2, 954-967, 10.1038/ismej.2008.51, 2008.

Moisander, P. H., Zhang, R., Boyle, E. A., Hewson, I., Montoya, J. P., and Zehr, J. P.: Analogous nutrient limitations in unicellular diazotrophs and *Prochlorococcus* in

748 the South Pacific Ocean, Isme J, 6, 733-744, 10.1038/ismej.2011.152, 2012.
749 Montoya, J. P., Voss, M., Kähler, P., and Capone, D. G.: A simple, high-precision, high-
750 sensitivity tracer assay for N_2 fixation, Appl. Environ. Microbiol., 62, 986-993,
751 10.1128/AEM.62.3.986-993.1996, 1996.
752 Needoba, J. A., Foster, R. A., Sakamoto, C., Zehr, J. P., and Johnson, K. S.: Nitrogen
753 fixation by unicellular diazotrophic cyanobacteria in the temperate oligotrophic
754 North Pacific Ocean, Limnol. Oceanogr., 54, 1317–1327,
755 10.4319/lo.2007.52.4.1317, 2007.
756 Rodriguez, F., Lillington, J., Johnson, S., Timmel, C. R., Lea, S. M., and Berks, B. C.:
757 Crystal structure of the bacillus subtilis phosphodiesterase PhoD reveals an iron and
758 calcium-containing active site, J. Biol. Chem., 289, 30889-30899,
759 10.1074/jbc.M114.604892, 2014.
760 Rubin, M., Berman-Frank, I., and Shaked, Y.: Dust- and mineral-iron utilization by the
761 marine dinitrogen-fixer *Trichodesmium*, Nat Geosci, 4, 529-534,
762 10.1038/ngeo1181, 2011.
763 Saito, M. A., Goepfert, T. J., and Ritt, J. T.: Some thoughts on the concept of colimitation:
764 Three definitions and the importance of bioavailability, Limnol. Oceanogr., 53, 276-
765 290, 10.4319/lo.2008.53.1.0276, 2008.
766 Saito, M. A., Bertrand, E. M., Dutkiewicz, S., Bulygin, V. V., Moran, D. M., Monteiro, F.
767 M., Follows, M. J., Valois, F. W., and Waterbury, J. B.: Iron conservation by

reduction of metalloenzyme inventories in the marine diazotroph *Crocosphaera watsonii*, Proc. Natl. Acad. Sci. U.S.A., 108, 2184-2189, 10.1073/pnas.1006943108, 2011.

Sañudo-Wilhelmy, S. A., Kustka, A. B., Gobler, C. J., Hutchins, D. A., Yang, M., Lwiza, K., Burns, J. A., Capone, D. G., Ravenk, J. A., and Carpenter, E. J.: Phosphorus limitation of nitrogen fixation by *Trichodesmiun* in the central Atlantic Ocean, Nature, 411, 66-69, 10.1038/35075041, 2001.

Sargent, E. C., Hitchcock, A., Johansson, S. A., Langlois, R., Moore, C. M., LaRoche, J., Poulton, A. J., and Bibby, T. S.: Evidence for polyploidy in the globally important diazotroph *Trichodesmium*, FEMS Microbiol. Lett., 363, 10.1093/femsle/fnw244, 2016.

Schlosser, C., Klar, J. K., Wake, B. D., Snow, J. T., Honey, D. J., Woodward, E. M. S., Lohan, M. C., Achterberg, E. P., and Moore, C. M.: Seasonal ITCZ migration dynamically controls the location of the (sub)tropical Atlantic biogeochemical divide, Proc. Natl. Acad. Sci. U.S.A., 111, 1438-1442, 10.1073/pnas.1318670111, 2014.

Shiozaki, T., Kodama, T., and Furuya, K.: Large-scale impact of the island mass effect through nitrogen fixation in the western South Pacific Ocean, Geophys. Res. Lett., 41, 2907-2913, 10.1002/2014GL059835 2014a.

Shiozaki, T., Nagata, T., Ijichi, M., and Furuya, K.: Nitrogen fixation and the diazotroph

788 community in the temperate coastal region of the northwestern North Pacific,
789 Biogeosciences, 12, 4751-4764, 10.5194/bg-12-4751-2015, 2015a.
790 Shiozaki, T., Chen, Y. L. L., Lin, Y. H., Taniuchi, Y., Sheu, D. S., Furuya, K., and Chen,
791 H. Y.: Seasonal variations of unicellular diazotroph groups A and B, and
792 *Trichodesmium* in the northern South China Sea and neighboring upstream
793 Kuroshio Current, Cont. Shelf Res., 80, 20-31, 10.1016/j.csr.2014.02.015, 2014b.
794 Shiozaki, T., Furuya, K., Kodama, T., Kitajima, S., Takeda, S., Takemura, T., and Kanda,
795 J.: New estimation of N_2 fixation in the western and central Pacific Ocean and its
796 marginal seas, Global Biogeochem. Cycles, 24, GB1015, 10.1029/2009gb003620,
797 2010.
798 Shiozaki, T., Takeda, S., Itoh, S., Kodama, T., Liu, X., Hashihama, F., and Furuya, K.:
799 Why is *Trichodesmium* abundant in the Kuroshio?, Biogeosciences, 12, 6931-6943,
800 10.5194/bg-12-6931-2015, 2015b.
801 Shiozaki, T., Bombar, D., Riemann, L., Hashihama, F., Takeda, S., Yamaguchi, T.,
802 Ehama, M., Hamasaki, K., and Furuya, K.: Basin scale variability of active
803 diazotrophs and nitrogen fixation in the North Pacific, from the tropics to the
804 subarctic Bering Sea, Global Biogeochem. Cycles, 31, 996-1009
805 10.1002/2017gb005681, 2017.
806 Snow, J. T., Schlosser, C., Woodward, E. M., Mills, M., Achterberg, E. P., Mahaffey, C.,
807 Bibby, T. S., and Moore, C. M.: Environmental controls on the biogeography of

diazotrophy and *Trichodesmium* in the Atlantic Ocean, Global Biogeochem. Cycles, 29, 865-884, 10.1002/2015GB005090, 2015.

Sohm, J. A., Webb, E. A., and Capone, D. G.: Emerging patterns of marine nitrogen fixation, Nature reviews. Microbiology, 9, 499-508, 10.1038/nrmicro2594, 2011.

Sperfeld, E., Raubenheimer, D., and Wacker, A.: Bridging factorial and gradient concepts of resource co-limitation: Towards a general framework applied to consumers, Ecol. Lett., 19, 201-215, 10.1111/ele.12554, 2016.

Stenegren, M., Caputo, A., Berg, C., Bonnet, S., and Foster, R. A.: Distribution and drivers of symbiotic and free-living diazotrophic cyanobacteria in the western tropical South Pacific, Biogeosciences, 15, 1559-1578, 10.5194/bg-15-1559-2018, 2018.

Tanita, I., Shiozaki, T., Kodama, T., Hashihama, F., Sato, M., Takahashi, K., and Furuya, K.: Regionally variable responses of nitrogen fixation to iron and phosphorus enrichment in the Pacific Ocean, Journal of Geophysical Research: Biogeosciences, 126, e2021JG006542, 10.1029/2021jg006542, 2021.

Thompson, A. W., Carter, B. J., Turk-Kubo, K. A., Malfatti, F., Azam, F., and Zehr, J. P.: Genetic diversity of the unicellular nitrogen-fixing cyanobacteria UCYN-A and its prymnesiophyte host, Environ. Microbiol., 16, 3238-3249, 10.1111/1462-2920.12490, 2014.

Turk-Kubo, K. A., Achilles, K. M., Serros, T. R., Ochiai, M., Montoya, J. P., and Zehr, J.

P.: Nitrogenase (*nifH*) gene expression in diazotrophic cyanobacteria in the Tropical North Atlantic in response to nutrient amendments, Front Microbiol, 3, 386, 10.3389/fmicb.2012.00386, 2012.

Wang, W. L., Moore, J. K., Martiny, A. C., and Primeau, F. W.: Convergent estimates of marine nitrogen fixation, Nature, 566, 205-211, 10.1038/s41586-019-0911-2, 2019.

Ward, B. A., Dutkiewicz, S., Moore, C. M., and Follows, M. J.: Iron, phosphorus, and nitrogen supply ratios define the biogeography of nitrogen fixation, Limnol. Oceanogr., 58, 2059-2075, 10.4319/lo.2013.58.6.2059, 2013.

Watkins-Brandt, K. S., Letelier, R. M., Spitz, Y. H., Church, M. J., Böttjer, D., and White, A. E.: Addition of inorganic or organic phosphorus enhances nitrogen and carbon fixation in the oligotrophic North Pacific, Mar. Ecol. Prog. Ser., 432, 17-29, 10.3354/meps09147, 2011.

Welschmeyer, N. A.: Fluorometric analysis of chlorophyll-a in the presence of chlorophyll-B and pheopigments, Limnol. Oceanogr., 39, 1985-1992, 1994.

Wen, Z., Browning, T. J., Cai, Y., Dai, R., Zhang, R., Du, C., Jiang, R., Lin, W., Liu, X., Cao, Z., Hong, H., Dai, M., and Shi, D.: Nutrient regulation of biological nitrogen fixation across the tropical western North Pacific, Sci. Adv., 8, eabl7564, 10.1126/sciadv.abl7564, 2022.

White, A. E., Watkins-Brandt, K.S., and Church, M. J.: Temporal variability of *Trichodesmium* spp. and diatom-diazotroph assemblages in the North Pacific

Subtropical Gyre, Front. Mar. Sci., 5, 10.3389/fmars.2018.00027, 2018.

Wu, C., Fu, F. X., Sun, J., Thangaraj, S., and Pujari, L.: Nitrogen fixation by *Trichodesmium* and unicellular diazotrophs in the northern South China Sea and the Kuroshio in summer, Sci Rep-Uk, 8, 2415, 10.1038/s41598-018-20743-0, 2018.

Wu, J., Chung, S. W., Wen, L. S., Liu, K. K., Chen, Y. L. L., Chen, H. Y., and Karl, D. M.: Dissolved inorganic phosphorus, dissolved iron, and *Trichodesmium* in the oligotrophic South China Sea, Global Biogeochem. Cycles, 17, 1008, 10.1029/2002gb001924, 2003.

Xu, M. N., Zhang, W., Zhu, Y., Liu, L., Zheng, Z., Wan, X. H. S., Qian, W., Dai, M., Gan, J., Hutchins, D. A., and Kao, S. J.: Enhanced ammonia oxidation caused by lateral Kuroshio intrusion in the boundary zone of the northern South China Sea, Geophys. Res. Lett., 45, 6585-6593, 10.1029/2018gl077896, 2018.

Yong, S. C., Roversi, P., Lillington, J., Rodriguez, F., Krehenbrink, M., Zeldin, O. B., Garman, E. F., Lea, S. M., and Berks, B. C.: A complex iron-calcium cofactor catalyzing phosphotransfer chemistry, Science, 345, 1170-1173, 10.1126/science.1254237, 2014.

Zehr, J. P. and Capone, D. G.: Changing perspectives in marine nitrogen fixation, Science, 368, eaay9514, 10.1126/science.aay9514, 2020.

Zhang, J. Z.: Shipboard automated determination of trace concentrations of nitrite and nitrate in oligotrophic water by gas-segmented continuous flow analysis with a

liquid waveguide capillary flow cell, Deep Sea Research Part I, 47, 1157-1171, 10.1016/S0967-0637(99)00085-0, 2000.

Zhang, R., Zhu, X., Yang, C., Ye, L., Zhang, G., Ren, J. L., Wu, Y., Liu, S. M., Zhang, J., and Zhou, M.: Distribution of dissolved iron in the Pearl River (Zhujiang) Estuary and the northern continental slope of the South China Sea, Deep Sea Research Part II, 167, 14-24, 10.1016/j.dsr2.2018.12.006, 2019.