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Key Points:

- Limiting factors for nitrogen fixation as examined by enrichment experiments differed among the different regions in the Pacific Ocean
- Limitation of N_2 fixation by Fe and P and their colimitation were shown in the western North Pacific subtropical gyre for the first time

Supporting Information:

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

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Regionally Variable Responses of Nitrogen Fixation to Iron and Phosphorus Enrichment in the Pacific Ocean

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Abstract Iron and phosphorus are major nutrients limiting marine nitrogen fixation. We directly assessed iron and phosphate enrichment using the surface water of the western and central North Pacific subtropical gyre (NPSG), central equatorial Pacific, and eastern South Pacific subtropical gyre (SPSG). Diazotrophic phylotypes showed regionally distinct distribution patterns. The abundances of *nifH* in UCYN-A, *Trichodesmium, Crocosphaera,* and γ-24774A11 and filaments of *Richelia* were significantly higher in the central and western NPSG than in the eastern SPSG. The surface dissolved iron concentration was <0.2 nmol l^{-1} in most of the regions; however, nitrogen fixation was enhanced by the addition of iron only in the western NPSG and equatorial upwelling. In the western NPSG, where surface phosphate was almost exhausted, and levels varied below 7 nmol l^{-1}, diazotrophs also exhibited either phosphate limitation or colimitation by iron and phosphate. The present study demonstrated the limitation of nitrogen fixation by iron and phosphorus individually and colimitation by iron and phosphorus in the western NPSG for the first time, and the data showed that the nutrients limiting nitrogen fixation in the Pacific Ocean differed among the regions, indicating that the response of nitrogen fixation to environmental changes is not uniform.

Plain Language Summary While nitrogen is an essential element for biota, nitrogenous nutrients tend to be scarce in the ocean. Therefore, biological nitrogen fixation is important for providing nitrogen in a bioavailable form to fuel marine ecosystems. For nitrogen-fixing organisms, the availability of iron and phosphorus are key factors, and iron is reported to be limiting in North Pacific subtropical waters (NPSG). However, although the surface phosphate concentration is extremely low in the western NPSG compared to the eastern NPSG, phosphorus limitation in the western NPSG has not been examined to date. We used enrichment experiments to investigate iron and phosphorus limitation of nitrogen fixation at a basin scale in the Pacific Ocean. Our results showed that nitrogen fixation was limited by iron and/or phosphate in the western NPSG and by iron in the central equatorial area but not by either iron or phosphate in the central NPSG and eastern South Pacific. Phosphorus limitations are considered to become severe when the upward phosphate supply is weakened by increased ocean stratification caused by global warming. Thus, the limiting nutrients for nitrogen fixation vary among regions, and the response of nitrogen fixation to environmental changes is not uniform in the ocean.

1. Introduction

Nitrogen fixation is a major source of new nitrogen in the ocean, where nitrogen limits primary production and drives carbon flux from the surface water to the ocean interior (Gruber & Galloway, 2008). Iron and phosphorus are well-known key factors for marine nitrogen fixation (Sohm, Webb, & Capone, 2011; Zehr & Capone, 2020). Iron is contained in the reaction center of nitrogenase, the key enzyme for nitrogen fixation, and is essential to the dimeric protein of nitrogenase that donates electrons to the catalytic sites (Dixon & Kahn, 2004). Since diazotrophs require a substantial amount of iron (Kustka et al., 2003), iron availability controls the distribution of nitrogen fixation in the ocean (Falkowski, 1997). Nitrogen fixation

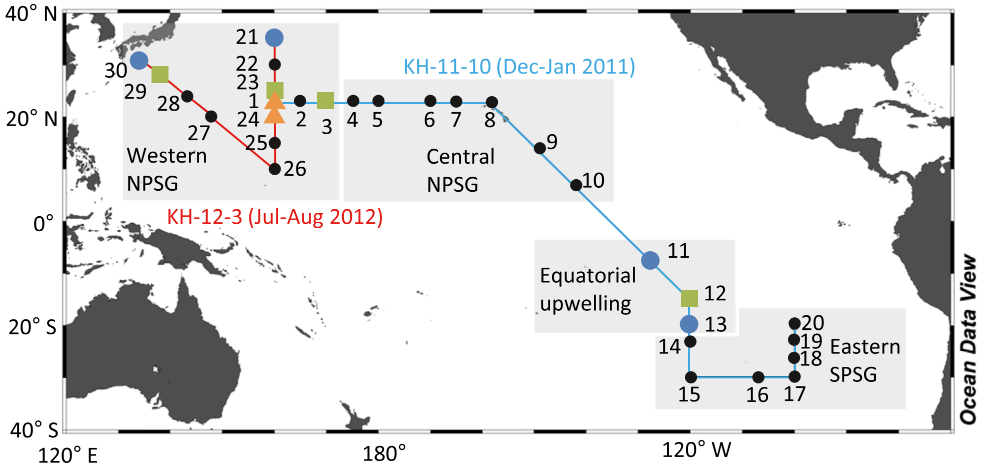

Figure 1. Station locations established during the KH-11-10 (blue line) and KH-12-3 (red line) cruises. Symbols indicate nutrients limiting nitrogen fixation: blue circles (iron), orange triangles (phosphate), and green squares (both iron and phosphorus). NPSG, North Pacific subtropical gyre; and SPSG, South Pacific subtropical gyre.

by diazotrophs also requires phosphorus. When phosphorus is depleted in the environment, the growth of diazotrophs is limited.

It is important to investigate regional factors limiting nitrogen fixation to understand the present condition of nitrogen feedback, that is, the balance between nitrogen fixation and fixed nitrogen loss at a basin scale (Deutsch et al., 2007; Weber & Deutsch, 2014). The limitation of marine nitrogen fixation by either iron, phosphorus, or both has been demonstrated by nutrient enrichment experiments or by investigations of the elemental ratio in the cells of diazotrophs (e.g., Krupke et al., 2015; Langlois et al., 2012; Mills et al., 2004; Paerl et al., 1994; Sañudo-Wilhelmy et al., 2001). Based on these studies, marine ecosystem models have been used to assess the factors limiting nitrogen fixation in each oceanic region and to simulate the distribution of nitrogen fixation activity across global oceans (Dutkiewicz et al., 2012, 2014; J. K. Moore et al., 2004). However, direct observations of the factors controlling nitrogen fixation by enrichment experiments have been confined to some regions.

In the Pacific Ocean, the factors limiting nitrogen fixation have been examined by iron and phosphorus enrichment experiments in the waters around Hawaii and the eastern North Pacific subtropical gyre (NPSG) (Grabowski et al., 2008; Needoba et al., 2007; Watkins-Brandt et al., 2011), in the eastern South Pacific subtropical gyre (SPSG) (Bonnet et al., 2008; Dekaezemacker et al., 2013), and in the western SPSG (Moisander et al., 2012, not effects on nitrogen fixation but on *nifH* abundance). However, there remains an uncertainty concerning the basin-scale limiting factors across the Pacific Ocean including the western NPSG. A west-east gradient of iron supply resulting from Asian dust deposition in the NPSG is considered to control the distribution of nitrogen fixation activity (Kitajima et al., 2009; Shiozaki et al., 2009, 2010), and the surface phosphate concentration is known to be extremely low in the western NPSG compared to the eastern SPSG (Hashihama et al., 2009; Martiny et al., 2019). Such spatial gradients of iron and phosphorus could affect the nutrient physiology of diazotrophs and act as limiting factors for nitrogen fixation. However, to date, there is no direct evidence that iron and/or phosphorus enhances nitrogen fixation in the western NPSG. In the present study, we aimed to elucidate regional response patterns of bulk nitrogen fixation activity to iron and/or phosphorus enrichment across the Pacific Ocean.

2. Materials and Methods

2.1. Study Area and Sampling

Surveys were conducted in four regions of the Pacific Ocean: The western NPSG (Stns. 1–3), the central NPSG (Stns. 4–10), the equatorial upwelling (Stns. 11–13), and the eastern SPSG (Stns. 14–20) during the R/V Hakuho Maru KH-11-10 cruise in the boreal winter (December 2011–January 2012) (Figure 1). The western NPSG (Stns. 21–30) was also investigated during the R/V Hakuho Maru KH-12-3 cruise in summer (July–August 2012). Seawater was sampled for incubation experiments and nutrient analyses from the depth corresponding to 50% of surface light intensity (4–11 m), representative of the surface mixed water, and acid-cleaned Niskin-X samplers were used to minimize Fe contamination. Photosynthetically active radiation was vertically profiled around local noon using a HyperProfiler (Satlantic) before water sampling. Samples for DNA and microscopic analysis were collected from the surface using an acid-cleaned bucket.

2.2. Nutrient Analyses

Concentrations of nitrate + nitrite (N + N) and phosphate were determined with a highly sensitive method (Hashihama et al., 2009). For the sensitivity analysis of phosphate, phosphate-free seawater purified by the MAGIC method (Karl & Tien, 1992) was used as a blank sample (Hashihama et al., 2009). The detection limit for N + N and phosphate was 3 nmol l^{-1}, which was three times the standard deviation of the blank measurement on land ($n = 3$). When the concentrations of N + N and phosphate were higher than 100 nmol l^{-1}, a conventional autoanalyzer (TRAACS 2000, Bran + Luebbe) was used.

Seawater samples for the analysis of dissolved iron (DFe) were gravity-filtered with a 0.2 μm AcroPak 200 capsule filter cartridge and collected in a trace-metal-free 125-ml bottle made of low-density polyethylene. Samples were acidified to a pH of 1.7 with high-purity hydrochloric acid (Tamapure-AA-100, Tama Chemicals Co.) in a class-100 clean room and stored at room temperature for more than half a year to let iron dissociate from organic ligands. Milli-Q water acidified in the same manner was used as a blank. Concentrations of DFe were determined by catalytic cathodic stripping voltammetry (Obata & van den Berg, 2001) with the substitution of the pH buffer from HEPPS (3-[4-(2-hydroxyethyl) piperazin-1-yl] propane-1-sulfonic acid) to POPSO (piperazine-N,N′-bis(2-hydroxypropanesulfonic acid)) (Sato et al., 2007). The detection limit was determined once a day as three times the standard deviation of blank measurements (≤0.12 nmol l^{-1}). All data shown here were above the detection limit.

2.3. Dust Deposition

Dust deposition was estimated by the Spectral Radiation-Transport Model for Aerosol Species (SPRINTARS) (Takemura et al., 2000) and averaged for 15 days prior to sampling at each station.

2.4. Abundance of Diazotrophs

The *nifH* genes of *Trichodesmium*, UCYN-A, *Crocosphaera*, and γ-proteobacteria 24774A11 (γ-24774A11), which are commonly detected in tropical and subtropical oligotrophic oceans were quantified by quantitative polymerase chain reaction (qPCR). For the DNA analysis, particles in one liter of seawater were collected onto a 47 mm Nuclepore membrane (0.2 μm pore size, Whatman, <270 mbar vacuum), flash-frozen in liquid nitrogen, and stored at −80°C. DNA on the filters was extracted and purified with a Charge Switch Forensic DNA Purification Kit (Invitrogen) by following the manufacturer's protocol and preserved at −20°C. Forward and reverse primers, probes and standard templates, and qPCR conditions have been described elsewhere (Moisander et al., 2008; Shiozaki et al., 2014, 2017). The amplification efficiencies of the PCR assays for *Trichodesmium*, UCYN-A, *Crocosphaera*, and γ-24774A11 were 93.6%–100.0%, 90.8%–107.1%, 91.8%–105.9%, and 91.8%–110.0%, respectively. The determination coefficients of the regression of the standard curves were >0.99. The detection limit was 1×10^2 copies l^{-1} on the basis of the extracted volumes, assuming that PCR can amplify DNA from one copy in template (Shiozaki et al., 2014). *Richelia intracellularis* filaments were enumerated using the Utermöhl method under an inverted microscope after samples fixed with acid Lugol's iodine solution were concentrated.

2.5. Iron and Phosphate Enrichment Experiments

Seawater for incubation experiments was introduced to acid-cleaned 4.5-l polycarbonate bottles. Potassium dihydrogen phosphate (KH_2PO_4) and iron chloride ($FeCl_3$) (JIS Special Grade, Wako Pure Chemical Industries) were added to the bottles, singly or in pairs, to establish final concentrations of 200 nmol l^{-1} and 2 nmol l^{-1}, respectively. Bottles without any addition were treated as controls. Quadruplicate bottles were prepared for each treatment, and therefore, a total of 16 bottles were prepared at each station. Phosphate limitation was not assessed at Stns. 16–20 because phosphate is generally replete in the equatorial upwelling region and eastern SPSG (Moutin et al., 2008). After sealing the bottles with thermoplastic elastomer septum caps (Nalgene) without headspace, 2 ml of $^{15}N_2$ gas (SI Science, 99.8% ^{15}N, made-to-order, and no lot No.) was injected using a gas-tight syringe, the stainless needle of which was covered with polyether ether ketone tubing (Nirei Industry) to avoid contamination from the needle (Needoba et al., 2007). The bottles were shaken 20 times to aid $^{15}N_2$ gas dissolution. The bottles were covered with 50% light-decreasing black mesh screens and incubated for 48 h in on-deck tanks cooled by running seawater taken in from an intake on the bottom of the ship. Incubations were terminated by gentle filtration (<270 mbar vacuum) onto precombusted glass microfiber filters (GF/F, Whatman), and the filters were stored at −20°C. For the initial $\delta^{15}N$ samples, 4 l of seawater was filtered in the same manner as described above. During the whole procedure, the samples were strictly isolated from tracer samples to prevent contamination. The amounts and isotopic ratios of nitrogen in the samples were analyzed as described by Shiozaki et al. (2009), and nitrogen fixation rates were calculated according to Montoya et al. (1996).

Enrichment effects on nitrogen fixation were judged against controls by Welch's *t*-test at a significance level of $p = 0.05$ after normality of data was confirmed by the Kolmogorov-Smirnov test. When an abnormal rate of nitrogen fixation was observed in one of the replicate bottles, the outlier was examined by the Smirnov-Grubbs test and excluded when p was <0.05. Although the $^{15}N_2$ bubble injection method has been shown to underestimate nitrogen fixation, which results from low dissolution of $^{15}N_2$ gas (Mohr et al., 2010; White et al., 2020), we used this method because it was impossible to avoid possible iron contamination when degassing seawater for the $^{15}N_2$ dissolution method (Klawonn et al., 2015). Our estimates also carried possible underestimation due to the use of GF/F filters (Bombar et al., 2018; Konno et al., 2010). Contamination of inorganic nitrogen has been reported from some commercial $^{15}N_2$ gases (Dabundo et al., 2014), but contamination from the gas purified by SI Science's method has been confirmed to be negligible (Shiozaki et al., 2015). The limit of detection was defined as a 0.00146 atom % difference between the initial and final isotopic ratios (Gradoville et al., 2017; Montoya et al., 1996), corresponding to nitrogen fixation of 0.0273–0.204 nmol l^{-1} d^{-1}. The amount of particulate organic nitrogen on a filter ranged from 13.6 to 118 μg with an average of 23.8 μg per 4.5 l filtration and was within the linear range of calibration lines. The minimal quantifiable rate (MQR) of nitrogen fixation (Gradoville et al., 2017) was calculated for each enrichment series and ranged from 0.0602–1.38 nmol N l^{-1} d^{-1}. Values below the MQR were retained in the analysis but were not used in the significance test.

2.6. Statistical Analysis

DFe, dust deposition, and nitrogen fixation in the control bottles were compared among the regions (Figure 1). Normality and homogeneity of variance were tested for each data point using the Kolmogorov-Smirnov test and Levene's test, respectively. For DFe and nitrogen fixation, analysis of variance (ANOVA) was conducted. For comparison of dust deposition, pairwise Welch's *t*-test was conducted with the Bonferroni correction because the data were not homogenous in variance. In addition, the correlation between each data point was tested by Spearman's method using all cruise data.

3. Results

3.1. Environmental Conditions, Abundances of Diazotrophs, and Nitrogen Fixation

The surface N + N concentration was consistently low at the nanomolar level, varying from below the detection limit to 17 nmol l^{-1}, except for at the equatorial upwelling region (>229 nmol l^{-1}, Figure 2a). In particular, N + N was exhausted in almost the entire region of the western NPSG. In contrast, surface phosphate levels varied considerably according to region, from below the detection limit to 709 nmol l^{-1} (Figure 2b). At stations west of 160°E, surface phosphate levels were low, varying at less than 7 nmol l^{-1} except for Stns. 25 and 26. It increased eastwardly in the central NPSG and exceeded 98 nmol l^{-1} in the equatorial upwelling and SPSG.

Surface DFe concentrations were variable, ranging from 0.063 nmol l^{-1} at Stn. 19 to 0.90 nmol l^{-1} at Stn. 28 (Figure 2c). High concentrations also occurred at Stn. 8 (0.73 nmol l^{-1}), and levels were below 0.5 nmol l^{-1} at

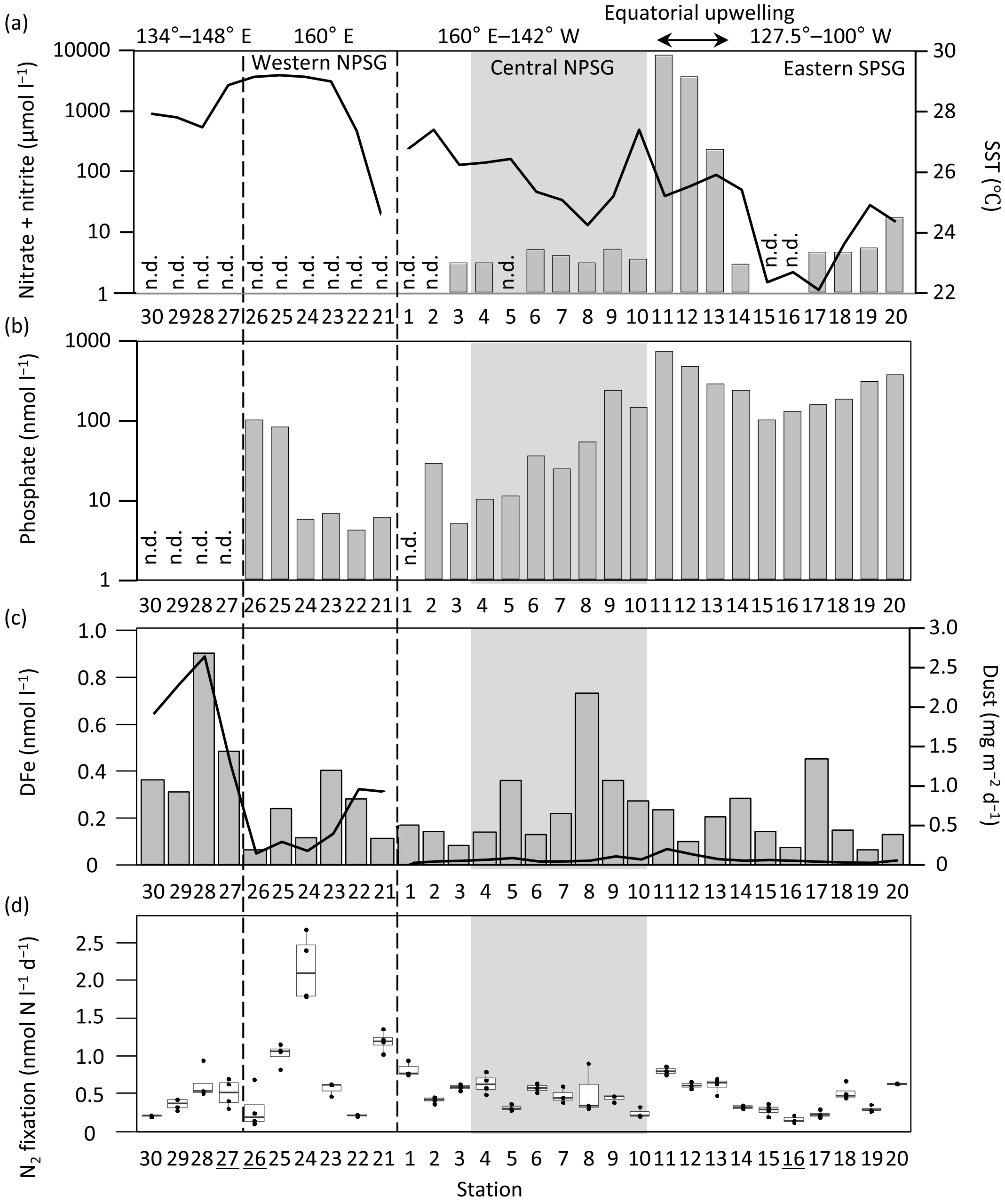

Figure 2. (a) Concentrations of nitrate + nitrite (bars) and sea surface temperature (SST, lines), (b) concentrations of phosphate, (c) dissolved iron (DFe, bars) and dust deposition (lines), and (d) nitrogen fixation rates in control bottles, expressed as activity per day. Nitrogen fixation is expressed by dots showing all individual values and boxplots, where the lower and upper boundaries of the boxes indicate the 25th and 75th percentiles, and thick horizontal lines within the box denote the median. Error bars at the top and bottom of the boxes show the maximum and minimum values no further than 1.5 times the difference between the 75th and 25th percentiles from the box, respectively. Note that stations are arranged in an eastward order. Vertical dashed lines separate different transects. Nutrients below the detection limit are shown as "n.d." White and gray background colors and the top horizontal arrow indicates oceanic regions defined in Figure 1. Nitrogen fixation rates at Stns. 27, 26, and 16 were below the minimal quantifiable rate, as indicated by underlining of station labels. Stns. 1–20 and Stns. 21–30 were investigated during boreal winter and summer, respectively.

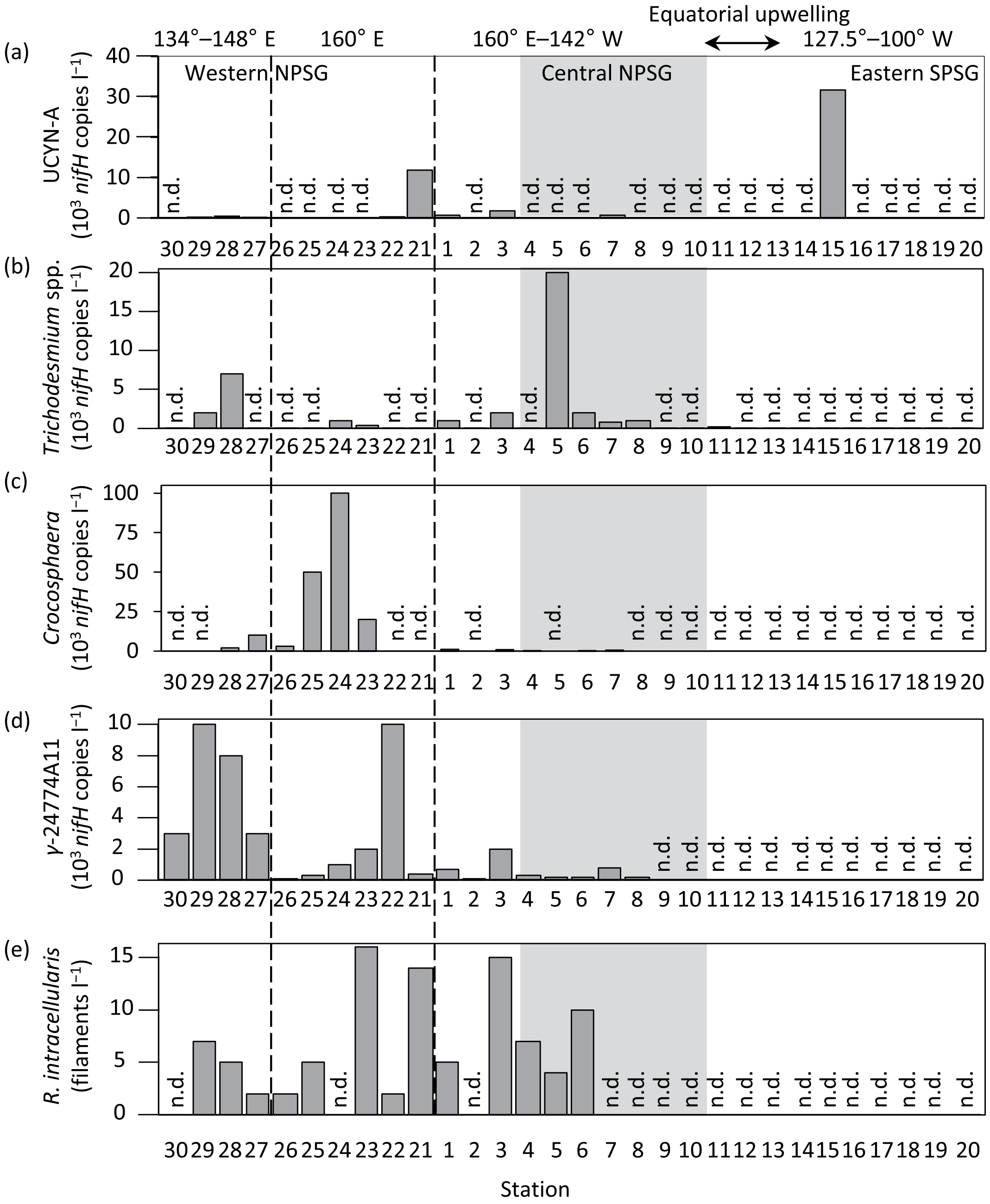

Figure 3. Densities of *nifH* copies of (a) UCYN-A, (b) *Trichodesmium* spp., (c) *Crocosphaera*, and (d) γ-proteobacteria 24774A11. (e) Densities of filaments of *Richelia intracellularis.* n.d.: not detected. Stns. 1–20 and Stns. 21–30 were investigated during boreal winter and summer, respectively.

stations other than Stns. 8 and 28. No significant differences were observed among regional mean DFe concentrations (ANOVA, $p > 0.05$). In contrast, dust deposition was significantly higher in the western NPSG than in the eastern SPSG (pairwise *t*-test, $p < 0.05$), reflecting dust originating from East Asia (Figure 2c).

Nitrogen fixation was detected at all stations, mostly varying below 1 nmol N l^{-1} d^{-1}. A high activity of 2.22 nmol N l^{-1} d^{-1} was observed at Stn. 24. No significant difference was noted in the regional means (ANOVA, $p > 0.05$) (Figure 2d).

Diazotrophs showed regionally distinct distribution patterns. The diazotrophs targeted for qPCR and microscopic analyses were found almost exclusively in the NPSG (Figure 3). *Trichodesmium, Crocosphaera,*

Table 1

Correlation Coefficients Between Environmental Parameters, Diazotroph Abundances, Nitrogen Fixation, and Nutrient Enrichment Effects, as Determined by Spearman's Test

	Temperature	N + N	Phosphate	DFe	Dust	*Trichodesmium*	UCYN-A	*Crocosphaera*	γ-24774A11	*Richelia*	Nitrogen fixation	P effect	Fe effect
N + N	−0.54**												
Phosphate	−0.54**	0.76***											
DFe	0.24	−0.16	−0.31										
Dust	0.59***	−0.42*	−0.40*	0.38*									
Trichodesmium	0.22	−0.15	−0.44*	0.23	0.05								
UCYN-A	−0.04	−0.37*	−0.60***	−0.09	0.16	0.25							
Crocosphaera	0.62***	−0.38*	−0.49**	0.03	0.19	0.37*	0.16						
γ-24774A11	0.63***	−0.63***	−0.93***	0.35	0.53**	0.51**	0.46**	0.56**					
Richelia	0.42*	−0.43*	−0.61***	0.02	0.30	0.45*	0.45*	0.53**	0.65***				
Nitrogen fixation	0.15	0.17	−0.05	−0.16	0.10	0.29	0.15	0.45*	0.15	0.34			
P Effect	0.12	−0.13	−0.14	−0.13	0.05	0.27	−0.11	0.06	0.11	0.17	0.21		
Fe Effect	0.09	−0.01	−0.07	0.27	0.34	0.10	0.03	−0.31	0.17	0.13	0.02	0.15	
P + Fe Effect	−0.13	0.19	−0.03	0.16	0.08	0.18	0.00	−0.29	0.05	−0.00	0.13	0.55**	0.48*

Note. Significance is shown as *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

γ-24772A11, and *Richelia* were frequently observed in the western NPSG. In contrast, UCYN-A occurred sporadically at Stns. 15 and 21, where temperatures were as low as 24.4°C and 22.4°C, respectively. *Crocosphaera* occurred in high-temperature areas (27.5–29.2°C) between 10 and 25°N, and its peak abundance, which coincided with the highest nitrogen fixation activity, was found at Stn. 24 (Figures 2d and 3c). While nitrogen fixation was observed rather constantly, the diazotrophs targeted by our assay were not detected at most stations in the equatorial upwelling and SPSG.

Analysis with Spearman's rank correlation showed that the abundances of all diazotrophic phylotypes showed a negative correlation with N + N and phosphate concentrations ($p < 0.05$), except for that between *Trichodesmium* and N + N. In contrast, the nitrogen fixation rate showed no correlation with either N + N, phosphate or DFe. Positive relationships were noted in some diazotrophs: the abundance of *Crocosphaera* was positively correlated with temperature and nitrogen fixation, and the abundance of γ-24772A11 was positively correlated with dust deposition and temperature ($p < 0.05$, Table 1). Temperature was also positively correlated with *Richelia* abundance ($p < 0.05$, Table 1).

3.2. Response of Nitrogen Fixation to Iron and Phosphate Enrichment

The response of nitrogen fixation to the addition of iron and phosphate varied greatly depending on the oceanic regions (Figures 1 and 4). Single enrichment of iron significantly enhanced nitrogen fixation in the western NPSG north of 25°N (Stns. 21, 23, and 30) and in the equatorial upwelling region (Stns. 11 and 13). A single addition of phosphate was found to stimulate nitrogen fixation only in the western NPSG between 20 and 25°N (Stns. 1, 23, and 24). Phosphorus limitation, including colimitation with iron, was observed only at stations where surface phosphate was ≤7 nmol l^{-1} throughout our study. Among these stations, colimitation was observed only at Stn. 23, and either iron or phosphate was singly limiting at the other stations. Enrichment of both iron and phosphate generally supported the results obtained by single enrichment, with variations found only at Stns. 13 and 30 (Figure S1). At Stns. 12 and 29, despite the lack of enhancement by single enrichment, enrichment of both iron and phosphate elevated nitrogen fixation, indicating colimitation. Statistical comparison was not applicable at Stns. 1, 3, 5, 10, 17, and 20 because of a shortage of replications between the control and iron addition at Stns. 1, 3, 17, and 20 and between the control and addition of both iron and phosphate at Stns. 5 and 10. Error bars are not shown for these data in Figures S1 and 4. There was no response to iron and/or phosphate addition by nitrogen fixation in the central NPSG east of 175°E and eastern SPSG. Nitrogen fixation was below the MQR for the following samples: phosphate addition and addition of both phosphate and iron at Stn. 10; the control at Stn. 16; the control, iron addition, and phosphate addition at Stn. 26; and the control and phosphate addition at Stn. 27 (Figures S1 and 4). The enrichment response by nitrogen fixation did not show any significant relationship with environmental factors or diazotroph abundance in the Spearman's rank correlation analysis (Table 1, $p > 0.05$).

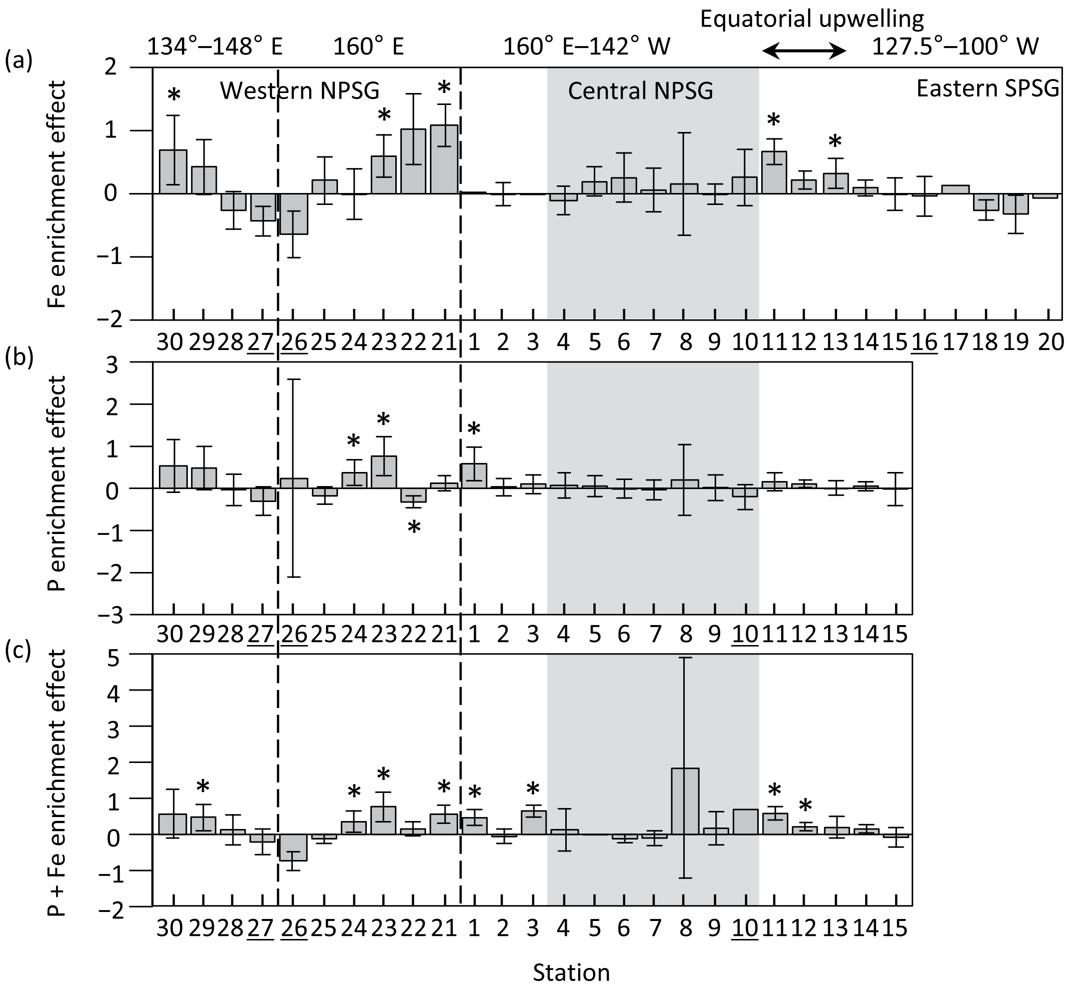

Figure 4. Response of nitrogen fixation to iron and phosphate enrichment. Bars show relative differences in nitrogen fixation with enrichment of (a) iron (Fe), (b) phosphate (P), and (c) both iron and phosphate (P + Fe) compared to the control. A value of 0 means equal to the control, and a value of 1 corresponds to doubling of nitrogen fixation relative to the control. Error bars indicate standard deviations, and the lack of an error bar means that there were fewer than three replicate bottles. Asterisks (*) indicate significant differences compared with the control (*t*-test, $p < 0.05$). Note that phosphate and phosphate + iron enrichment was not conducted at Stns. 16–20. The response at each treatment is shown in Figure S1. Nitrogen fixation rates of either enriched samples or controls were below the minimal quantifiable rate at Stns. 10, 16, 26, and 27, as indicated by underlining of station labels. Stns. 1–20 and Stns. 21–30 were investigated during boreal winter and summer, respectively.

Interestingly, in the western NPSG, nitrogen fixation tended to respond to iron addition, either singly or with phosphate in high dust-deposition areas (25–35°N), while other stations located at 10–24°N did not yield enhanced nitrogen fixation upon the addition of iron (Figures 2c, 4a and 4c).

4. Discussion

4.1. Distributions of Nitrogen Fixation and Diazotrophs

Nitrogen fixation was detected in all the regions examined (Figure 2d). However, the diazotroph community varied among regions (Figure 3). The NPSG was characterized by autotrophic diazotrophs *Crocosphaera*, *Trichodesmium*, and *Richelia* (Figure 3), supporting previous reports (Church et al., 2008; Shiozaki et al., 2017). Their occurrence in the NPSG was consistent with a high aeolian iron supply and N + N depletion (Figures 2a and 2c), which is advantageous for autotrophic diazotrophs in resource competition against nondiazotrophic autotrophs (Dutkiewicz et al., 2014; Ward et al., 2013). The positive correlation between the abundance of γ-24774A11 *nifH* and dust deposition implies that their distribution was restricted by iron supply, which is compatible with previous reports indicating that their distribution is limited in the western and central Pacific (Halm et al., 2012; Moisander et al., 2012; Shiozaki et al., 2018).

In contrast, diazotrophs were not detected in the equatorial upwelling region and eastern SPSG, except for *Trichodesmium* at Stn. 11 and UCYN-A at Stn. 15 (Figure 3), indicating that unexamined diazotrophs were responsible in these regions. This is consistent with previous reports attributing nitrogen fixation in these areas to noncyanobacterial diazotrophs and UCYN-A (Halm et al., 2012; Shiozaki et al., 2018). Noncyanobacterial diazotrophs, together with the autotrophic diazotrophs, might have contributed to nitrogen fixation in not only the SPSG but also the NPSG (Figure 2d).

4.2. Iron Limitation of Nitrogen Fixation

In the western NPSG, iron enrichment enhanced nitrogen fixation during both winter and summer. Since iron limitation of nitrogen fixation had already been confirmed at Stn. ALOHA (Grabowski et al., 2008), nitrogen fixation was considered to be limited by iron supply in a wide area across the central and western NPSG. This inference was consistent with model studies (Dutkiewicz et al., 2014; J. K. Moore et al., 2004).

In the central NPSG, nitrogen fixation did not respond to iron enrichment. Nonetheless, Grabowski et al. (2008) reported an iron enrichment effect for Stn. ALOHA in February and March. Since our observation was made in December, the difference in enrichment effects was related to temporal variability of the occurrence of iron limitation. This is related to the unsteady nature of the iron supply, which results from the island mass effect (Sohm, Subramaniam, et al., 2011), internal waves (Wilson, 2011), or seasonal variation in iron concentrations associated with mixed layer depth in this region (Boyle et al., 2005; Dore et al., 2002). Furthermore, depending on the strength and position of Aleutian low pressure, dust deposition varies interannually in the central NPSG, resulting in temporal variability in iron limitation of nitrogen fixation (Letelier et al., 2019). In addition to iron supply, solubility and bioavailability of deposited iron is also dependent on a range of environmental factors; solubility of iron from dust particles can be affected by origin, size, atmospheric processing of dust, and organic ligands in seawater (Baker & Croot, 2010; Buck et al., 2013; Wu et al., 2001), and bioavailability of iron is related to ligands, redox state, and size fraction (Chen & Wang, 2001; Kustka et al., 2005; Lis et al., 2015; Rue & Bruland, 1997). Therefore, variabilities in iron sources and their seasonal and interannual dynamics may in part explain the absence of an enrichment effect in the central NPSG in the present study (Figure 4).

Significant iron enrichment was observed in the central equatorial upwelling region. Previously, Dekaezemacker et al. (2013) reported that nitrogen fixation was limited by both iron and organic matter in the HNLC region located far east of our study area (10–15°S, 82–95°W). While their study area was in proximity to the coastal upwelling, our results further revealed that iron widely limited nitrogen fixation in equatorial upwelling. Because diazotrophs were mainly noncyanobacterial in this region (Halm et al., 2012; Shiozaki et al., 2018), iron enrichment might have alleviated the deficiency of organic matter through the enhancement of primary production (Coale et al., 1996; Martin et al., 1994), other than a possibility of direct deficiency of iron for diazotrophs (Dekaezemacker et al., 2013). The present study was conducted in January and that by Dekaezemacker et al. (2013) was conducted in February and March–April; thus, these studies confirmed the effects of iron on nitrogen fixation in boreal winter. However, it remains uncertain whether this tendency is seasonal or year-round.

In the eastern SPSG, there was no response of nitrogen fixation to iron enrichment, confirming previous studies (Bonnet et al., 2008; Dekaezemacker et al., 2013). We do not have a suitable explanation for the lack of a response to iron enrichment in iron-deficient waters. Studies of the physiology of iron requirements and metabolism in diazotrophs in this region are needed.

4.3. Phosphorus Limitation and Phosphorus-Iron Colimitation of Nitrogen Fixation

This study is the first to demonstrate that phosphate availability controls nitrogen fixation in the western NPSG during both summer and winter. The limitations of iron and phosphate were confirmed during both seasons. The surface phosphate level in the western NPSG was <15 nmol l^{-1}, regardless of the season (Hashihama et al., 2009, 2019, 2021; Kodama et al., 2011). Similarly, low levels were observed in the North Atlantic subtropical gyre (Mather et al., 2008), where diazotrophs are under phosphate limitation and colimitation of iron and phosphate (Langlois et al., 2012; Mills et al., 2004; Sañudo-Wilhelmy et al., 2001; Turk-Kubo et al., 2012). In addition to inorganic phosphate, labile organic phosphorus, which is an alternative source of phosphorus for organisms, is also depleted in the western NPSG (Hashihama et al., 2013; Yamaguchi et al., 2021). Because of the thorough depletion of phosphorus sources across the western NPSG, it is not surprising that diazotrophs were phosphate-limited and/or phosphate-iron colimited in the western NPSG as predicted by Weber and Deutsch (2014).

While the phosphate concentration is relatively higher in the central NPSG than in the western NPSG (Hashihama et al., 2009; Karl & Tien, 1997), phosphorus limitation on nitrogen fixation was also reported in the central NPSG (Grabowski et al., 2008; Watkins-Brandt et al., 2011). Grabowski et al. (2008) showed that nitrogen fixation at Stn. ALOHA increased by phosphate enrichment when ambient concentrations of soluble reactive phosphorus were around or below 40 nmol l^{-1}. Further, Watkins-Brandt et al. (2011) reported that phosphorus enrichment enhanced nitrogen fixation in northern Hawaii where surface phosphate varied below 50 nmol l^{-1}. Interannual variability in water column stratification is also relevant to the occurrence of phosphorus limitation in the eastern NPSG, and the ecosystem oscillates on sub-decadal scales between inorganic phosphorus sufficiency and limitation, when concentration in surface waters decreases below 50–60 nmol l^{-1} (Letelier et al., 2019). In our study, the effect of phosphate was observed only when the ambient concentration was ≤ 7 nmol l^{-1} suggesting that nanomolar-level variations in phosphate determined the regional distribution of phosphorus limitation in the western NPSG. While phosphorus is not considered a primary limiting nutrient for nitrogen fixation across the NPSG (Dutkiewicz et al., 2014; J. K. Moore et al., 2004), our results indicate that nitrogen fixation in the western NPSG, where phosphate is exhausted (Hashihama et al., 2009), tended to be more phosphorus limited than in the central NPSG. Thus, taking the ambient phosphate concentrations into account, diazotrophs were under severe phosphorus limitation compared to those in the central and eastern NPSG. Martiny et al. (2019) showed that existing models (Fu et al., 2016) systematically overestimate surface phosphate concentrations. This may explain why previous model studies failed to predict the limiting nutrient factors in this region.

4.4. Biogeochemical Implications of the Nutrient Limiting Pattern of Nitrogen Fixation

Biogeochemical estimates suggest that nitrogen fixation in the Pacific Ocean is driven by nitrogen loss relative to phosphorus caused by high denitrification in the eastern equatorial upwelling (Deutsch et al., 2007). Weber and Deutsch (2014) argued that phosphate-rich water originating from denitrification becomes phosphate-depleted through phosphate consumption by diazotrophs during shallow circulation in the Pacific Ocean. The present study supports their hypothesis by providing evidence for phosphorus limitation of nitrogen fixation in the western NPSG.

Weber and Deutsch (2014) suggested that even if the amount of dust deposition changes, it would have little effect on the total amount of nitrogen fixed over the Pacific basin because of phosphorus limitations. However, it should be noted that changes in dust deposition likely alter the compositions of diazotrophs, as inferred from enrichment experiments on natural diazotroph assemblages (Langlois et al., 2012). Moreover, since global warming physiologically alleviates iron limitation in *Trichodesmium* (Jiang et al., 2018), phosphorus limitation can be potentially critical in *Trichodesmium* productivity. Thus, although the alleviation of iron limitation is not known for other diazotrophs, global warming could impact diazotroph composition and limiting factors of nitrogen fixation through changes in dust deposition on the ocean.

On the other hand, it is anticipated that the phosphate supply from below the phosphocline to the euphotic layer will be weakened by global warming due to the strengthening of ocean stratification (Sallée et al., 2021). Furthermore, since the phosphocline is deeper in the western NPSG than in the central and eastern NPSG (Dore et al., 2008; Yamaguchi et al., 2021) and winter vertical mixing does not reach the phosphocline (Dore et al., 2008), the strengthening of ocean stratification could potentially accelerate the depletion of surface phosphate in the western NPSG. Therefore, it is highly probable that phosphorus limitation of nitrogen fixation will become even more serious with global warming. Thus, limiting factors in the NPSG are strongly influenced by climate change, and the impact of climate change on nutrient limitation of nitrogen fixation in the NPSG will require strict attention.

5. Conclusions

The present study demonstrated the factors limiting nitrogen fixation in the Pacific Ocean. Nitrogen fixation was limited by deficiencies of iron and phosphate in the western NPSG and by a deficiency of iron in the equatorial upwelling. Nitrogen fixation did not respond to the addition of iron in the central NPSG and eastern SPSG. Although nitrogen fixation has been shown to be limited by iron deficiency over the Pacific Ocean (Dutkiewicz et al., 2014; J. K. Moore et al., 2004; Sohm, Webb, & Capone, 2011), this study revealed that the limiting factors vary depending on the region. In particular, it is notable that nitrogen fixation was limited by a low supply of phosphate alone or with iron in the western NPSG. Although phosphate limitations of microbes, including diazotrophs, have been well studied in the North Atlantic subtropical gyre (e.g., Browning et al., 2017; Mills et al., 2004; C. M. Moore et al., 2013; Sañudo-Wilhelmy et al., 2001; Turk-Kubo et al., 2012), information on the western NPSG is extremely scarce (C. M. Moore et al., 2013; Sato et al., 2013). Given the insufficient representation by numerical models (Martiny et al., 2019), further studies in the western NPSG are required to understand the response of ecosystems to environmental changes.

Data Availability Statement

Data presented in this article are available through the UTokyo repository (http://hdl.handle.net/2261/00080172).

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