Reviewer 2

In this paper, Wen et al. reported the patterns and limiting factors of nitrogen fixation activity and diazotroph community in the South China Sea and western North Pacific. Through the iron and phosphate amendment experiments, they delineated the nutrient limitations of nitrogen fixation and diazotrophs for different water bodies in the study region. These findings may facilitate our understanding of the bottom-up controls of the diazotrophs in the study region, while I have some concerns regarding the methods, interpretations, and discussions in the current manuscript.

This manuscript gives me a general impression that the authors kept mentioning another paper of their group (Wen et al., 2022), instead of sticking to the findings of the current study. For example, they discussed a lot about the iron to nitrogen supply ratio, which seems like a highlight in Wen et al., 2022, but this ratio was not analyzed in the current study. Also, the dissolved iron to dissolved inorganic nitrogen ratio was not available in this manuscript. I suggest the authors focus more on the direct and interactive effects of iron and phosphorus availability on the diazotrophs for the discussion.

We thank the Reviewer for this suggestion., and fully agree with the Reviewer that although it appears that the iron to nitrogen supply ratio was important in regulating diazotroph biogeography in this study, this ratio was not directly analyzed and thus should not be overly highlighted. We have now reduced discussion on Fe:N supply ratio and shifted the focus further to the diazotroph nutrient limitations and their potential causes.

Given that the iron and phosphorus limitations of diazotrophs are the major focus of this study, the authors should describe the iron and phosphorus availability in the study region based on their real data, like ambient concentrations of iron and phosphate, instead of speculating. Also, the iron and phosphate concentrations in the nutrient amendment experiments should also be reported. This information is particularly important when the authors discuss the reasons and biogeochemical implications for the experimental results.

Surface low-level inorganic nutrients have now been added in Table 1 and also discussed in the text. Unfortunately, in this study, iron concentrations in surface seawater were not measured, nor were nutrient concentrations within the individual incubation bottle. However, the experimental results we have obtained in the present study can be explained very well by using previously reported nutrient concentrations in the similar region.

Besides, the authors should be aware that they only analyzed some commonly observed diazotroph groups (i.e., part of the diazotroph community) with qPCR assays. In other words, some of the unanalyzed diazotrophs might be important N-fixers at some stations. Some unanalyzed diazotrophs might even pop up during the 3-day incubation. So, they need to be cautious when comparing the patterns of nitrogen fixation rates and diazotroph abundances. The authors may also consider conducting *nifH* amplicon sequencing for reconstructing the whole diazotroph community in the study region. Also, initial diazotroph abundances of the incubations should be reported as well.

We fully agree with the Reviewer that our qPCR-based analysis of *nifH* community may neglect some other diazotrophs. We have now revised the text to note this caveat (alongside the polyploidy comment made by Reviewer 1) when discussing diazotroph abundances and community structure.

However, we also note that an exhaustive analysis of diazotroph community structure using high-throughput sequencing has been done in the similar region of our study (Ding et al., 2021). The results showed that *Trichodesmium*, UCYN-A and B, and γ-24774A11 were indeed the main species that contributed >80% of the diazotroph community. Thus, we believe that our qPCR analysis has nevertheless captured the main diazotroph phylotypes that commonly exist in the NSCS and WNP.

Unfortunately, the initial diazotroph abundances at the beginning (i.e., t=0) of the nutrient amendment experiments were not available. The *nifH* abundances of the seawater from the CTD deployed at exactly the same locations as the nutrient amendment experiments were reported, which we assume represented the initial conditions.

L28-30: It is better to avoid hypothesis/speculation in the abstract. The iron to nitrogen supply ratio was not directly measured in this study as the authors stated in L416.

Thanks for the suggestion. The hypothesis has been removed.

L34-L35: "the largest" and "always" seem subjective and inaccurate based on Figure 5. Also, there was no significant response at some stations where *Trichodesmium* dominated.

The sentence has been rephrased as "the largest responses of *nifH* gene abundances were dominated by either *Trichodesmium* or UCYN-B in 6 out of 8 experiments".

L38-40: Why? I did not see any evidence from this study supporting this speculation directly.

We realized that the hypothesis that we put forward was speculative, so the sentence has been changed to "This study provides comprehensive evidence of nutrient controls on diazotroph biogeography in the margin of western North Pacific Ocean."

L136-137: This sentence is not informative, as the depths are not labeled in the figure. Nevertheless, the depths of the seafloor are not important here.

Sentence deleted.

L152-156: Were the water samples collected from different depths (2-5m) exposed to different degrees of dissolved iron contamination from the research vessel? Did the authors measure dissolved iron concentration for these water samples?

The stated 2-5 m depth range refers to the range that the tow-fish moved continuously during sailing (i.e., with movement of the vessel, passage of waves etc.).

Dissolved Fe concentrations were not measured during these cruises. However, in a cruise during summer 2019, surface waters were sampled using the same method for the measurements of dFe, and the results showed values of 0.43 nM at a near-shelf station and 0.27 nM at the basin station SEATS (Wen et al., 2022). These concentrations were comparable to 0.2-0.3 nM previously reported in the NSCS basin (Wu et al., 2003). Thus, we believe our sampling approach meets trace metal clean standards, and that the water samples were not contaminated by the research vessels.

L160: The results of primary production were not described or discussed. Was primary production also measured in the nutrient amendment experiment? The Chl-a and primary production from the experiments may be helpful when the authors discuss/speculate about the competition between diazotroph and non-diazotrophic phytoplankton (L28; L403).

Chl *a* from the experiments were measured and the results and discussions have now been added in the supplementary material Fig. S3 and the main text. Briefly, Chl *a* concentrations were not significantly affected by the amendments of nutrients, which in combination with the low concentrations of surface DIN implies that the overall phytoplankton community in both NSCS and western boundary of North Pacific was N-limited.

L226: Did you measure iron concentration in the $^{15}N_2$ enriched water? The preparation of $^{15}N_2$ enriched water may introduce iron contaminants (Klawonn et al., 2015).
Klawonn, I., Lavik, G., Böning, P., Marchant, H. K., Dekaezemacker, J., Mohr, W., & Ploug, H. (2015). Simple approach for the preparation of $^{15}N_2$-enriched water for nitrogen fixation assessments: evaluation, application and recommendations. Frontiers in microbiology, 6, 769.

We did not measure iron concentration in the enriched water. We note that all the materials including the degas unit and Tedlar®PVF bag coming in contact with the $^{15}N_2$ enriched water were acid-washed in a Class-100 cleanroom before use. We therefore believe restricted iron contaminants were introduced into the enriched water. In addition, we observed enhancement of N_2 fixation rates after Fe addition in this study, suggesting that any contaminating Fe (if there was) was not enough to stimulate N_2 fixation. This was also found in in a previously study in which the same approach was used (Wen et al., 2022).

L307: The description of S3 is confusing. Does “ab” in Figure 4 mean no significant difference with a and b? If that’s the case, S3 seems not “independent co-limited”. Please clarify.

Yes, rates in +P and +Fe+P were not significantly different from control. However, the averaged rates were increased by 1.64 and 1.44 times relative to control, which were comparable to the degree of enhancement in +Fe (1.70 times). Thus, N_2 fixation at this station can also cautiously be recognized as independently limited. We have carefully revised the description in the revised manuscript to: “In the experiments conducted at station S3, N_2 fixation was also recognized to be independent co-limited, the rates in all nutrient-amended groups increased by 1.44-1.70 times compared to control, although statistical significances were not observed in +P and +Fe+P (Fig. 4).”

L317-323: Please clarify the exact number of replicates for each treatment. Also, I doubt the statistical significance based on duplicates (n=2). The limitation of replication should be stated clearly in the manuscript. It is also the same for Figure 5. Were initial nitrogen fixation rates (i.e., the rates of the seawater from the pump) measured?

Treatments for most of the bioassay experiments (7 out of 8) were conducted with 3 replicates. However, there were three cases when one of the triplicate samples was lost due to filtration errors (e.g., one +Fe+P carboy at station S1, one NFR/PP sample of +Fe+P at station WP, and one +P sample at station S3). In addition, for the bioassay 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 groups retained 3 replicates. Further details outlining the above have now been added to the Methods section and also in the figure legends of Figures 4 and 5.

Unfortunately, the initial N_2 fixation rates of the seawater from the pump were not measured in this study. The rates we measured (with seawater from the CTD) were at exactly the same locations as where the bioassay experiments were set up, which we assume can represent the initial rates.

L350: As you only analyzed part of the diazotroph community, you may consider revising "diazotroph community structure" to "abundances of analyzed diazotrophs".

Revised accordingly.

L367 and Figure 5: How about UCYN-A1? UCYN-A1 was also abundant at K1 and WP based on Figure 3, while they disappeared in the nutrient amendment experiment (Figure 5). Also, the initial diazotroph abundances should also be displayed in Figure 5.

Unfortunately, the initial diazotroph abundances at the very beginning of the nutrient amendment experiments were not available. We measured he *nifH* abundances of the seawater from the CTD deployed at exactly the same locations as where the bioassay experiments were setup, which we assume can represent the initial condition. The shift of diazotroph composition in the bioassay incubations at K1 and WP could be attributed to the three-days incubation times and thus "bottle effects" (Göran et al., 2003). More discussions have been included in the revised manuscript.

L382: There is no doubt about Kuroshio being a hotspot of nitrogen fixation, while the low rate at K1 is not the "increasing evidence" as stated here.

We thank the Reviewer for this comments. We think that the "hot spots of N_2 fixation" in the Kuroshio not only represents high N_2 fixation rates but also the abundant diazotrophs in this region. In our study, higher N_2 fixation rate was not found at station K1, but much higher diazotroph biomass were observed here compared to that in the NSCS basin. We agree with the Reviewer (see the comment below) that "Abundance of diazotrophs do not necessarily mean their contribution to nitrogen fixation". However, the abundant diazotrophs here may imply a potential of high N_2 fixation activities supported by the favorable environmental conditions of this "hot spot". The coincidental lower rate we observed at K1 could have been caused by the

environmental conditions during our investigation, which do not necessarily mean the loss of high N_2 fixation potential, given the abundant diazotrophs we observed.

L385: Abundances of diazotrophs do not necessarily mean their contribution to nitrogen fixation.

Please see the response above.

L412-431, 453-464, etc.: The contents (mostly iron to nitrogen supply ratio) of Wen et al. 2022 are worth mentioning, but, they should be reduced significantly in the discussion. As said, the iron to nitrogen supply ratio was not analyzed in this study.

We thank for the Reviewer for this suggestion. As mentioned, although it appears that the iron to nitrogen supply ratio was important in regulating diazotroph biogeography in this study, this ratio was not directly analyzed and thus should not be overly highlighted here. We have now reduced discussion on Fe:N supply ratio and shifted the focus further to the diazotroph nutrient limitations and their potential causes.

L440: I think another reason would be that the analyzed groups did not represent the entire diazotroph community. There could be other diazotroph groups, which were not analyzed in this study, influenced by treatments.

We agree. A discussion of this possibility has been added as follow. "Other diazotrophs which were not analyzed by the qPCR assay, may be responsible for the enhanced N_2 fixation rates after nutrient additions"

L473-475: However, the nitrogen fixation at S3 and S4 was mostly iron-limited, while the *Trichodesmium* abundances there were not affected by iron addition treatment. All these pieces of finding should be considered when you discuss the relationship between iron and *Trichodesmium* in the NSCS.

We agree with this comment. In fact, significant enhancement of *Trichodesmium* abundance after Fe addition was observed in the experiment conducted at station S4 (see Fig. S2). However, at station S3, Fe-stimulation effect was only observed with N_2 fixation rate but not *Trichodesmium* abundance. This probably reflects a decouple of N_2 fixation rate and diazotroph abundance under specific environmental conditions. Nevertheless, regulation of Fe supply on diazotroph community structure remains a hypothesis that is difficult to test directly with the available experimental data.

References:

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