Reviewer 1

General comments
In their manuscript bg-2022-155, Wen et al. present the results of a series of nutrient additions experiments conducted in the South China Sea and western North Pacific, where in addition to bulk N_2 fixation rates, species composition based on *nifH* gene abundance was analyzed in response to Fe and P amendments.

Overall, I enjoyed reading the manuscript, and I find the dataset is a useful addition to our understanding of the regulation of N_2 fixation rates and how it is linked to species composition. The manuscript is well written, and the discussion insightful. Yet, there are a few points that I believe should be discussed/improved, specifically I have some concerns/queries with regard to replication and interpretation of qPCR results, as outlined below.

We thank the Reviewer for the positive evaluation of our manuscript, and provide responses to specific comments below.

Specific comments
Apparently, in several of the experiments there were only 2 replicates – I wonder whether the statistical analysis procedures are valid for two replicates? Could the authors at least indicate in each of the figures which data are averages of 2?

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 (i.e., 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 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 groups retained 3 replicates. Further details outlining the above information have now been added to the Methods section and also in the figure legends of Figures 4 and 5.

The authors acknowledge that ^{15}N label% was not measured, which is indeed a shortcoming, but as they state that the experimental procedure and the results were comparable to their previous study, I believe it is acceptable. An average and stdev of label% in the previous study are given, but could the authors add any further details to help us understand how reproducible the approach was (number of replicates etc)?

We sampled the $^{15}N_2$ atom% for N_2 fixation measurements using exactly the same approach during a cruise in 2020. The $^{15}N_2$ atom% values in the incubation bottles from the cruise are shown in table below. Only one replicate was measured for the $^{15}N_2$ atom% for each of the station depths. However, the relatively narrow range of 1.28% to 1.56% (mean ± s.d. of 1.40 ± 0.08, n = 17) suggests that the preparation, sampling, and measurement of $^{15}N_2$ were stable and reproducible.

Measured $^{15}N_2$ atom% in incubation bottles in a subsequent cruise to the western North Pacific during winter 2020.

Station	Longitude (degrees_east)	Latitude (degrees_north)	Depth (m)	$A^{15}N_2$ (atom %)
K11	118.5	21.5	5	1.37
K11	118.5	21.5	15	1.41
K11	118.5	21.5	25	1.43
K11	118.5	21.5	40	1.33
K11	118.5	21.5	90	1.41
K11	118.5	21.5	140	1.42
K11<10 µm	118.5	21.5	5	1.53
K11<10 µm	118.5	21.5	25	1.52
K11<10 µm	118.5	21.5	140	1.56
K8a	155.0	12.5	5	1.28
K8a	155.0	12.5	25	1.31
K13a	131.0	11.0	5	1.41
K13a	131.0	11.0	150	1.35
UW-127	137.6	13.4	Surface	1.39
ZH-56	126.0	20.2	Surface	1.39
ZH-58	124.0	20.4	Surface	1.35
ZH-61	121.0	21.0	Surface	1.33

I wonder how the reports on *Trichodesmium* polyploidy (ca 100 genome copies per cell in field samples, e.g. Sargent et al. 2016 https://doi.org/10.1093/femsle/fnw244) affect the estimates of species composition based on *nifH* gene copies, as well as the trends observed in bioassays. Was polyploidy taken into account when the 'dominant species (e.g., l. 351)' were determined? And, taking into account the high level of polyploidy in *Trichodesmium* compared to the other species, couldn't shifts in the species composition explain the mismatch in responses of N_2 fixation vs *nifH* abundance (e.g., l. 343)?

We agree with the Reviewer that polyploidy may have an important impact on the estimates of diazotroph compositions. 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 do not attempt to account/correct for this in calculations of proportions of the different diazotrophs. We however have made revisions to the text to stress that polyploidy might impact the assumed proportions of the different diazotrophs under both in situ and nutrient amended conditions (with associated references).

Related to this, I would suggest being more cautious about the use of the terms 'abundance' (e.g. abstract l. 26 'abundances of specific diazotrophs', l. 119 'abundances of specific diazotroph phyla') and 'growth' where actually gene copy number was measured. Specifically, in supplementary figure 1, I would suggest replacing 'growth rate' by 'gene abundance', since growth rate might imply that measurements of cell density or C concentration were made.

Thanks for this suggestion. Changes have now been made to the manuscript. For example, we emphasize it was *nifH* gene abundance that we measured. Also, the "growth rate" in Figure S1 has been replaced by "increase rate".

Minor comments

34-35 'the largest responses were always dominated by either *Trichodesmium* or UCYN-B': this is not clear (responses in what?) – can it be clarified?

This has now been clarified as "The largest responses in *nifH* gene abundances…".

110-112 it is not completely clear from this why high spatial resolution is necessary - can this be justified better?

The Kuroshio intrusion generates a frontal zone with a unique diazotrophy regime in the NSCS (Lu et al., 2019). Therefore, the lower spatial resolution of the experiments in Wen et al. (2022) and other studies (Chen et al., 2019; Shiozaki et al., 2014b) remains insufficient to delineate Fe and P controls on diazotrophy at finer spatial scales between the neighboring NSCS and the western boundary of the North Pacific. This justification has now been further refined in the revised manuscript.

164 can more details on the gas-tight plastic bags (supplier) be added?

Details added: "Tedlar®PVF, Dalian Delin Gas Packing Co., Ltd".

221 why were those Fe and P concentrations chosen – are there any references to add on how these relate to in situ concentrations in this area?

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-h experimental period, approximately 5 times higher concentrations were added into the incubation bottles. Details have now been added to the Methods.

223 Can the authors supply some more details on the incubation system? Do I understand correctly that 10L carboys were placed in the on-deck incubator, and there was some kind of water jacket flushed with seawater for temperature control?

The 10 L carboys were placed in a ~400-L clear on-deck incubator with inflow and outflow. Surface seawaters were then pumped into the incubator for temperature control. Details have now been added to the revised Methods.

Please supply more detail or a reference on the method for chl measurement.

More details and reference (Welschmeyer 1994) have now been added in the Methods.

441 I believe the biochemical substitution of Fe and P deserves some more explanation (either here or at a later stage) - how could this work, are there specific mechanism/enzymes that can substitute Fe for P?

We suggested that this substitution was not directly between Fe and P, but indirectly between the resources or the abilities of resource acquisition controlled by Fe and P. For instance, in addition to serving as cofactors of nitrogenase, Fe is also cofactors of alkaline phosphatases (Rodriguez et al., 2014; Yong et al., 2014). Thus, the addition of Fe may enhance the utilization of dissolved organic P (DOP) in the face of low level dissolved inorganic P (DIP) (Browning et al., 2017). Explanations have now been added to the revised text.

450-451 also the 'serial limitation' of N_2 fixation by another resource deserves a few more words for explanation I believe – it is not clear how this would work

A serial limitation, also termed secondary limitation, is the scenario where only the addition of one resource shows a positive response (either resource 1 or 2) and the addition of both resources together shows a bigger response than the primary, single limiting resource. This means, for example, the addition of resource 1 has led to growth which leads to depletion of resource 2 in the system. Thus, further addition of resource 2 leads a secondary growth response. In our case, N_2 fixation at stations SEATS2016 and S3 were independently co-limited by Fe and P, and the addition of Fe and P simultaneously should have led an additive rate response of N_2 fixation rates (Sperfeld et al., 2016). However, the absent of this additive response in our study may reflects that either addition of Fe or P has depleted other secondary limitation nutrients (e.g., Ni), or that the overall light level during our incubation has set an upper limit of N_2 fixation rate, which both have prevented further rates enhancement after nutrient additions.

476 I think there might be more specific refences for the Fe demand of *Trichodesmium* (e.g., Kustka et al., https://doi.org/10.1016/S0923-2508(02)01325-6, 10.4319/lo.2003.48.5.1869, https://doi.org/10.1046/j.1529-8817.2003.01156.x)

We thank the Reviewer for providing this information. This reference has now been added to the revised manuscript.

Could it be specified how the data in Fig. S1 was calculated? Is the growth rate in units d-1 the total increase in gene abundance over the 3 days bioassay experiment divided by 3?

The relative growth rates (now change to increase) were defined as the relatively changes of N_2 fixation rates or *nifH* abundances after nutrient additions compared to the control. The equation for the increase rate calculation is Ln (*nifH*_treatment / *nifH*_control) / time, where time is in days. Details were added in the main text and figure legend of Figure S1.

In the supplementary table showing parameters involved in nitrogen fixation rate calculations, the units for several of the parameters (e.g., depth, chl a, PON, NFR) are missing in the headers, please add these.

Units have been added in the supplementary table.

Technical corrections
62-63 check the order of references: in sequence of publishing year?

Corrected.

69 *did* not quantitatively match

Corrected.

72 *is* potentially crucial

Corrected.

76 I assume this should mean 'contribute differently to the sinking flux carbon that small unicellular species (i.e., delete 'that of')'?

Corrected.

110 remain*s*

Corrected.

242 metal bound (remove hyphen)

Corrected.

266 below

Corrected.

313 and others: remove brackets around station names in the text

Corrected.

References:

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, 8, 15465, 10.1038/ncomms15465, 2017.

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.

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, Nature Communications, 12, 10.1038/s41467-021-24299-y, 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.

Rodriguez, F., Lillington, J., Johnson, S., Timmel, C. R., Lea, S. M., and Berks, B. C.: Crystal structure of the bacillus subtilis phosphodiesterase PhoD reveals an iron and calcium-containing active site, J. Biol. Chem., 289, 30889-30899, 10.1074/jbc.M114.604892, 2014.

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.

Shiozaki, T., Chen, Y. L. L., Lin, Y. H., Taniuchi, Y., Sheu, D. S., Furuya, K., and Chen, H. Y.: Seasonal variations of unicellular diazotroph groups A and B, and *Trichodesmium* in the northern South China Sea and neighboring upstream Kuroshio Current, Cont. Shelf Res., 80, 20-31, 10.1016/j.csr.2014.02.015, 2014.

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.

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, Science Advances, 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, Frontiers in Marine Science, 5, 10.3389/fmars.2018.00027, 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.

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, 345, 1170-1173, 10.1126/science.1254237, 2014.

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.