

“Changes in diazotrophic community structure associated with Kuroshio succession in the northern South China Sea” by Han Zhang et al.

We have taken all the comments of the Reviewers into account in the revision. Our point-by-point responses are provided below in blue fonts. Please note that all the line numbers mentioned in the response refer to the Marked-up Manuscript.

**General comments:**

**Reviewer #2 (Comments for the Author):**

1. The study explores the impact of interannual Kuroshio intrusions (KIs) on diazotrophic communities in the northern South China Sea. The research identifies a correlation between diazotroph variations and KI-induced changes in environmental factors. Notably, *Trichodesmium* abundance and N<sub>2</sub> rate increases in strongly affected stations, while unicellular N<sub>2</sub>-fixing cyanobacteria (UCYN-B) thrive in less affected stations. UCYN-C and non-cyanobacterial  $\gamma$ -proteobacteria dominate moderately affected stations. The findings suggest KI succession shapes diazotrophic communities as a stochastic process, potentially influencing region-scale redistribution of diazotrophs and nitrogen budgets in a warming ocean. I find the work excellent and should be considered for acceptance, however some minor revision is needed.

**Response:**

We thank the Reviewer for the comments. We are thankful for considering this manuscript for acceptance. We hope that our point-by-point response below will address your concern.

2. **Introduction:** As there is some excellent work done with non-cyanobacteria diazotrophs I recommend a deeper introduction into those. As a start, investigate “non-cyanobacterial diazotrophs: global diversity, distribution, ecophysiology, and activity in marine waters” by Kendra A. Turk-Kubo to get an overview.

**Response:**

We are grateful for the Reviewer’s comments. We agree with the Reviewer that there is some excellent work done with non-cyanobacteria diazotrophs, and that non-cyanobacterial diazotrophs could play an important role in global marine N<sub>2</sub> fixation. Therefore, we have added further description about non-cyanobacterial diazotrophs in Introduction as below:

Line 52-54: “NCDs have been reported to be ubiquitous in ocean ecosystems and contribute to global marine N<sub>2</sub> fixation (Moisander et al, 2014; Chakraborty et al, 2021; Turk-Kubo et al 2022).”.

**Method:**

3. Regarding N<sub>2</sub> fixation and productivity rate. What elemental analyzer were used (i.e. model).

**Response:**

We are grateful for the Reviewer’s comments. We have added further description about elemental analyzer in Method as below:

Line 136-138: “To estimate the natural and tracer-enriched  $^{15}\text{N}$  and  $^{13}\text{C}$  abundance, the samples were first acid fumed to remove the inorganic carbon and then analyzed using an elemental analyzer coupled to a mass spectrometer (Flash HT 2000-Delta V Plus, Thermo Fisher Scientific).”

4. You used *nifH* ARB database from John Zehr. However, this is from 2017 and has not been updated. I suggest that you use the new *nifH* database, based on ARB and John Zehr but updated in 2023 according to the NCBI. See link <https://github.com/moyn413/nifHdada2>

Response: We are thankful for the Reviewer’s suggestion. We have used the updated *nifH* database to conduct the analysis and got the same results. We have added further description about elemental analyzer in Method as below:

Line 187-189: “OTUs were annotated down to the genus level using a DADA2 formatted *nifH* gene database (<https://github.com/moyn413/nifHdada2>) that was updated in 2023 according to NCBI and Zehr Lab (<https://www.jzehrlab.com/nifH>).”.

5. It would be helpful to know if you did qPCR on all stations and if you did sequence of all stations or only some.

Response:

We are thankful for the Reviewer’s comments. The DNA samples on all stations (Fig. 1) were used as templates to quantify the *nifH* gene copies using TaqMan qPCR technique targeting 10 major diazotrophic phylotype, and nested PCR was also performed to amplify *nifH* genes sequence from DNA samples on all stations using the nested, degenerate *nifH* primers.

#### **Discussion:**

6. At section 4.1, I think that the paper could benefit from a deeper discussion about non-cyanobacterial diazotrophs.

Response:

We are thankful for the Reviewer’s suggestion. We have added a deeper discussion about non-cyanobacterial diazotrophs as below:

Line 312-315: “In contrast to UCYN-C, the abundance of non-cyanobacterial diazotrophic  $\alpha$ - and  $\gamma$ -proteobacteria was much higher in 2017 than in 2018, suggesting that heterotrophic diazotrophs could potentially be transported by Kuroshio to the nSCS. The close abundance of non-cyanobacterial diazotrophic  $\alpha$ - and  $\gamma$ -proteobacteria (Fig. 3) indicates that these two groups occupy similar ecological niches (Turk-Kubo *et al.* 2022).”.

#### **Specific comments:**

7. Title: Should it not be “changes in diazotrophic community.....”

Response:

We thank the Reviewer for the suggestion, and we have revised the Title as “Changes in diazotrophic community structure associated with Kuroshio succession in the northern South China Sea”

8. In line 18 at the abstract, you write “..non-cyanobacterial gamma-proteobacteria..”, you can delete either non-cyanobacteria or gamma-proteobacteria. As it is evident that a gamma-proteobacteria is a NCD.

Response:

Thanks for the suggestion and we have deleted “non-cyanobacterial”. in Abstract as below:

Line18-19: “.....,whereas UCYN-C and the non-cyanobacterial  $\gamma$ -proteobacteria were prevalent at stations moderately affected by KI.”

9. Line 20 - remove “more”.

Response:

Thanks for the suggestion and we have removed it. Please refer to Line 20.

10. Line 20-21 – Rephrase “..in 2017 when KI was strong than in 2018 when KI appeared to have retreated.” Or perhaps just say “..when KI was stronger compared to 2018 where KI retreated”. If I understood it correctly

Response:

We are thankful for the Reviewer’s suggestion. We have revised the description as bellow:

Line 19-21: “Neutral community model further demonstrated that dominant diazotrophic subcommunities were more significantly affected by environmental factors in 2017 when KI was stronger compared to 2018 when KI retreated.”

11. Line 50-52 - Perhaps expand the importance of these new groups? e.g., they have been models and measurement of NCDs contribution to  $N_2$  fixation.

Response:

We thank the Reviewer for the comments. We have added the description about the importance of these new groups as below:

Line 50-54: “Particularly, diazotrophs genomes reconstructed from global metagenomic data have extended the PCR-based amplicon surveys, revealing new diatom-diazotroph symbioses (Schvarcz et al., 2022) and new species of non-cyanobacterial diazotrophs (Bombar et al., 2016; Delmont et al., 2018). NCDs have been reported to be ubiquitous in ocean ecosystems and contribute to global marine  $N_2$  fixation (Moisander et al, 2014; Chakraborty et al, 2021; Turk-Kubo et al 2022).”

12. Line 180 - were relative abundance of OTU determined from UPARSE? Or how was this determined?

Response:

We thank the Reviewer for the comments. We determined relative abundance of OTU using UPARSE with a 97% similarity cutoff.

Line 180-187: “Raw demultiplexed paired-end reads were processed into Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1) (<http://drive5.com/uparse/>) with a novel ‘greedy’ algorithm that performs chimera filtering and OTU clustering simultaneously. OTUs were annotated down to the genus level using a DADA2 formatted

nifH gene database (<https://github.com/moyn413/nifHdada2>) that was updated in 2023 according to NCBI and Zehr Lab(<https://www.jzehrlab.com/nifH>).”.

Sequence data were not rarified before analysis as this may lead to a loss of information and our minimum sequence depth of ca. 36519 reads per sample exceeded the depth considered acceptable for comparing microbiome composition between samples. Functions within the package ‘phyloseq’ (v. 1.34.0; McMurdie and Holmes, 2013) were used to generate a phyloseq object containing the filtered count table, taxonomy table, and contextual sample data. Statistical analyses were performed with compositional data based on the “total sum” relative read abundances.

13. Line 184 - you mention “some samples”. What is the threshold? How many samples? below or above e.g. 10?

Response:

We are thankful for the Reviewer’s comments. The total number is 10, so the threshold is 10. Here, “some” means the number of samples is below the threshold 10. We have clarified this in the revised manuscript.

Line 193-199: The OTUs were classified into six categories: (1) OTUs with abundance >1% in all samples (n=10) were classified as abundant taxa (AT); (2) OTUs with abundance <0.01% in all samples were classified as rare taxa (RT); (3) OTUs with abundance between 0.01 and 1% in all samples were classified as moderate taxa (MT); (4) OTUs with abundance below 1% in all samples and <0.01% in some samples (n<10) were classified as conditionally rare taxa (CRT); (5) OTUs with abundance ≥0.01% in all samples and ≥1% in some samples were classified as conditionally abundant taxa (CAT); (6) OTUs with abundance between 0.01% and 1% were classified as conditionally rare and abundant taxa (CRAT).”.

14. Line 191 - why not transform pH?

Response:

We thank the Reviewer for the comments. The pH data in this study was close to normal distribution. Therefore, the pH data was not transformed.

15. Line 268-269 - I would recommend to include station number, to help the reader.

Response:

We are thankful for the Reviewer’s comments. We have added the station number in the manuscript as below:

Line 283-284: “Specifically, the two most abundant diazotrophs were *Trichodesmium* and UCYN-B, which dominated the Kuroshio (stn11) and nSCS (stn4\*), respectively (Fig. 3).”

16. Line 275 - Regarding sinking *Trichodesmium*. Check this reference “Sinking *Trichodesmium* fixes nitrogen in the dark ocean” by Mar Benavdie.

Response:

We thank the Reviewer’s for the reminder. We have revised the reference.

Please see Line 292-293: “*Trichodesmium* with gas vacuoles can migrate vertically to the deeper waters to optimize the uptake, utilization, and even storage of P, fulfilling the growth requirement (Sohm et al., 2011; Benavides et al., 2022).”.

17. Line 343 - Regarding salinity affecting diazotrophs. Look into “Salinity as a key control on the diazotrophic community composition in the southern Baltic Sea” by Christian Reeder and “Diversity, structure, and distribution of bacterioplankton and diazotroph communities in the Bay of Bengal during the winter monsoon” by Chao Wu.

Response:

We are thankful for the Reviewer’s comments. We have added further discussion about the effect of salinity on diazotrophs as below:

Line 377-379: “Salinity is another important environmental factor affecting the osmoregulation, metabolism, and community composition of diazotrophs (Zehr and Turner, 2001; Reeder et al., 2022). *Trichodesmium* was reported to be abundant in the area with high salinity (e.g., the Luzon Strait) (Wu et al, 2018), which is consistent with our analysis.”

18. Figure 2 - “b” is needed. Which figures belong to which year? and secondly, legend title is needed. E.g. depths for b,c and temperature for d,e. Also legend title and unit for a

Response:

We thank the Reviewer for the comments. We have added “b”, legend title and unit in Figure 2. In addition, we have revised the figure caption to clarify the sampling year for each panel in Figure 2 as below:

Line 713-717: “Figure 2. Potential temperatures, salinities and density anomalies modeled for the cruises in 2017 and 2018. (a) Plot of  $\theta$ - $S$  showing potential temperatures ( $\theta$ , °C) and salinities ( $S$ ) of water parcels resulting from mixing of the Kuroshio and SCS water masses. Potential density anomalies ( $\sigma_\theta$ , kg m<sup>-3</sup>), shown in grey lines, are imposed on the  $\theta$ - $S$  plot. (b–e) Profiles of depths (b, c) and temperatures (d, e) of isopycnal surface along the  $\sigma_\theta$  of 23 kg m<sup>-3</sup> of water masses depicted for the cruises in 2017 (b, d) and 2018 (c, e).”

## 19. References

Benavides, M., Bonnet, S., Le Moigne, F.A.C. et al. Sinking *Trichodesmium* fixes nitrogen in the dark ocean. ISME J 16, 2398–2405 (2022). <https://doi.org/10.1038/s41396-022-01289-6>

Wu C, Narale DD, Cui Z, Wang X, Liu H, Xu W, Zhang G, Sun J. Diversity, structure, and distribution of bacterioplankton and diazotroph communities in the Bay of Bengal during the winter monsoon. Front Microbiol. 2022 Nov 30;13:987462. doi: 10.3389/fmicb.2022.987462. PMID: 36532434; PMCID: PMC9748438.

Reeder, C. F., Stoltenberg, I., Javidpour, J., and Löscher, C. R.: Salinity as a key control on the diazotrophic community composition in the southern Baltic Sea, Ocean Sci., 18, 401–417, <https://doi.org/10.5194/os-18-401-2022>, 2022.

Kendra A Turk-Kubo, Mary R Gradoville, Shunyan Cheung, Francisco M Cornejo-Castillo, Katie J Harding, Michael Morando, Matthew Mills, Jonathan P Zehr, Non-cyanobacterial

diazotrophs: global diversity, distribution, ecophysiology, and activity in marine waters, FEMS Microbiology Reviews, 2022; fuac046, <https://doi.org/10.1093/femsre/fuac046>

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Response:

We thank the Reviewer for providing the references. They are helpful and we have cited these references in the revised manuscript.