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"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.

Reviewer comments:

Reviewer #1 (Comments for the Author):

1. N_2 fixation plays important role in food-web process, carbon sequestration and export of organic carbon to the deep ocean. Kuroshio intrusion and associated environmental alteration may profoundly affect biogeography and N_2 fixation rate of diazotrophs. This study demonstrated changes in diazotrophic community structure and N_2 fixation because of Kuroshio intrusion in the northern South China Sea (nSCS) based on two cruises in 2017 and 2018. The authors found that Trichodesmium was more abundant and N_2 fixation rate was higher at stations strongly affected by Kuroshio intrusion, whereas UCYN-B were more abundant at stations least affected by Kuroshio intrusion. UCYN-C and γ-proteobacteria were mainly distributed at stations moderately affected by the Kuroshio. These results suggested that diazotrophic community composition and nitrogen fixation rate in nSCS are highly regulated by Kuroshio intrusion, which will contribute to our understanding of how Kuroshio affect diazotrophic diversity and nitrogen fixation. Overall, I appreciate their excellent work. However, the presentation of manuscript (particularly Results and Discussion) needs to be improved. I will recommend consideration of its acceptance for potential publication after a minor revision.

Response:

We thank the Reviewer #1 for a very elaborated and professional review. We are thankful for the helpful comments that helped us update and improve the manuscript. We believe that the revised version is in a better form, as we did our best to address all the comments. Our point-by-point response is provided below.

2. Introduction should review what is already known about effects of Kuroshio intrusion on diazotrophs (particularly *Trichodesmium*) and nitrogen fixation in the in marginal seas of NW Pacific. Also, there is a lack of hypothese on the effects of Kuroshio intrusion on the diazotrophic community composition and N_2 fixation in the nSCS as well as the intrusion intensity.

Response:

We are grateful to the Reviewer for providing constructive comments. We have incorporated the Reviewer's suggestions and have made specific modifications to the Introduction accordingly. These revisions encompassed two key aspects.

First, we focused on the impacts of KI on diazotrophs (particularly *Trichodesmium*) and nitrogen fixation in the in marginal seas of western North Pacific.

Second, we focused on the hypothesis regarding the impact of Kuroshio intrusion on the composition of diazotrophic communities, nitrogen fixation, and intrusion intensity in the nSCS.

We have revised the Introduction as below:

Line 75–77: "In contrast to the well studied diazotrophic biogeography in these regions, less effort has focused on the impacts of KI on marginal seas of the northwestern Pacific (Jiang et al., 2023)."

3. Prefilteration using a 100 μm pore-size nylon mesh can remove large zooplankton, but probably remove *Trichodesmium*, particularly large-sized colonial trichomes, resulting in a potential underestimation of *Trichodesmium* abundance.

Response:

We completely agree with the reviewer that prefiltration using a 100 μm pore-size nylon mesh might potentially remove colonial trichomes of *Trichodesmium* larger than 100 μm. To be more rigorous, we clarified the rationale of prefiltration and revised the Materials and Methods and Discussion section as below:

Line 99–102: "At each station, 1.5–3 L of seawater was prefiltered through a 100-μm pore-size nylon mesh to remove large zooplankton and fish, and then filtered through a 0.22-μm pore-size 47-mm diameter polycarbonate membrane (Millipore, USA) with low pressure (<100 mm Hg pressure) for subsequent DNA extraction."

Line 293–295: "It is worth noting that, since we did not observe colonial *Trichodesmium* in our sampling stations, prefilteration using a 100-μm pore-size nylon mesh was not likely to underestimate *Trichodesmium* abundance."

4. The authors don't describe how to collect qPCR samples for daytime and nighttime in Materials and Methods, is it to take parallel samples at the same time, one of which is filtered during the daytime (nighttime) to obtain the samples, and the other is put into the nighttime (daytime) to be filtered again? Discussion also does not address in detail why the *nifH* gene abundance of the diazotrophic groups differed largely between daytime and nighttime. In general, the *nifH* gene expression in diazotrophic groups should be determined using RT-qPCR rather than qPCR.

Response:

We are thankful to the Reviewer for the comments. We have addressed your concerns regarding the collection of samples and the analysis conducted in our study.

We have revised the description in Materials and Methods as below:

Line 97–99: "Samples were collected using a Seabird SBE 911Plus rosette sampling system (Sea-Bird Electronics, USA) at 14 stations (Fig. 1 and Table S1), seven of which were chosen for both daytime and nighttime sampling, whereas the other seven stations were used only for either daytime or nighttime sampling.".

The difference in the *nifH* gene abundance of the diazotrophic groups between daytime and nighttime can be attributed to temporal and spatial variations in environmental factors across different sampling times and stations. We agree with the Reviewer that the *nifH* gene expression in

diazotrophic groups should be determined using RT-qPCR rather than qPCR. We noted that the *nifH* transcripts could be influenced by various external factors (e.g., light intensity, temperature and nutrients). In addition, the *nifH* gene expression can exhibit large variability over time within each diazotroph (Church et al., 2005; Wilson et al., 2017). Therefore, conducting comparisons across different organisms based on the *nifH* gene expression data would not be appropriate. Furthermore, given that the timing of sampling differed across stations, comparisons among these stations using the *nifH* gene expression data would not be valid, even within a single organism. We appreciate your understanding of these limitations and their implications for our study.

5. Nitrogen fixation rate and primary production are missing in Results. The authors can compare nitrogen fixation rates and primary production in the Kuroshio water, mixed water and SCS water. This analysis should be useful in further exploring the influences of changes in diazotrophic composition and nitrogen fixation induced by the Kuroshio intrusion on the carbon and nitrogen biogeochemical cycling of the nSCS as well as the implications.

Response:

We thank the Reviewer for the comments. We have added the description about nitrogen fixation and primary production rates in Results as below:

Line 228-233: "The water column-integrated rates of N_2 fixation (I_{NFR}) in the KC (129.42 ± 100.86 μmol N m^{-2} d^{-1}) was higher than that in the nSCS (97.85 ± 6.98 μmol N m^{-2} d^{-1}). Surface N_2 fixation rates (1.88 ± 0.85 nmol N L^{-1} d^{-1}) in the nSCS was higher than those in the KC (0.60 ± 0.25 nmol N m^{-2} d^{-1}, stn 1 to stn 4) as observed along the large-scale transect. However, the surface primary productivity was found to be similar between the nSCS (0.37 ± 0.12 μmol C L^{-1} d^{-1}) and the KC (0.21 ± 0.11 μmol C L^{-1} d^{-1})."

We also have incorporated this into the Discussion in the marked-up Manuscript as below:

Line 316-321: "the nitrigen fixation rate at surface (S_{NFR}) in the KC was lower than that at nSCS, which may be due to the deeper nitracline in the KC inhibiting the upward transport of nutrients (Chen et al., 2008). The relatively higher nitrogen fixation rate of the entire water column (I_{NFR}) was observed. This special distribution pattern of I_{NFR} and S_{NFR} might be explained by the distinct niches of different diazotrophs, where KI-transported *Trichodesmium* typically dominates shallow warm water (< 50 m) (Jiang et al., 2015), whereas UCYN-B prefers to live in lower temperature and deeper water (50–75 m) (Moisander et al. 2010)."

6. Conclusions are not simple repetitions of the results, please revise them.

Response:

We are grateful for the Reviewer's suggestion. We have revised Conclusions as below:

Line 407-417: "In this study, we surveyed the diversity and abundance of diazotrophic phylotypes in the nSCS in two consecutive years based on molecular approaches targeting the nitrogenase gene *nifH* and associated the patterns in changes of diazotrophic community structure in the upper 100 m with the degrees of KI quantified using the isopycnal mixing model. Our study reveals a lineage-specific niche adaptation to environmental changes associated with KI in the

overall diazotrophic community structure in the nSCS. Four diazotrophic groups, *Trichodesmium*, UCYN-B, UCYN-C, and γ-proteobacteria, appear to be highly correlated with KI leading to variations in a range of physicochemical parameters. KI has a dominant dilution effect on the nutrient inventory and is responsible for the enhanced N_2 fixation on one hand, it also causes redistribution of the diazotrophic taxa and reallocation of the nutrients on the other hand. Neutral model simulation suggests that the diazotrophic community is more likely affected by KI primarily as a stochastic process. As KIs are projected to intensify in a future warming ocean, Kuroshio may transport diazotrophs (especially the UCYN-A) in the upstream warmer regions including SCS northward to higher latitudes, resulting in a wider distribution of N_2 fixation in the global ocean."

Specific comments below:

7. I suggest delete some unnecessary connecting adverbs in the text, such as "Moreover" and "Collectively" in Abstract.

Response:

We thank the Reviewer for the suggestion. We have removed several unnecessary connecting adverbs from the text. We have revised Abstract as below:

Line 10-23: "Kuroshio intrusion (KI) is a key process that transports water from the Western Pacific Ocean to the northern South China Sea (nSCS), where KI-induced surface water mixing often causes variations in microbial assemblages. Yet, how interannual KIs affect biogeography of diazotrophs and associated environmental factors, remains poorly characterized. Here, by quantifying the degree of KIs in two consecutive years, coupled with monitoring the diversity and distribution of nitrogenase-encoding *nifH* phylotypes with quantitative PCR and high-throughput sequencing, we show that changes in the diazotrophic community structure in the nSCS are highly correlated with KI leading to variations in a range of physicochemical parameters. Specifically, the filamentous cyanobacterium *Trichodesmium* was more abundant at stations strongly affected by KI, and hereby with deeper mixed layer, higher surface salinity, and temperature; the unicellular N_2-fixing cyanobacteria in group B (UCYN-B) were more abundant at stations least affected by KI and correlated with nutrient availability, whereas UCYN-C and the γ-proteobacteria were prevalent at stations moderately affected by KI. Neutral community model further demonstrated that dominant diazotrophic subcommunities were significantly affected by environmental factors in 2017 when KI was stronger compared to 2018 when KI retreated. Our analyses provide insightful evidence in the role of KI succession in shaping diazotrophic community structure primarily as a stochastic process, implying a potential region-scale redistribution of diazotrophs and nitrogen budget, given that KIs are projected to intensify in a future warming ocean."

8. L45: Please replace "UCYN-B is mostly free-living with Crocosphaera watsonii being a cultivated representative" with "UCYN-B (Crocosphaera watsonii) is mostly free-living, being a cultivated representative".

Response:

We thank the Reviewer for the suggestion. We have revised Introduction as below:

Line 43-46: “Phylogenetically distinct clades of UCYN have been detected. The “*Candidatus* Atelocyanobacterium thalassa” in group A (UCYN-A) appears to be an obligate endosymbiont of the single-celled prymnesiophyte algae (Thompson et al., 2012), whereas UCYN-B is mostly free-living and a culturable representative, *Crocosphaera watsonii*, has been successfully isolated.”

9. L124-125: Light gradient should be provided.

Response:

We appreciate the Reviewers’ comments and suggestions regarding our manuscript. We have added the description about light gradient as below:

Line 131-133: “Light conditions were recorded at 5 fixed depths (5, 25, 50, 60-75, 115-150 m) within the water column, corresponding to approximately 50%, 30%, 10%, 1%, and 0.1% of the surface irradiance, respectively.”

10. L207: Please delete “Du et al., 2013”.

Response:

We thank the Reviewer for the suggestion. We have deleted “Du et al., 2013” from the text. Please see Line 219.

11. L295: This paragraph is not directly related to nitrogen fixation. I suggest the authors emphasize the changes in physical parameters and nutrients in nSCS caused by Kuroshio intrusion, in addition the description of dynamic process. How and why these changes affect N_2 fixation?

Response:

We are thankful for the Reviewer’s suggestion. We have integrated into the discussion about the changes in physical parameters and nutrients in nSCS caused by Kuroshio intrusion and their effects on N_2 fixation.

Line 354–357: "The dilution effect of KI on surface nutrient budget in nSCS could provide favorable ecological niches for diazotrophs, limiting other phytoplankton which can not conduct N_2 fixation (Zehr & Capone 2020). This might partially elucidate why KI has the potential to enhance N2 fixation rates in the nSCS.".

12. L314-315: In addition to the results of Kao et al., 2012, how diazotrophs contributed nitrogen budget and primary/new production.

Response:

We thank the Reviewer for the comment. According to previous studies conducted by Chen et al (2014) and Wu et al (2018), it has been suggested that unicellular diazotrophs play a significant role in N_2 fixation in the South China Sea (SCS) and the Kuroshio. Wu et al (2018) reported that unicellular diazotrophs contribute approximately 75% of N_2 fixation in the northern SCS and the Kuroshio. Similarly, Chen et al (2014) demonstrated that unicellular diazotrophs contribute 65% and 50% of N_2 fixation in the SCS and the Kuroshio, respectively.The contribution of N_2 fixation to primary production has been found to be less than 20% in studies conducted by Lu et al (2019) and Wu et al (2018). The estimated amount of new nitrogen introduced by *Trichodesmium*

contributed up to 0.14% of the total primary production and 0.41% of the new production in the Luzon Strait (Wu et al 2018). We revised the text as below:

Line 343–345: "N_2 fixation in the nSCS was estimated to be ~20 mmol N m^{-2} yr^{-1}, which accounts for less than 10% of the current nutrient inventory variation (~250 mmol N m^{-2} yr^{-1}) (Kao et al 2012). Unicellular diazotrophs were reported to contribute more than 50% of N_2 fixation (Chen et al 2014, Wu et al 2018). "

13. L318-319: These values of 75.98 ±48.77 μmol N m^{-2} d^{-1} and 74.98 ± 26.55 μmol N m^{-2} d^{-1} were the average NFR in nSCS during 2017 and 2018, respectively.

Response:

We thank the Reviewer for the comment. We have revised the text as below:

Line 351-352: "The KI was conspicuously responsible for the higher water column-integrated rates of N_2 fixation (I_{NFR}) in KC than in nSCS".

14. Table S4: I suggest remove Table S4 from supplementary materials to the text.

Response:

We thank the Reviewer for the suggestion. We have removed Table S4 from supplementary materials to the text (Table 1).

15. Table S5: Why are there two sampling times for the same station in Table S5, is it to compare the effects of daytime and nighttime on diazotrophs? If so, RT-qPCR should have been used to determine *nifH* gene expression in different diazotrophic groups.

Response:

We thank the Reviewer for the question. In Table S4 (Table 5 in the previous version), we removed the *nifH* gene copies data of nighttime. We primarily focused on surface daytime data to conduct our subsequent analysis (including determination of abundant and rare taxa, correlation of microbial communities with environmental factors and geographical distance, and diazotrophic community assembly modeling) in the main text. We agree with the Reviewer that anlysis on the effects of daytime and nighttime on diazotrophs should be based on the *nifH* gene expression data using RT-qPCR. However, this might not be the main purpose of this study.

Furthermore, we conducted quantitative and high-throughput sequencing experiments at the DNA level due to the potential influence of external factors on gene expression at the transcriptional level. In order to maintain consistency and minimize such influences, we specifically selected samples collected during the day for our analysis. We appreciate your feedback and hope that this explanation addresses your concerns satisfactorily.

16. Figure 1: Please give the full names of nSCS an KI.

Response:

We thank the Reviewer for the suggestion. We have give the full names of nSCS and KI in the caption of Figure 1.

17. Figure 2: "(b)" is lacking.

Response:

We appreciate the Reviewer's comment. We have added "(b)" in Figure 2.

18. Figure 6: Please give the full name of RDA. In addition, the relative contribution of different parameters to variation in diazotrophic community composition needs to be quantified.

Response:

We thank the Reviewer for the suggestion. We have given the full name of Redundancy analysis in the caption of Figure 6. In addition, the relative contribution of different parameters to variation in diazotrophic community composition have been quantified in supplementary materials Table S8, S9.

19. The station numbers in Fig. 3 and Table S5 did not match. Figure 3 and Table S5 showed that the abundance of Richelia in Kuroshio water was higher than that in SCS water, which is similar to the findings of Tuo et al (2014). Chen et al. (2014) has showed that nitrogen fixation rate was much higher in Kusoshio than in nSCS during warm seasons. Also, they found that relative contribution (59%) of filamentous diazotrophs (>10 or 20 mm, mostly *Trichodesmium* and *Richelia*) to nitrogen fixation was higher than that (41%) of unicellular filamentous during warm seasons. Similarly, result of Jiang et al. (2023) suggested that Kuroshio intrusion stimulated growth of *Trichodesmium* and enhanced nitrogen fixation in the East China Sea during summer. Therefore, the present study on diazotrophic abundance and nitrogen fixation was highly consistent with earlier studies in marginal seas of NW Pacific suffered from intrusion of Kuroshio. I suggest the authors analyze more deeply about the mechanism of Kuroshio intrusion on the community composition of diazotrophs and nitrogen and carbon fixation.

Response:

We appreciate the Reviewer's valuable comments. Figure 3 presented the surface daytime abundance of the ten major diazotrophic groups, as determined by the TaqMan qPCR assay of the *nifH* gene copies. In Table S4 (Table 5 in the previous version), we had included the diel patterns in abundance of the targeted diazotrophic groups. We apologized for any confusion caused by the absence of nighttime data in Table S4. We have revised the Table S4 in supplementary materials. We have revised the text as below:

Line 286-288: "The high abundance of *Trichodesmium* was previously reported in the East China Sea affected by KI during summer (Jiang et al., 2023). This may be attributed to *Trichodesmium*'s higher temperature preference and the coastal input of iron when Kuroshio flows past some islands (Cheung et al., 2017)."

Table S8. The importance components, eigenvalue proportion explained and cumulative proportion in redundancy analysis (RDA)

Components	Eigenvalue	Proportion	Cumulative
RDA1	0.1640	0.5770	0.5770
RDA2	0.0615	0.2164	0.7935
RDA3	0.0309	0.1086	0.9021
RDA4	0.0121	0.0426	0.9447
RDA5	0.0065	0.0228	0.9675
RDA6	0.0025	0.0087	0.9761
RDA7	0.0011	0.0040	0.9802
RDA8	0.0009	0.0031	0.9833
RDA9	0.0003	0.0012	0.9845
RDA10	0.0001	0.0003	0.9847
RDA11	0.0000	0.0001	0.9848
RDA12	0.0000	0.0000	0.9849
RDA13	0.0043	0.0151	1

Table S9. The contribution of variables in the environments factors matrix and major diazotrophic groups to the variation in the species matrix explained by the RDA. The environmental factors used in this study are shown in Table S4.

		RDA1	RDA2
Environments factors	SST	-0.0952	0.5214
	SSS	-0.1040	0.7065
	MLD	0.1829	0.4228
	DCM	0.0851	0.8747
	I_{DIN}	-0.8404	-0.2569
	I_{DIP}	-0.9077	-0.1455
	Nit	-0.8394	0.1058
	I_{NFR}	-0.3828	0.4466
	I_{PP}	-0.3233	-0.5956
	S_{NFR}	-0.6608	0.0791
	S_{PP}	-0.4627	-0.5505
	R_{K_100}	0.1800	0.6767
Major diazotrophic groups	*Trichodesmium*	0.0110	0.5879
	UCYN-B	-0.7612	-0.1394
	UCYN-C	0.5982	-0.1114
	γ-proteobacteria	0.2126	-0.1731