Biogeosciences

Discussions

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Interactive comment on "Seasonal dynamics of nitrogen fixation and the diazotroph community in the temperate coastal region of the northwestern North Pacific" *by* T. Shiozaki et al.

T. Shiozaki et al.

shiozaki@aori.u-tokyo.ac.jp

General comments: Nitrogen fixation rate measurements and nifH gene based molecular studies in temperate regions of western North Pacific Ocean is relatively rare, comparing with the intensively studied Eastern Pacific Ocean and Atlantic Ocean. The authors reported nitrogen fixation rate and some nifH sequences of potential nitrogen fixers in the temperate coastal region of the western North Pacific Ocean, which can provide some missing knowledge in this field. In general, the patterns and explanations of nitrogen fixation rate presented in this study are good and making sense, while the part of nifH gene based molecular study is too weak to reveal the community structures

in the studied regions. The authors only included less than 200 clones of nifH gene amplicons from the six cruises and tried to discuss "diazotroph community structure", in which any statements made are not convince enough. Besides that, there are numbers of unclear issues, related to the methodologies of both rate measurement and molecular works, needed to be clarified or addressed. The authors tried to link up the 2011 Tohoku-oki tsunami with the diazotroph community structure in discussion part and conclusion part. However, without comparing the diazotroph community structures before and after the tsunami, it is inappropriate to make any related conclusions.

// The number of obtained sequences was not enough to describe diazotroph diversity. As you suggested below, some diazotrophs could not be captured from the small number of clones. The clone library analysis showed that UCYN-A, Trichodesmium, and γ-24774A11 were likely to be important diazotrophs from early summer to fall when nitrogen fixation occurred. Therefore, to make up the deficiency, we have quantified these groups by a qPCR analysis. In addition, UCYN-B which is considered a major diazotrophs in the tropical and subtropical oligotrophic ocean (Moisander et al., 2010) has been quantified. The qPCR analysis demonstrated that the target groups were quantified even at stations where these clones were not recovered from the clone library analysis, suggesting that the number of clones was not sufficient to capture the diazotroph community structure on each cruise. Despite this limitation, the sequences more frequently recovered in the clone library generally corresponded to the most abundant group revealed by the qPCR analysis. For example, UCYN-A was frequently recovered in the library during the KT-12-20_Aug, KK-13-1_Jun, and KK-13-6_Sep cruises; for these samples, the qPCR results showed that UCYN-A was the most abundant group among the four examined. Similarly, qPCR data indicated that Trichodesmium was the most abundant group during fall, when this group was frequently recovered in the library (during the KT-12-27_Oct cruise). This consistency in the general results obtained by the clone library and qPCR suggests that both of these approaches captured a similar seasonal trend in community composition changes for at least the major diazotroph groups. (L163-179, 390-402) Regarding the issues related

to the methodologies of both rate measurement and molecular works, we have replied to the following comments (see below). We would have to compare the diazotroph community before and after the tsunami to discuss the influence of the 2011 Tohoku-oki tsunami as you suggested. Therefore we have deleted the related sentences from the Discussion and Conclusion. Meanwhile, we have left the related sentences in the Introduction for the future research. //

Specific Comments: A. nifH gene based molecular works: The amount of nifH clones (200 clones) sequenced is really too few to reveal the community structure in 6 cruises. If the clones sequenced were evenly selected from the samples of 6 cruises, there should be approximately 33 clones sequenced per cruise and 8 sequences representing the diazotroph community in each sampling stations. In this case, the conclusions about distribution of diazotrophs will be very inaccurate. For examples, absence of Trichodesmium and other cyanobacterial diazotrophs in most of cruises may be due to the low coverage of sequencing and PCR primer induced bias(Langlois, Hümmer et al. 2008).

// As you suggested, the number of nifH clones could not be enough to reveal diazotroph community structure. The qPCR analysis showed that the target groups were quantified even at stations where these clones were not recovered from the clone library analysis. Meanwhile, as I mentioned above, the qPCR analysis indicated that the clone library analysis could capture dominant group in diazotroph community. In the revised manuscript, on the basis of these results, we have shown the major diazotroph (UCYN-A from early summer to late summer, Trichodesmium in fall, and Cluster III from winter to spring), and discussed why they thrive in a particular season. (L390-466) //

P.2, l.4: As mentioned by another referee, the authors should not use the term "diversity" here. The author can use "diazotroph community" or "identities of potential nitrogen fixers" to replace the term "diversity".

// We have rephrased the word to "diazotroph community". (L14) //

C1452

P.5 l.9-14: The authors mentioned that DNA was extracted from the samples collected in Stns. OT4, ON1, ON5, and ON7. However, it is unclear that how the data of relative abundances of diazotroph species in different cruises was generated. Did they mix the DNA samples or PCR products or sequence data of different stations in the same cruise? The authors should clarify this part.

// The nifH sequences were separately obtained from each station. We have written the number of obtained sequences at each station in Table 1 in the revised manuscript. (Table 1). //

P.5 l.26-27: The bootstrap values of most important branches (dividing the clusters of nifH) in the phylogenetic tree were lower than 50

// The bootstrap values did not divide the groups as you suggested. In a phylogenetic tree of nifH, a cluster including known sequences is named specific name even though the bootstrap value was lower than 50 (Zehr et al., 2003a). The obtained sequences were assigned to bacterial groups based on known sequences in a cluster within the phylogenetic tree (Zehr et al., 2003a). (L158-160) //

P.9 l.6-22, fig.6 : the authors should describe the diazotroph community structure in different stations of the same cruise separately (if they would like to sequence more clones), rather than just presenting the total sequencing results of each cruise as one community. Inconsistent nutrient concentration and nitrogen fixation rate were detected in different stations during the same cruise (fig. S2), therefore, the diazotroph community in these stations may also be different.

// We agree. We have deleted Figure 6 in the previous manuscript. //

B. Nitrogen fixation rate measurement: P.6 l.7-8: Recently study reported contamination of commercial stock 15-N2 gas with 15N-labeled ammonia and nitrate, which could affect the results of nitrogen fixation rate measurement significantly (Dabundo, Lehmann et al. 2014). Therefore, the authors should ensure the purity of their 15-N2

C1453

gas.

// We recognize this important issue. In the present study, we used 15N2 gas produced by SI Science Co., Ltd. Our recent study demonstrated that the 15N2 gas of SI Science did not show significant contamination of nitrate, nitrite, and ammonium at nanomolar level (Shiozaki et al., submitted to PLoS one). This could be due to different production method of 15N2 gas from it indicated by Dabundo et al. (2014). The 15N2 gas of SI Science is produced by oxidation of 15N-labeled ammonium sulfate with potassium hypobromite, and to avoid generation of ammonia and NOx gas, they added surplus potassium hypobromite (Shiozaki et al., submitted to PLoS one). We have added these statements in L104-108. //

C. Nitrogen Fixation and environmental data P.2 l15-16: Previous study showed that ammonia is stronger inhibitor than nitrate (Ito and Watanabe 1983), and the inhibitory effect of nitrate to different diazotrophs is still not clear (Cejudo and Paneque 1986). However, the authors were just caring about nitrate in this paper. It seems that the data of ammonia was also included in the supplementary figures. Why did not the authors make use of the ammonia data?

// In the revised manuscript, we have added ammonium data. There was no noticeable seasonal difference in surface ammonium concentration such as those seen in nitrate and phosphate (Fig. 4b). Furthermore, ammonium concentration was not negatively correlated with nitrogen fixation in this study ($p > 0.05$) (Table 2), suggesting that ammonium did not influence nitrogen fixation. We have stated this in L214-218, 252-253. //

P.9 l1-2: As exceptions were found in subsurface layer of OT4, statistical analysis (e.g. principal component analysis) is needed to find out the important and significant environmental variables. As mentioned before, I suggest the author to include ammonia as one of the environmental variables. Besides the concentrations of DIN, they can use N:P ratio as a better indicator of nitrogen limitation.

// In the revised manuscript, we examined relationship between nitrogen fixation rates and the related environmental variables (temperature, nitrate, ammonium, phosphate, N/P ratio) (Table 2). Nitrogen fixation was positively correlated with temperature and was negatively correlated with nitrate and phosphate concentration ($p < 0.01$) (Table 2). Meanwhile, nitrogen fixation was not significantly related with ammonium concentration and with N/P ratio ($p > 0.05$) (L245, 251-255). //

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