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Interactive
Comment

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.

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Currently, dinitrogen (N2) fixation in temperate coastal regions is considered inconsequential due to the surplus of dissolved inorganic N and the scarceness of known oceanic diazotrophs. However, recent data suggest that N2 fixation can occasionally contribute with significant amounts of reactive N to these systems with unknown implications for the marine N budget. In this paper, Shiozaki et al. present N2 fixation rates of up to 13.6 nmol N L-1 d-1 in coastal regions in the northwestern North Pacific. They also report N2 fixation at depths in the presence of significant amounts of dissolved inorganic N. Through analyses of nitrogenase reductase gene sequences they suggest

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that unicellular cyanobacteria group A (UCYN-A) diazotrophs may be responsible for the observed N2 fixation.

General comments: Overall this paper presents valuable data on coastal N2 fixation rates and adds to the growing body of data suggesting that the role of N2 fixation in temperate coastal environments need to be re-evaluated. However, the accompanying nitrogenase reductase gene composition analyses are difficult to evaluate as it is unclear from where the samples are taken. Only 26 – 38 sequences were analyzed per cruise; each cruise representing multiple sampling stations. How many sequences were obtained per station is unknown, but it must be very few since the approximately 30 sequences per cruise represent multiple sampling stations. Hence, it is impossible to talk about "diazotrophic diversity". Also the obtained sequences are divided into phylogenetic subgroups without reporting sequence similarities. Furthermore, contamination of PCR reagents by DNA containing nitrogenase reductase genes is a well-known phenomenon. The authors state that "no DNA was detected from negative controls", but they do not say how they performed these tests. Cloning negative controls that supposedly did not contain amplicons have yielded clones in the past. Were the negative controls here checked by mere concentration measurements or gel electrophoresis? Or were no clones obtained when cloning with the negative control?

In M&M the authors are stating that ammonium concentrations were determined, but in the results these data are omitted. Nitrate assimilation acquires the mobilization of eight electrons and some bacteria lack the ability to utilize nitrate. Hence, ammonium may be a better predictor of N2 fixation than nitrate. Furthermore, it has been shown for some coastal diazotrophic communities that N2 fixation is negatively correlated with ammonium concentrations and not nitrate. Since the authors have determined ammonium concentrations, I will strongly suggest that they include these data. Also, I advise the authors to present the data in the same order in which they present the materials and methods to facilitate comprehension.

// Samples for the clone library analysis were taken from surface water at Stns. OT4,

ON1, ON5, and ON7 during the KT-12-20_Aug and KT-12-27_Oct cruises, at Stns OT4, ON1, and ON5 during the KT-13-2_Jan and KS-14-2_Mar cruises, at Stns OT4, ON1, ON5, and ON8 during the KK-13-1_Jun cruise, and at Stns OT4, ON5, ON7 during the KK-13-6_Sep cruise (L139-144). The number of recovered sequences ranged from 1 to 15. We have added number of recovered sequences at each station in every cruise in Table 1. Since our obtained clones were included the known nifH cluster (Zehr et al., 2003a), they were labeled (L158-160). As you mentioned, we could not discuss diazotroph diversity based on the number of sequences, and thus, we have deleted the word "diversity" from the revised manuscript. Sterile distilled water was used as the negative control. After the PCR analysis, we confirmed there was no band in agarose gel of electrophoresis from the negative control. Therefore, we considered that there was no contamination in PCR reagents (L147-149). Nevertheless, the sequences with similarity (>97%) at the amino acid level to the contaminants were recovered from samples obtained during the KK-13-6_Sep and KS-14-2_Mar cruises (10 clones in total), and we do not include these sequences in our data analysis (L266-270). Furthermore, we have added the data of ammonium concentration in the revised manuscript (Fig. 4b and S2). Ammonium concentration was not significantly correlated with nitrogen fixation ($p > 0.05$) (Table 2). We have presented the data in the same order in which we present the materials and methods in the revised manuscript. //

Specific comments: P.2, l.4: Avoid using "diversity" here as you only have approximately 30 sequences per cruise. You can talk about composition at best with these numbers.

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

P.2, l. 14-15: Here, the authors suggest that Cluster III diazotrpohs rarely have been reported to be abundant in surface waters. This is not really true. In coastal regions, cluster III sequences are often recovered. See for instance the following papers: Short et al. 2004, Appl Environ Microbiol 70, Moisander et al. 2008, ISME J 2, Farnelid et al. 2009, Environ Microbiol Rep 1, Farnelid et al. 2011 PLOS ONE 6, Mulholland et

al. 2012 Limnol Oceanogr 57, Farnelid et al. 2013 ISME J 7, Bentzon-Tilia et al. 2015 ISME J 9

// We have changed the related sentences in the abstract as follows. “In contrast, when nitrogen fixation was undetectable (winter to spring), many sequences affiliated with Cluster III diazotrophs (putative anaerobic bacteria) were recovered.”(L23-24) //

P. 4, l. 1-2: The paper does not examine seasonal diazotrophy in the temperate ocean as such, but it does examine diazotrophy in a temperate coastal region during different seasons as Mulholland et al. 2012 Limnol Oceanogr 57, but in the northwestern north Pacific.

// We have changed the sentence as follows. “the present study is the first to examine diazotrophy during all seasons in the temperate ocean.” (L66-67) //

P. 4 l. 6-13: the cruise names are confusing to me, and I have to revisit this section of the paper every time a cruise is mentioned to see at what season the cruise corresponds to. I recommend renaming the cruises to include the month in which they were conducted. Possibly just by a subsequent letter (June = J).

// In the revised manuscript, we have changed the cruise ID as follows

KT-12-20: KT-12-20_Aug KT-12-27: KT-12-27_Oct KT-13-2: KT-13-2_Jan KK-13-1: KK-13-1_Jun KK-13-6: KK-13-6_Sep KS-14-2: KS-14-2_Mar //

P. 4, l. 19-20: In the results you have nitrate data for several depths. Here you write that you took samples for nutrient analyses at 7 – 15 m depth at stations outside the bay and at 1 – 13 m inside the bay. Please clarify.

// We wrote incorrectly this sentence. We have revised it as follows. “At offshore stations, samples for nutrient analysis were collected from 7–15 different depths in the upper 200 m, while at shallower (<200 m) bay stations, samples were collected from 4–9 different depths in the entire water column” (L86-89) //

P. 4, l. 19-20: I don't understand sentence. Please revise.

// We have revised it as follows. "except at Stn. OT1 where only surface water samples were collected"(L88-89) //

P. 4, l. 21-24: Here you state that samples for DNA analyses and incubation experiments were taken at all stations in the surface and at two stations in deeper waters. Later on you write that nifH composition is only analyzed in DNA from four samples per cruise. Please revise to avoid confusion.

// As I mentioned in the previous manuscript, samples for DNA analyses were collected from waters in which incubation experiments were performed. Meanwhile, the clone library analysis was only applied to the samples collected from surface water at Stns. OT4, ON1, ON5, and ON7 during the KT-12-20_Aug and KT-12-27_Oct cruises, at Stns OT4, ON1, and ON5 during the KT-13-2_Jan and KS-14-2_Mar cruises, at Stns OT4, ON1, ON5, and ON8 during the KK-13-1_Jun cruise, and at Stns OT4, ON5, ON7 during the KK-13-6_Sep cruise (Table 1). In the revised manuscript, we have newly performed a qPCR analysis using all the DNA samples. We have added this information in L139-144, 163-179. //

P. 5, l. 2: Here the authors mention that they determine ammonium concentrations, but it is omitted in the rest of the paper, unfortunately. I would strongly suggest adding these data considering their implications for N2 fixation.

// We have added the result of ammonium concentration in the revised manuscript (Fig. 4b and S2). Ammonium concentration was not negatively correlated with nitrogen fixation ($p>0.05$) (Table 2), suggesting that it did not influence nitrogen fixation in this study. //

P. 5, l. 19: How did the authors determine that the nested PCR did not produce amplicons? Did they clone the negative control? Did they compare sequences from their dataset to those of known contaminants?

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// Sterile distilled water was used as the negative control. After the PCR analysis, we confirmed there was no band in agarose gel of electrophoresis from the negative control. In the revised manuscript, these results are now clearly described (L147-149). We compared our sequence results with those of known contaminants (Zehr et al. 2003b). The sequences with similarity (>97%) at the amino acid level to the contaminants were recovered from samples obtained during the KK-13-6_Sep and KS-14-2_Mar cruises. Since we do not include these sequences in our data analysis, those do not influence our results. (L266-270) //

P. 5, l. 20: I suggest mentioning here that you have 197 sequences.

// We have added this number of sequences as suggested (L150-151). //

P. 6, l. 3-10: Was the incubations done in replicates? Was the T0's done in replicates?

Was the incubations done in replicates.

// Yes. Triplicate bottles were used for mannitol enrichment experiment and for the experiments to compare the bubble and the dissolution methods. For other experiments, duplicate bottles were used. This information is now clearly described in the text (L102-103, 128-129, 133). //

Was the T0's done in replicates?

// No it wasn't. This is now stated in the text (L103) //

P. 6, l. 20: How long did you store these bags? Tedlar bags are not completely impermeable to gas and 15N2 will equilibrate with the atmosphere over time.

// These bags were stored no more than 1 h after the preparation. We consider that the exchange of the N2 gas was minimal during this period. In the revised manuscript, we now clearly state this. (L126-127) //

P. 7, l. 24 – p. 8, l. 2: Consider moving this part to the Discussion.

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// We have moved this part to the Discussion (L376-381). //

P. 8, l. 12-21: Here the authors present N2 fixation rates in vertical profiles. I suggest adding the actual rates and referring to figure 3.

// According to the suggestion, actual rates are now reported (L239-240) and the Fig. 4 (Fig.3 in the previous manuscript) is referred (L232). //

P. 9, l. 6: How many sequences were obtained from each cruise and each sample?

// The number of recovered sequences ranged from 1 to 15. We have added the number of sequences at each station in every cruise in Table 1. //

P. 9, l. 5-22: How did you assign sequences to groups? At what AA sequence similarity level?

// Our obtained clones were included the known nifH cluster (Zehr et al., 2003a), and hence they were labeled in the present study. (L158-160) //

P. 10, l. 12: How did they compare to rates from the NE Atlantic coastal waters (e.g. Rees et al., 2009 Aquat Microb Ecol 374, Bentzon-Tilia et al., 2015 ISME J 9)

// We have now compared our data with those reported by Rees et al. and Bentzon-Tilia et al. in the revised manuscript (L337-340). "Higher nitrogen fixation rates have been determined in other temperate oceans, including the western English Channel (18.9±0.01 and 20.0 nmol N l-1 d-1; Rees et al., 2009) and the Baltic Sea estuaries (47–83 nmol N l-1 d-1; Bentzon-Tilia et al., 2015)." //

P. 11, l. 1-4: Iron concentrations were not determined in this study, hence it could limit N2 fixation. What concentrations of iron are usual in this particular environment?

// Average dissolved iron concentration in the surface mixed layer in this region in spring was reported to be 0.79–8.46 nM (Nishioka et al., 2007) which is higher than in areas where nitrogen fixation is limited by iron (<0.4 nM) (Sohm et al., 2011 and reference therein). Hence, iron would not limit nitrogen fixation in this region. We have added the

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iron concentration in L356-359. //

P. 12, l. 6: Delete oligotrophic.

// Deleted as suggested. //

P. 12, l. 12-19: The original -24774A11 sequence from the South China Sea is closely related (95% AA sequence similarity) to many Pseudomonas stutzeri-like sequences, which are continuously reported from most waters including temperate coastal regions. At what level do the sequences obtained in this study resemble the original -24774A11 sequence relative to known temperate Pseudomonas stutzeri-like nifH sequences?

// The nifH sequences that we obtained (KK-13-6_ON7-4 and KT-12-27_ON5-13) resembled the γ-24774A11 sequence at a >97% similarity level, whereas the corresponding similarity level for P. stutzeri was 94%, except that a sequence obtained at Stn. OT4 during the KT-13-2_Jan cruise (KT-13-2_OT4-14) displayed >97% similarity to P. stutzeri. (L431-433, 436-438) //

P. 12, l. 20-28: It is not rare for Cluster III sequences to make up a substantial part of the surface community. See references listed above.

// We have deleted this sentence. //

P. 13, l. 21-24: UCYN-A and the gamma-Proteobacterium -24774A11 are speculated to be responsible for N2 fixation. Consider -24774A11 being a Pseudomonas stutzeri–like sequence: How does the conclusion presented here relate to previous findings from temperate coastal regions (e.g. Bentzon-Tilia et al., 2015 ISME J 9)?

// The qPCR results, which we have newly added in the revised manuscript, demonstrated that γ-24774A11 was detected from all the cruises except the KS-14-2_Mar (Fig. 7, 8 and S5). Although the P. stutzeri-like nifH sequence (>97% similarity at the AA level) was recovered from Stn. OT4 during the KT-13-2_Jan cruise, γ-24774A11 was not detected there by the qPCR analysis (Fig. S5). Therefore, P. stutzeri-like sequence was not detected as the γ-24774A11 by the qPCR analysis. Bentzon-Tilia et al

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(2015) found that UCYN-A was one of the major diazotroph in temperate estuaries by their one year observation, which was consistent with our study. Although they recovered many sequences of γ-proteobacteria throughout a year, sequence of γ-24774A11 was not major in the group. They reported that P. stutzeri-like sequence (99% similarity at nucleotide level) was the most abundant in their sample, which was not consistent with our results. We have added these statements in L408-410, 429-440. //

P. 13, l.2: Here you talk about ammonium. Include these data in Results, please.

// We have added the data of ammonium in the revised manuscript (in detail, see above). //

Figure 2: How many replicates? $P<0.05$, n=?

// We conducted this comparative experiment in triplicate. We have added the sample number in the text (L102-103, 128-129, 133). //

Figure 4: Add ammonium to this figure Make symbols identical for each parameter/nutrient you present instead of having circles, triangles, stars, diamonds etc. representing the same things in the different sub-panels. Why are there just straight lines in the last panel? Is this the station where the CTD was not cast? If so provide this info in figure legend.

// We have added ammonium in Figure 5 (Fig. 4 in the previous manucript), and changed the color to distinguish from the other parameters. The straight lines in the last panel were temperature and nitrate obtained by a CTD cast, which were ascribable to strong mixing. We have stated this in the figure legend. //

Figure 5: In this figure as anywhere else in the paper it would facilitate comprehension a lot if the cruise names were given names corresponding to sampling time/season/month

// We have added information of sampling month and season in all revised figures. //

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Figure 6: Consider indicating at what transects you saw high N2 fixation rates, or consider adding the mean rate in relation to the bar so you can couple community composition and the N2 fixation rate. Clarify that the number of sequences is total for that cruise in the legend.

// We have deleted this figure in the revised manuscript as suggested by reviewer #2. //

Technical comments: P. 2, l. 6: Add a space between "nmol" and "N" here and throughout the paper including figures.

// We have added a space as suggested. //

P. 5, l. 10: Correct the degree-sign in "-80 C" to "-80 C" here and throughout the paper.

// We have corrected as suggested. //

P. 10, l. 14: Correct to "through".

// We have corrected the word. //

P. 14, l. 2-3: Correct to "Bentzon" and 2014b to "2015"

// We have corrected the reference. //

P. 15, l. 7: Correct reference to "ISME J 9, 273-285"

// We have corrected the reference. //

Interactive comment on Biogeosciences Discuss., 12, 865, 2015.