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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.

Anonymous Referee #1

Received and published: 10 February 2015

Referee’s comments to the paper:

”Seasonal dynamics of nitrogen fixation and the diazotroph community in the temperate coastal region of the northwestern North Pacific”

by Shiozaki et al.

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

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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 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

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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.

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.

P.2, l. 14-15: Here, the authors suggest that Cluster III diazotrophs 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, Moisaner 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

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.

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).

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

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bay and at 1 – 13 m inside the bay. Please clarify.

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

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.

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.

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?

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

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

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.

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

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.

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

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

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)

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Interactive Discussion

Discussion Paper



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?

P. 12, l. 6: Delete oligotrophic.

P. 12, l. 12-19: The original γ -24774A11 sequence from the South China Sea is closely related ($\geq 95\%$ 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 γ -24774A11 sequence relative to known temperate *Pseudomonas stutzeri*-like nifH sequences?

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.

P. 13, l. 21-24: UCYN-A and the gamma-Proteobacterium γ -24774A11 are speculated to be responsible for N₂ fixation. Consider γ -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)?

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

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

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.

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

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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.

Technical comments:

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

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

P. 10, l. 14: Correct to “through”.

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

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

End of review

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

BGD

12, C131–C136, 2015

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