We have carefully studied the various points raised by the three reviewers and revised the manuscript accordingly. For the revised manuscript, we have newly performed a qPCR experiment as reviewer #3 suggested. Combining with the results of the qPCR analysis, we found that the clone library analysis could capture a major group in diazotroph community in each cruise. We also added the results of ammonium concentration in the revised manuscript. Ammonium concentration was not significantly correlated with nitrogen fixation rate ($p < 0.05$). Since phosphate concentration during the KS-14-2_Mar cruise was miscalculated in the previous manuscript, we have revised the data. This correction did not affect our major conclusions. Our responses to the reviewer's comments are detailed below.

Referee #1

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 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?

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 N_2 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.

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 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 (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.

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.

Referee #2

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)

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 gas.

We recognize this important issue. In the present study, we used $^{15}N_2$ gas produced by SI Science Co., Ltd. Our recent study demonstrated that the $^{15}N_2$ 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 $^{15}N_2$ gas from it indicated by Dabundo et al. (2014). The $^{15}N_2$ gas of SI Science is produced by oxidation of ^{15}N-labeled ammonium sulfate with potassium hypobromite, and to avoid generation of ammonia and NO_x 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).

Cejudo, F. J. and A. Paneque (1986). "Short-term nitrate (nitrite) inhibition of nitrogen fixation in Azotobacter chroococcum." Journal of bacteriology 165(1): 240-243.

Dabundo, R., M. F. Lehmann, et al. (2014). "The Contamination of Commercial 15N2 Gas

Stocks with 15N–Labeled Nitrate and Ammonium and Consequences for Nitrogen Fixation Measurements." PLoS One 9(10): e110335.

Ito, O. and I. Watanabe (1983). "THE RELATIONSHIP BETWEEN COMBINED NITROGEN UPTAKES AND NITROGEN FIXATION IN AZOLLAâˇARˇ ANABAENA SYMBIOSIS." New phytologist 95(4): 647-654.

Langlois, R. J., D. Hümmer, et al. (2008). "Abundances and distributions of the dominant nifH phylotypes in the Northern Atlantic Ocean." Applied and environmental microbiology 74(6): 1922-1931.

Referee #3

General comments: This manuscript provides the seasonal variability of nitrogen fixation and diazotrophs in the coastal region of the western North Pacific Ocean where was seriously affected by the 2011 Tohoku earthquake-induced tsunami. The authors showed that the high nitrogen fixation rate was observed in summer and fall, and the nifH sequence of UCYN-A and -proteobacteria was detected at the same time. They concluded that the origin of these diazotroph is Tsugaru warm current and the findings will be re-evaluate the nitrogen fixation in temperate ocean. They also recovered nifH sequences assigned with benthic strains or terrestrial strains in this region and discussed the influence of the 2011 Tohoku earthquake-induced tsunami. The observation field is relatively interesting. The authors have made a good attempt at the understanding of nitrogen fixation in the temperate region. However, I feel that this study is not adequately describe.

Specific comments:

1. The meaning of the transect sampling is not clear. There are some hydrographic difference between coastal and offshore stations, due to Tsugaru water current, Kuroshio and Oyashio. The authors sometimes mentioned terrestrial effects. However authors showed the average of each line. I suggest the analysis region should be divide with each stations or coastal/offshore stations. It may be better if the authors would like to discuss about the influence of the 2011 Tohoku earthquake induced tsunami. The influence of the damage of benthic environment may be clear in coastal stations than those of offshore stations. 2. To discuss the seasonal variation of diazotrophic community, the number of recovered nifH sequences is not enough. Much more sequences should be analyzed. In addition, I wonder why the Richelia, UCYN-B and UCYN-C were not recovered. Were the sequence number and the volume of sampling water appropriate? Furthermore, if the authors describe the benthic strains or terrestrial strains, the data also should be shown as each stations. I think that the quantitative PCR also useful method for this study, such as Shiozaki et al (2014).

I encourage the authors to reanalyzing the nitrogen fixation rate, environmental factors and nifH sequences at each station.

Although we set the transect from inside bay to open ocean to examine the gradient of environmental variables and diazotrophy, there were no significant difference between inside and outside the bays (Fig. S2 and S5) except at the OT line during the KK-13-6_Sep when nitrate, ammonium, and phosphate concentration increased inside the bay. This similarity could be because intense exchange of surface waters often occurred between

inside and outside the bays (Furuya et al., 1993). According to the TS diagram (Fig. 3), surface waters collected during the same cruise in a particular season generally belonged to the same water system that was prevalent in the investigated region at the time of our sampling. Therefore, for simplicity, the average value of each variable was shown in each cruise. We have added these statements in L191-201.

The clone library analysis showed that UCYN-A, *Trichodesmium*, and γ-24774A11 were likely important diazotrophs from early summer to fall when nitrogen fixation occurred. Therefore, the present study quantified these *nifH* phylotypes by a qPCR analysis to examine their relative importance in these seasons. 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. We are now clearly stated in L390-402. Abundance of *Richelia* is generally lower than that of *Trichodesmium* and it inhabits tropical and subtropical regions as with *Trichodesmium* (Shiozaki et al., 2010). The UCYN-B and UCYN-C are also observed in tropical and subtropical regions (Moisander et al., 2010; Taniuchi et al., 2012), and not in the temperate regions (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012). Our results thus were consistent with the previous ones. We have added number of recovered sequences at each station in every cruise in Table 1.

P. 2, l. 11 The nifH sequences were only recovered, not quantified. It is hard to say as 'played key role' in this study.

In the revised manuscript, we have quantified the four diazotroph group as mentioned above. According to the results of the qPCR analysis. We have revised the description as

follows.

"Quantitative PCR analysis revealed that UCYN-A was relatively abundant from early to late summer compared with *Trichodesmium* and γ-24774A11, whereas *Trichodesmium* abundance was the highest among the three groups during fall." (L25-27).

P. 7, l. 25 This sentence belongs to the 'discussion'.

We have moved the related sentences to the Discussion (L376-381).

P. 8, l. 10-11 The horizontal distributions of nitrogen fixation and nutrients should be shown.

We have shown these horizontal distributions in Figure S2.

P. 9, l. 5-22 It would be shown the stations where the nifH sequences were recovered.

The obtained clone number has been shown at each station in every cruise in Table 1.

P. 10, l. 4-24 It would be added the discussion about the influence of eddy which seems bring the water mass from Kuroshio. The sea surface temperature showed some currents from south to north around offshore stations in KT-12-27 and KK-13-6 (Fig. S1). And the nitrogen fixation rate of offshore station OT7 was higher than the other stations (Fig.2).

Although the geostrophic current fields showed that Stn. ON7 during the KT-12-27_Oct cruise was located in anticyclonic eddy, the nitrogen fixation rate was similar with that at the other stations. Furthermore, according the TS diagram, water belonged to the Kuroshio water system did not exist (Fig. 3).

P. 10, l. 25-P. 11 l. 4 The approach is interesting. But the authors should compere the community structure to Blais et al (2012) and discuss the different usage of organic materials of bacteria.

Blais et al (2012) suggested this hypothesis on the basis of their observation that gradient of nitrogen fixation rate occurred from the river mouth to the open ocean, and did not mention the specific diazotroph groups.

P. 11, l. 5-23 This sense should be improved to clarify the why the low nitrogen fixation rates

were observed.

High DIN concentration are generally regarded to inhibit nitrogen fixation (Falkowski, 1983). We wrote this in L53-54.

P. 11, l. 20 Add the nitrogen fixation rate along the OT transect line to the Fig. S3. It would be clear the influence of Typhoon Man-yi.

Nitrogen fixation rate in the OT line has been shown in Fig. S2.

P. 12, l. 7-11 This part is not convincing. There is no evidence that the cluster III was not exist in this region before the tsunami. It is not clear that the cluster III recovered in this study is the same as those living in sediment or not.

We agree. Since we do not have data before the tsunami, we have deleted these sentences.

P. 12, l. 11-15 It would be better to show the horizontal distribution of diazotroph.

We have added this information in Table 1 and Fig. S5.

P. 12, l. 24 The experiments were conducted to surface layer, and did not consider any bottom structure or re-suspending. If the authors would like to evaluate the damage of tsunami, the study should be include the bottom layer and sediments.

We have deleted the sentences related with the damage of tsunami from the Discussion.

Fig. 4 (b) The line of N2 fixation is hard to see in KK13-6 and KS14-2. To help the reader, seasons should be added with the cruise name, such as Fig.3.

In the revised figure, we have used color to distinguish each parameter. Further, we have added information of season in each cruise.

Fig. 6 The data should not be summarized with different stations.

We have deleted this figure from the revised manuscript.

614

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~~Seasonal dynamics of n~~Nitrogen fixation and the diazotroph community in the temperate coastal region of the northwestern North Pacific

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Abstract

Nitrogen fixation in temperate oceans is a potentially important, but poorly understood process that may influence the marine nitrogen budget. This study determined seasonal variations in nitrogen fixation and the ~~*nifH* gene diversity~~diazotroph community within the euphotic zone in the temperate coastal region of the northwestern North Pacific. Nitrogen fixation as high as 13.6 nmol N L^{-1} d^{-1} was measured from early summer to fall when the surface temperature exceeded 14.2º C (but was lower than 24.3º C) and the surface nitrate

concentration was low (≤0.30 µM), although we also detected nitrogen fixation in subsurface layers (42–62 m) where nitrate concentrations were high (>1 µM). Clone library analysis results indicated that *nifH* gene sequences were omnipresent throughout the investigation period. During the period when nitrogen fixation was detected (early summer to fall), the genes affiliated with UCYN-A, *Trichodesmium*, and γ-proteobacterial phylotype γ-24774A11 were frequently recovered. In contrast, when nitrogen fixation was undetectable (winter to spring), many sequences affiliated with Cluster III diazotrophs (putative anaerobic bacteria) were recovered. Quantitative PCR analysis revealed that UCYN-A was relatively abundant from early to late summer compared with *Trichodesmium* and γ-24774A11, whereas *Trichodesmium* abundance was the highest among the three groups during fall.

1. Introduction

The amount of bioavailable nitrogen introduced into the global ocean via nitrogen fixation is considered to be roughly balanced~~,~~ at the large spatiotemporal scale~~,~~ by nitrogen loss through denitrification, as indicated by the sedimentary nitrogen isotope record during the Holocene epoch (Brandes and Devol, 2002; Deutsch et al., 2004). However, ~~the data of~~ rate measurement data~~s~~ have revealed that denitrification far exceeds nitrogen fixation (Codispoti, 2007). This discrepancy in the nitrogen balance has raised the possibility that the current

estimate of marine nitrogen fixation, which is primarily based on data collected in tropical and subtropical oceans where large cyanobacterial diazotrophs (e.g., *Trichodesmium* spp. and *Richelia intracellularis*) are considered to be mainly responsible for nitrogen fixation (e.g., Capone et al., 1997), might be too low (Codispoti, 2007). This is supported by the results of recent studies using molecular approaches that have increasingly revealed that marine diazotrophs are more diverse and widespread than previously thought (Riemann et al., 2010; Zehr, 2011). Recently discovered marine diazotrophic taxa, including those belonging to unicellular cyanobacteria and heterotrophic bacteria, are abundant in oceanic regions where large cyanobacterial diazotrophs are scarce (Needoba et al., 2007; Moisander et al., 2010; Halm et al., 2012; Bonnet et al., 2013; Rahav et al., 2013; Shiozaki et al., 2014a), suggesting that a failure to account for nitrogen fixation mediated by these diazotrophs might result in underestimation of marine nitrogen fixation.

The temperate coastal ocean is one of the regions where nitrogen fixation rates have been understudied and potentially underestimated. Conventionally, nitrogen fixation in temperate oceans has been assumed to be low because of the relatively low temperatures (<~20ºC), which generally inhibits the growth of large cyanobacterial diazotrophs (Breitbarth et al., 2007), and development of high dissolved inorganic nitrogen (DIN) concentrations (>1 μM). High DIN concentrations are generally regarded to inhibit nitrogen fixation (Falkowski,

1983), especially during mixing periods. However, recent studies have indicated that nitrogen fixation, presumably mediated by unicellular cyanobacteria and heterotrophic bacteria, is detectable even in the relatively cold (<10ºC) and DIN-rich waters (>1 µM) of the Atlantic coast (Mulholland et al., 2012) and the Baltic Sea estuaries (Bentzon-Tilia et al., ~~2014a~~2015). These results highlight the necessity of re-evaluating the extent, variation, and control mechanisms of nitrogen fixation in temperate oceans, with recognition of the widespread occurrence of diverse diazotrophic microbes.

This study examined the seasonal variation in nitrogen fixation in the temperate inside bays and open ocean located in the interfrontal zone of the northwestern North Pacific. In this region, physical, chemical, and biological properties vary widely between seasons (Shiozaki et al., 2014b)~~,~~ due to the confluence of three currents: the Kuroshio (warm current), the Tsugaru Warm Current, and Oyashio (cold current). Data on nitrogen fixation rates in the temperate Pacific are limited (Needoba et al., 2007), and~~,~~ to the best of our knowledge, the present study is the first to examine ~~seasonal~~ diazotrophy during all seasons in the temperate ocean. ~~The~~ This study was conducted as part of a project to monitor the dynamics of the coastal ecosystem and the recovery thereof after the 2011 Tohoku-oki tsunami, which struck the region on 11 March 2011.

2. Materials and Methods

The experiments were conducted during six cruises in the temperate coastal region of the western North Pacific. These cruises covered a full seasonal cycle, including spring (KS-14-2 Mar, 14–19 March 2014), early summer (KK-13-1 Jun, 24–29 June 2013), summer (KT-12-20 Aug, 7–12 August 2012), late summer (KK-13-6 Sep, 14–21 September 2013), fall (KT-12-27 Oct, 15–22 October 2012), and winter (KT-13-2 Jan, 19–25 January 2013). Sampling stations were located along the transect lines OT (39°20'°N, 141°56'–142°50'°E) and ON (38°25°'N, 141°29'–142°20°'E). Eight stations were located offshore (OT4–6, ON4–8,–), while two stations were deployed in ~~with additional stations being deployed in~~ the Otsuchi (OT1) and Onagawa (ON13) bays (Fig. 1). Just before the KK-13-6 Sep cruise, Typhoon Man-yi passed from southwest to northeast in the study area (Fig. S1).

Temperature, salinity, and dissolved oxygen profiles ~~to~~ of regions near the bottom floor were measured using a SBE 911-plus ~~Conductivity~~conductivity-t~~T~~emperature-p~~P~~ressure (CTD) system (Sea-bird Electronics, Bellevue, WA, USA). Water samples were collected in an acid-cleaned bucket and Niskin-X bottles. At offshore stations, s~~S~~amples for nutrient analysis~~s~~ were collected from ~~a depth of~~ 7–15 different depths ~~m~~ in the upper 200 m, while at shallower (<200 m) bay stations, samples were collected from ~~2~~4 ~~at stations outside the bays~~

~~and from a depth of 1 13~~ 9 different depths ~~m~~ in the ~~upper bottom floor at stations inside the bays~~entire water column, except at Stn. OT1 where only surface water samples were collected. Samples for DNA analysis and incubation experiments were collected from the surface at almost every station, and from depths corresponding to 10% and 1% of the surface light intensities at Stns. OT4 and ON5. Light attenuation was determined using a submersible PAR sensor.

2.1. Nutrients

Samples for nutrient analysis were stored in 10-mL polyethylene tubes and kept frozen until onshore analyses. Nitrate, nitrite, ammonium, and phosphate concentrations were determined using an AACSII auto-analyzer (Bran+Luebbe, Norderstedt, Germany). The detection limits of nitrate, nitrite, ammonium, and phosphate ranged from 0.01–0.04 µM, 0.01–0.02 µM, 0.01–0.03 µM, and 0.01–0.02 µM, respectively. The nitracline was defined as the depth where nitrate concentrations increased above 1 µM.

~~2.2. DNA analysis~~

~~Samples (0.38–1 L) for DNA analysis were filtered through 0.2-µm-pore-sized Nuclepore filters and stored in a deep freezer (-80ºC) until onshore analysis. Total DNA was extracted using a ChargeSwitch Forensic DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) with slight modification of the manufacturers protocol (Shiozaki et al., 2014a). Partial *nifH*~~

~~fragments were amplified from samples collected from surface water at Stns. OT4, ON1, ON5, and ON7 using a nested PCR strategy (Zehr and Turner, 2001). PCR chemicals were applied as described by Shiozaki et al. (2014a). The first and second PCRs were run using the same cycling conditions: 95ºC for 30 s followed by 30 cycles of 98ºC for 10s, 52ºC for 30s, and 72ºC for 30 s; followed by a final extension at 72ºC for 7 min. The PCR products were cloned and sequenced according to Shiozaki et al. (2014a). No DNA was detected from negative controls of the PCRs. The *nifH* sequences were translated into amino acid sequences and searched against the protein database of the National Center for Biotechnology Information using the BLASTp algorithm. Clones with 100% amino acid sequence similarity were defined as the same operational taxonomic units (OTU) using the CD-HIT suite (Huang et al., 2010). The amino acid sequences were aligned using multiple sequence comparisons by the log-expectation (MUSCLE) module in the MEGA5 package (Tamura et al., 2011). A phylogenetic tree was constructed using the maximum likelihood method employing the Dayhoff matrix-based mode, and 1,000 bootstrap replicates were run. The sequences from this study were deposited in the DNA Data Bank of Japan (DDBJ) with accession numbers LC013480 to LC013676.~~

~~2.3.~~2.2. Nitrogen fixation activity and mannitol enrichment experiment

Nitrogen fixation was determined by the $^{15}N_2$ gas bubble method (hereafter, the bubble

method; Montoya et al., 1996). Samples for incubation were collected in duplicate acid-cleaned ~~2~~ 2-L polycarbonate (PC) bottles. ~~ and t~~The time-zero samples (n=1) were immediately filtered onto precombusted GF/F filters. Two milliliters of $^{15}N_2$ gas [SI Science Co. Japan, for this gas, contaminations of nitrate, nitrite, and ammonium were determined to be low (< nM level), indicating that the overestimation of nitrogen fixation rates due to the uptake of ^{15}N-labeled contaminants (Dabundo et al. 2014) was minimal (Shiozaki et al., unpublished data)] were injected directly into the incubation bottles through a septum using a gastight syringe. The tracer-added samples were covered with neutral-density screens to adjust the light level and incubated for 24 h in an on-deck incubator filled with flowing surface seawater. After the incubation, the samples were filtered onto precombusted GF/F filters. The isotopic analyses were performed as described previously (Shiozaki et al., 2009). The rate of nitrogen fixation was calculated using the equations of Montoya et al. (1996).

~~Because of~~To examine the possibility of underestimation of nitrogen fixation as determined by the bubble method (Mohr et al., 2010; Großkopf et al., 2012), we ~~also determine~~compared the~~d~~ nitrogen fixation rates determined using the $^{15}N_2$ gas dissolution method (hereafter, the dissolution method; Mohr et al., 2010) with those determined using the bubble method (see above) during the KK-13-6 Sep and KS-14-2 Mar cruises. For the dissolution method,~~.~~

145 $^{15}N_2$-enriched seawater was prepared according to Mohr et al. (2010) and Großkopf et al.
146 (2012). Briefly, filtered seawater was degassed using a Sterapore membrane unit
147 (20M1500A: Mitsubishi Rayon Co., Ltd., Tokyo, Japan) at a flow rate of ~500 mL min^{-1}
148 (recirculation period, 10 min). Degassed seawater was stored in ~~1~~ 1-L Tedlar bags without
149 headspaces and 10 mL $^{15}N_2$ gas was added. After complete dissolution, the $^{15}N_2$-enriched
150 seawater was added to seawater samples contained in 2-L PC bottles, which were incubated
151 and used for isotopic analyses as described above. The $^{15}N_2$-enriched seawater was
152 prepared at each station, and was added to the incubation bottles within 1 h after ~~the~~
153 preparation. The nitrogen fixation rate was calculated according to Mohr et al. (2010).
154 For this comparison, triplicate samples were used for both the dissolution and bubble
155 methods.
156 To examine if sugar addition affected nitrogen fixation rates (Bonnet et al., 2013; Rahav et al.,
157 2013; Moisander et al., 2011), we determined nitrogen fixation rates (the $^{15}N_2$ gas bubble
158 method, see above) for surface seawater samples (stations ON4 and OT6 during the
159 KS-14-2 Mar cruise) with and without addition of mannitol (final conc. 0.8 µM) (n=3).
160 **2.3. DNA analysis**
161 2.3.1. DNA extraction, sequencing, and phylogenetic analysis
162 Samples (0.38–1 L) for DNA analysis were filtered through 0.2-µm-pore-sized Nuclepore

filters and stored in a deep freezer (-80º C) until onshore analysis. Total DNA was extracted using a ChargeSwitch Forensic DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) with slight modification of the manufacturer's protocol (Shiozaki et al., 2014a). Partial *nifH* fragments were amplified using a nested PCR strategy (Zehr and Turner, 2001) from 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). PCR reagents were applied as described by Shiozaki et al. (2014a). The first and second PCRs were run using the same cycling conditions: 95º C for 30 s followed by 30 cycles of 98º C for 10 s, 52º C for 30 s, and 72º C for 30 s; followed by a final extension at 72º C for 7 min. Sterile distilled water was used as the negative control. After PCR analysis, we confirmed there was no band in agarose gel of electrophoresis from the negative control. The PCR products were cloned and sequenced according to Shiozaki et al. (2014a). The present study obtained 197 *nifH* sequences in total. The *nifH* sequences were translated into amino acid sequences and searched against the protein database of the National Center for Biotechnology Information using the BLASTp algorithm. Clones with 100% amino acid sequence similarity were defined as the same operational taxonomic unit (OTU) using the CD-HIT suite (Huang et al.,

2010). The amino acid sequences were aligned using multiple sequence comparisons by the log-expectation (MUSCLE) module in the MEGA5 package (Tamura et al., 2011). A phylogenetic tree was constructed using the maximum likelihood method employing the Dayhoff matrix-based mode, and 1,000 bootstrap replicates were run. The obtained sequences were assigned to bacterial groups based on known sequences included in a cluster within the phylogenetic tree (Zehr et al., 2003a). The sequences from this study were deposited in the DNA Data Bank of Japan (DDBJ) as accession numbers LC013480 to LC013676.

2.3.2. Quantitative PCR (qPCR) analysis

The clone library analysis showed that UCYN-A, *Trichodesmium*, and γ-proteobacterial phylotype γ-24774A11 (hereafter γ-24774A11) were likely important diazotrophs from early summer to fall when nitrogen fixation occurred (see below). Therefore, the present study quantified these *nifH* phylotypes by qPCR analysis to examine their relative importance duing these seasons. In addition, UCYN-B which is considered to be a major diazotroph in the tropical and subtropical oligotrophic ocean (Moisander et al., 2010), was quantified. TaqMan primer and probe sets previously designed for these four *nifH* phylotypes were used for quantification (Shiozaki et al., 2014a,c). The 20 µL qPCR reactions contained 10 µL 2 × Premix Ex Taq (Probe qPCR; Takara), 5.6 µL of nuclease-free water, 1 µL each of the

forward and reverse primers, 0.4 µL of TaqMan probe, and 2 µL of template DNA. The qPCR assays were performed using LightCycler 480 System (Roche Applied Science, Germany). The qPCR assays were run in triplicate reactions. Linear regression r^2 values for the standard curves were >0.99 for all reactions. The efficiency of the qPCR assays ranged from 90.9 to 98.4%, with an average of 95.1%. As the negative control, sterile distilled water was used, from which no amplification signals were detected. The detection limit was 75 copies L^{-1}.

3. RESULTS

3.1. Comparison of the bubble method and the dissolution method

Nitrogen fixation rates determined by the bubble and dissolution methods were compared during the KK-13-6 Sep and KS-14-2 Mar cruises (Fig. 2). Both methods failed to detect nitrogen fixation in samples collected during the KS-14-2 cruise. During the KK-13-6 Sep cruise, the nitrogen fixation rates determined by the dissolution method were significantly ~~(p <0.05)~~ higher (1.5–2.2 fold) than those determined by the bubble method at Stns. OT6 and ON5~~two of four stations~~ (p <0.05). At ~~other stations~~Stns. OT4 and ON7, the nitrogen fixation rates determined by the two methods did not differ significantly. ~~The following nitrogen fixation results were obtained by the bubble method; we sought to standardize~~

~~values among all cruises and to compare them with previous results. Hence, the levels are underestimates.~~

3.2. Seasonal variations in nitrogen fixation rates

According to the temperature-salinity (TS) diagram proposed by Hanawa and Mitsudera (1987), ~~–~~both the offshore and bay waters collected during this investigation primarily belonged to either the surface layer water system (SW) or ~~to~~ the Tsugaru Warm Current water system (TW) (Fig. 3), with the exception of waters collected from the 1% light depth (119 m) at Stn. ON5 during the KT-13-2_Jan cruise and those collected at the surface of OT5 during the KS-14-2_Mar cruise, which were classified as belonging to the Oyashio water system (OW) and the Coastal Oyashio water system (CO), respectively. These water classifications based on the TS diagram were generally consistent with the geostrophic current field of the investigated region (Fig.S1). Based on these results, it was considered that surface waters collected during the same cruise in a particular season generally belonged to the same water system that was prevalent in the investigated region at the time of our sampling.

Sea surface temperatures (SST) (range, 1.5 to 24.3º C) (Figs. ~~3a~~ 4a and S1) and surface ~~nutrient~~ nitrate and phosphate concentrations ~~(Figs. 3b and S2)~~ determined during each cruise were averaged to emphasize the seasonal variability of these parameters (Fig. 4b).~~.~~ In general, surface nitrate and phosphate concentrations were low (≤0.07 µM and ≤0.20 µM,

respectively) in the warmer seawaters (14.2–24.3º C) sampled in early summer (KK-13-1 Jun), summer (KT-12-20 Aug), and fall (KT-12-27 Oct), whereas they were relatively high (≥0.75 µM and ≥0.28 µM, respectively) in the colder seawaters (1.5–9.8º C) sampled ~~in~~ during winter (KT-13-2 Jan), and spring (KS-14-2 Mar). During the KK-13-6 Sep cruise (late summer), ~~, although~~ the nitrate concentrations were relatively high and variable (mean ± SD; 2.92 ± 7.90 µM) ~~in samples collected in summer during the KK-13-6 cruise~~. This was because ~~During the KK-13-6 cruise, low-salinity surface waters spread offshore along the OT transect line (Fig. S3). Concomitantly,~~ the highest nitrate concentration (22.6 µM) was determined at the near-shore Stn. OT1 (Fig. S2). Similar to nitrate, surface phosphate concentrations tended to be high during winter (KT-13-2 Jan) and spring (KS-14-2 Mar), while they were low during the warmer seasons. The seasonal variation pattern of the average ammonium concentration at the surface differed from those of nitrate and phosphate concentrations (Fig. 4b), characterized by –low concentrations (≤ ~1 µM) throughout the year. The high variation in surface ammonium concentration during the KK-13-6 Sep cruises were due to relatively high ammonium concentrations at Stn. OT1 (1.41 µM) (Fig. S2). ~~These results suggest that anomalously high nitrate concentrations were likely attributable to terrestrial surface discharge enhanced by Typhoon Man-yi that passed over the region immediately before the cruise.~~

Nitrogen fixation was detected in the surface waters of most samples collected during the four cruises conducted in early summer (KK-13-1 Jun), summer (KT-12-20 Aug ~~and KK-13-6~~), late summer (KK-13-6 Sep), and fall (KT-12-27 Oct), and varied in the range of 0.33–13.6 nmol N L^{-1} d^{-1} (Figs. ~~3c~~ 4c and S2). Relatively high nitrogen fixation rates were determined for samples collected during the KT-12-20 Aug cruise, although the highest value was obtained at ~~Sta~~Stn. ON7 during the KK-13-6 Sep cruise. Nitrogen fixation was not detected in seawater samples collected during the winter and spring cruises, even after addition of mannitol (KS-14-2 Mar). Furthermore, nitrogen fixation was not detected in DIN-replete water at Stn. OT1 in late summer (KK-13-6 Sep).

The rates of nitrogen fixation in samples collected at different depths (0–119 m) were examined at Stns. OT4 and ON5 (Fig. ~~4~~5). Nitrogen fixation was detectable only during the four cruises conducted in early summer (KK-13-1 Jun), summer (KT-12-20 Aug), late summer ~~and~~ (KK-13-6 Sep), and fall (KT-12-27 Oct), the same seasons during which surface nitrogen fixation was observed (Fig. 4). Nitrogen fixation rates tended to be higher at the surface than in the deeper layers ~~in~~ during summer (KT-12-20 Aug) and late summer ~~and(~~ KK-13-6 Sep (at Stn. OT4)), whereas this vertical trend was less evident ~~in~~ during fall (KT-12-27 Oct) and early summer (KK-13-1 Jun). At Stn. OT4, nitrogen fixation was detectable even in deeper layers below the nitracline, where nitrate concentrations were

relatively high (KT-12-27_Oct, depth = 62 m; KK-13-1_Jun, depth = 42 m). In this layer, the ammonium concentrations were 0.05 µM (KT-12-27_Oct) and 0.62 µM (KK-13-1_Jun). The nitrogen fixation rate below the nitracline (1.56 nmol N L^{-1} d^{-1}) was higher than that at the surface (0.87 nmol N L^{-1} d^{-1}) during the KK-13-1_Jun cruise. ~~.~~ ~~–~~The maximum depth-integrated nitrogen fixation (294 µmol N m^{-2} d^{-1}) was observed at Stn. OT4 ~~in~~ during summer (KT-12-20_Aug).

3.3. Relationship between nitrogen fixation rates and environmental variables

Nitrogen fixation rates tended to increase with temperature ($p < 0.01$) (Fig. 6a and Table 2). Nitrogen fixation was detected only when seawater temperatures exceeded 11.7º C, with higher rates (>6 nmol N L^{-1} d^{-1}) noted in waters warmer than 19.5º C. However, there were exceptions to this general relationship between the nitrogen fixation rate and temperature. For example, from the data collected during the KK-13-1_Jun cruise the nitrogen fixation rate was highest at 15.4º C, while it was low (undetectable) at higher temperatures.~~—~~ Nitrogen fixation rates were negatively correlated with nitrate and phosphate concentrations ($p < 0.01$) (Table 2). There was no significant correlation between nitrogen fixation rates and~~h~~ ammonium concentration ($p > 0.05$). We also found no significant correlation between nitrogen fixation rates and the ratio of total inorganic nitrogen (nitrate + nitrite + ammonium)

to phosphate (Table 2).

A plot of the nitrogen fixation against nitrate concentrations indicated that nitrogen fixation was generally detectable only when nitrate was depleted (Fig. ~~5b~~6b), except that relatively high nitrogen fixation rates were determined in the subsurface layer of Stn. OT4 (KT-12-27_Oct and KK-13-1_Jun). Active nitrogen fixation tended to occur at low ammonium concentration ≤ ~0.1 μM. However, seasonal variation in ammonium concentration was small and no statistically significant relationship with nitrogen fixation was observed (Fig. 6c).

3.4. Seasonal variation in the ~~phylogenetic compositions of the *nifH* gene~~diazotroph community

3.4.1. Diazotroph community

PCR reagents have been suggested to be a potential source of *nifH* genes during analysis of the diazotroph community (Zehr et al., 2003~~a~~b). Although we confirmed the absence of any bands from the negative control in agarose gel electrophoresis, sequences with similarity (>97%) at the amino acid level to contaminants in PCR reagents were recovered from samples obtained during the KK-13-6_Sep and KS-14-2_Mar cruises (10 clones in total). We did not include these sequences in our data analysis.

The *nifH* gene was recovered from all samples that we collected during this study across different stations and seasons (Table 1). Sixty-~~six~~ one ~~operational taxonomic units (~~OTUs~~)~~ were grouped from ~~197~~ 187 *nifH* clones, based on 100% amino acid sequence similarity. The OTUs were assigned to cyanobacteria, α-, β-, γ-, and δ-proteobacteria, and Cluster III diazotrophs (Zehr et al., 2003a) (Figs. ~~S4~~ S3 and S~~45~~).

The detected cyanobacterial sequences belonged to *Trichodesmium*, UCYN-A, and *Leptolyngbya*. The *nifH* sequences of UCYN-B, UCYN-C, and *Richelia intracellularis* were not recovered. The *nifH* sequence of *Trichodesmium* was recovered only during the KT-12-27_Oct cruise (~~Fig. 6~~Table 1). ~~The~~ UCYN-A was generally observed from early summer to fall, while ~~*Leptolyngbya*~~ *nifH* *of Leptolyngbya* was detected ~~in~~ during winter. ~~On~~ During the KS-14-2_Mar ~~spring~~ cruise, all recovered sequences were derived from heterotrophic bacteria, and were dominated by Cluster III diazotrophs at Stns. OT4 and ON5. The Cluster III diazotroph *nifH* sequences were recovered ~~on~~ on all cruises except the KK-13-1_Jun cruise. Note that ~~69~~ 58 out of ~~197~~ 187 sequences displayed >97% similarity, at the amino acid level, to terrestrial diazotroph sequences derived from soil, mudflats, and lakes (Fig. ~~S4~~ S3 and ~~S5~~S4). These sequences were mainly affiliated with α- and δ-proteobacterial diazotrophs, with ~~39~~ 29 of ~~49~~ 39 α-proteobacterial sequences and ~~21~~ 22 of 24 δ-proteobacterial sequences being similar to terrestrial diazotroph sequences.

3.4.2. Diazotrophs abundances

The *nifH* sequence of *Trichodesmium* was detected by qPCR assay during the KT-12-27_Oct and KK-13-6_Sep cruises (Fig. 7 and 8). During these two cruises, the abundance of *Trichodesmium* ranged from below the detection limit to 8.7×10^4 copies L^{-1} at all depths. *Trichodesmium* abundance at the surface was higher than those of UCYN-A, UCYN-B, and γ-24774A11 at most stations during the KT-12-27_Oct cruise (Fig. 7 and S5). UCYN-A was detected on all cruises except for the KS-14-2_Mar cruise (Fig. 7 and 8). The maximum abundance of UCYN-A generally occurred at the surface except at Stn. OT4 during the KK-13-6_Sep cruise where the peak (1.2×10^3 copies L^{-1}) was observed at 72 m (Fig. 8). The abundance of UCYN-A varied from below the detection limit to 2.6×10^5 copies L^{-1} at all depths. At the surface, UCYN-A was the most abundant among the four groups at ~~al~~most of the stations investigated during the KT-12-20_Aug, KT-13-2_Jan, KK-13-1_Jun, and KK-13-6_Sep cruises (Fig. 7 and S5). UCYN-B was detected only at Stn. ON7 during the KK-13-6_Sep cruise (Fig. 7, 8, and S5). γ-24774A11 was detected during~~in~~ all cruises except for the KS-14-2_Mar cruise (Fig. 7 and 8). The abundance of γ-24774A11 ranged from below the detection limit to 1.8×10^4 copies L^{-1}, with a tendency of a subsurface peak at both stations (Fig. 8).

4. DISCUSSION

4.1. Differences in nitrogen fixation rates between the bubble method and the dissolution method

The present study revealed that a significant difference between the bubble and the dissolution methods was not always present. Großkopf et al. (2012) indicated that the difference was smaller when *Trichodesmium* dominated in the diazotroph community than when unicellular cyanobacteria and γ-proteobacteria dominated presumably because *Trichodesmium* can float to the top of the bottle and directly use the added $^{15}N_2$ in the bubble method. Interestingly, *Trichodesmium* abundance was higher than or similar to those of UCYN-A, UCYN-B, and γ-24774A11 at Stns. OT4 and ON7, at which there was no significant difference detected between the two methods. On the other hand, *Trichodesmium* was not detected and UCYN-A was the most abundant among the four groups at Stns. OT6 and ON5, at which nitrogen fixation determined by the dissolution method was significantly higher than that by the bubble method. These results were consistent with the report by Großkopf et al. (2012). The larger variations in nitrogen fixation at Stns. OT4 and ON7 than at Stns. OT6 and ON5 were probably due to the heterogeneity of *Trichodesmium* abundance (Carpenter et al., 2004). Although nitrogen fixation rates determined by the bubble method in the present study were underestimated on all cruises, the level of

underestimation was relatively small during the KT-12-27_Oct cruise when *Trichodesmium* was dominant at most of the stations.

~~3.5.~~4.2. Seasonal variations in nitrogen fixation rates in the temperate coastal ~~region~~ocean

Nitrogen fixation rates were measurable mainly from early summer to fall when nitrate was generally depleted in sample seawaters, although there were some exceptions. Our estimates of the nitrogen fixation rates (0.33–13.6 nmol N l^{-1} d^{-1}) were significantly (p <0.05) higher than the corresponding values previously reported in the temperate region of the eastern North Pacific (0.15–0.31 nmol N l^{-1} d^{-1}; Needoba et al., 2007) and the oligotrophic region of the western and central North Pacific (0.17–3.62 nmol N l^{-1} d^{-1}; Shiozaki et al., 2010), whereas they were comparable to those determined in the Kuroshio (0.54–28 nmol N l^{-1} d^{-1}; Shiozaki et al., 2010) and the western Atlantic coastal regions (1.3–49.8 nmol N l^{-1} d^{-1}; Mulholland et al., 2012). 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).

In our study, spatiotemporal variability in ~~The relatively high~~ nitrogen fixation rates appeared

to be partly related to ~~s could be due to an influence of~~ the ~~–~~ Tsugaru Warm Current path. This current, which flows from the north (after passage th~~o~~rough the Tsugaru Strait) to the study region (Fig. S1), may carry active diazotrophs and therefore enhance nitrogen fixation in our study region. This is supported by the fact that nitrogen fixation rates during individual cruises tended to be higher at Stn. OT4 than at Stn. ON5. These stations were located up- and down-stream of the Tsugaru Warm Current, respectively. In addition, variations in nitrogen fixation rates among stations and seasons might also be related to the extent of vertical mixing ~~–~~in the Tsugaru Warm Current. It has been suggested that vertical mixing may introduce iron-rich subsurface water to the surface of the Tsugaru Strait (Saitoh et al., 2008). Such input of iron may enhance nitrogen fixation rates. Consistent with this notion, our results showed that the nitrogen fixation rate was relatively high at Stn. OT4, where the nitracline was relatively deep.

Blais et al. (2012) proposed that nitrogen fixation can proceed even in nutrient-replete waters, if large amounts of iron and organic materials are available for consumption by bacterial diazotrophs. In the present~~is~~ study, ~~we examined this~~ this possibility was examined by conducting mannitol addition experiments using ~~where mannitol was added to~~ surface seawaters collected ~~in~~ during spring. These waters, belonging to ~~when the iron supply was considered to be high due to intrusion of~~ the Oyashio Current system ~~–~~(Nishioka et al., 2007,

2011; Shiozaki et al., 2014b)~~,)~~. were considered to be rich in iron ~~in~~during spring, as indicated by a previous study (iron conc., 0.79–8.46 nM; Nishioka et al. 2007). Despite potentially high iron concentrations, our results showed that nitrogen fixation was undetectable even after the mannitol addition, suggesting that, contrary to the Blais et al. proposition, diazotrophs remained inactive under our experimental settings.

Our data showed that nitrogen fixation rates were below the detection limit during winter, spring, and late summer (KK-13-6 Sep), when nitrate concentrations were high. These results ~~These results are~~were consistent with the results of previous studies in the Pacific Ocean, which indicated that nitrogen fixation rates were low or undetectable in DIN-replete waters (Shiozaki et al., 2010). In contrast, Mulholland et al. (2012) reported that, in temperate regions of the Atlantic Ocean, nitrogen fixation rates were high even in DIN-replete (>1 μM) and cold (<10ºC) surface seawaters. Their study was conducted downstream of the Gulf Stream, where diazotrophs could be delivered from subtropical oceans where DIN is depleted. Previous studies have suggested that cyanobacterial diazotrophs can travel over long distances (>1,000 km) in currents, without losing their capacity for N_2 fixation (Shiozaki et al., 2013), and that activity is not lost immediately even after mixing with DIN-replete seawaters (Holl and Montoya, 2005; Dekaezemacker and Bonnet, 2011). In our region, because the Tsugaru Warm Current flows from north to south,

diazotrophs entrained by the current have little chance of meeting high-DIN water ~~in~~at~~at~~ the surface. DIN-replete water ~~in~~ during summer was observed at the inside bay station OT1 (Fig. S2). Concomitantly, low-salinity surface waters spread offshore along the OT transect line (Fig. S6), suggesting that anomalously high DIN concentrations were likely attributable to terrestrial surface discharge enhanced by Typhoon Man-yi, which passed over the region immediately before the cruise. ~~, with a low salinity, attributable to the washout of terrestrial water after the passage of a typhoon.~~ Subramaniam et al. (2008) reported that nitrogen fixation rates near the Amazon River estuary, with low salinity and high nitrate levels, were fairly low. Their results are ~~,~~ consistent with ours ~~results~~. Ammonium inhibits nitrogen fixation, especially when ammonium concentrations exceed 1 μM, as demonstrated by *Trichodesmium* (Mulholland et al. 2001). In our study, no negative relationship between nitrogen fixation and ammonium concentration was found. This can likely be explained by relatively low ammonium concentrations (≤ ~1 μM) throughout the year and across the investigated region~~s~~.

~~3.6.~~4.3. Seasonal variation in the diazotroph~~ic~~ community ~~structure~~ in the temperate coastal ~~region~~ocean

The qPCR analysis demonstrated that the target groups were quantifiable even at stations at

which their sequences were not recovered by 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. In the discussion below, we discuss possible factors responsible for seasonal variation in the diazotroph community by focusing on the major diazotroph groups.

UCYN-A was detected by qPCR in all seasons except spring (KS-14-2_Mar),~~Many recovered *nifH* sequences were similar to terrestrially derived sequences. These results agree with previous data collected in coastal regions, where terrestrially derived *nifH* sequences were also found (Rees et al., 2009; Mulholland et al., 2012; Blais et al., 2012). In~~

addition to the terrestrially derived *nifH* sequences, the cyanobacterium UCYN-A was regularly observed from early summer to fall, suggesting that this group of diazotrophs could be important agents of nitrogen fixation in this regionduring these periods (although we do not have direct evidence of nitrogen fixation by UCYN-A). Especially from early to late summer, the abundance of UCYN-A was generally higher than that of *Trichodesmium*, UCYN-B, and γ-24774A11. UCYN-A has been widely detected in oligotrophic temperate regions, and is considered to be one of the major diazotrophs of these locations (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012; Bentzon-Tilia et al., 2015). UCYN-A is known to be most abundant in relatively warm waters around ~20º C (Needoba et al., 2007; Moisander et al., 2010). UCYN-A was detected by qPCR even during winter at some stations, yet, was not observed during spring. This could be because UCYN-A abundance decreased from fall to winter with decreasing temperatures, eventually disappearing during in spring.

Trichodesmium was detected from late summer to fall by qPCR analysis, when water temperatures ranged from 19.1 to 23.4º C at the surface. Given that the optimal growth temperature for *Trichodesmium* has been reported to be high (24–30º C) (Breitbarth et al., 2007), *Trichodesmium* detected in the investigated region likely existed under suboptimum conditions. The relatively high abundance of *Trichodesmium* observed during fall, despite

the suboptimal temperature conditions, might indicate that *Trichodesmium* was transported from the adjacent subtropical region where seawater temperatures were high (>24° C). In the western North Pacific subtropical region, *Trichodesmium* is abundant from July to September (Marumo and Nagasawa, 1976; Chen et al., 2008). *Trichodesmium* that flourished in the subtropical region during summer could be transported by the Tsugaru Warm Current, displaying peak abundance during fall in the investigated region. This could support the above discussion that waters containing active nitrogen fixation were delivered to this region by the Tsugaru Warm Current.

We observed γ-24774A11 by qPCR analysis during all cruises except for the KS-14-2_Mar cruise. ~~The absence of UCYN-A in spring and winter was likely to be due to the low temperature (the SST in winter and spring ranged from 2.0 to 9.4°C), because UCYN-A is known to be most abundant in relatively warm waters around ~20°C (Needoba et al., 2007; Church et al., 2009; Moisander et al., 2010).~~

~~During the KT-12-27 (fall) and KK-13-6 (summer) cruises, we detected the γ-proteobacterial diazotroph closely related to phylotype γ-24774A11 (Fig. S4).~~ This phylotype has not been reported previously in other temperate ~~regions~~ oceans (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012). The *nifH* sequence of γ-24774A11 was similar to that of *Pseudomonas stutzeri* (94% similarity at the amino acid level), which was observed in waters

including temperate regions (Bentzon-Tilia et al., 2015). Bentzon-Tilia et al. (2015) reported that *P. stutzeri*-like *nifH* genes (99% similarity at the nucleotide level) were the most abundant sequences among their samples collected from the temperate Baltic Sea estuary. In the present study, we recovered *P. stutzeri*-like *nifH* genes (>97% similarity at the amino acid level) from Stn. OT4 during the KT-13-2_Jan cruise by the clone library analysis. However, γ-24774A11 was not detected on that occasion by qPCR analysis, suggesting that γ-24774A11 was not quantified as *P. stutzeri* and that *P. stutzeri* was not a major diazotroph in this study region. ~~, although this *nifH* gene is known to occur widely in the oligotrophic subtropical and tropical oceans, where it is considered to be one of the key agents of nitrogen fixation globally (Moisander et al., 2014).~~ The ecology of γ-24774A11 is still fairly unknown. It remains to be seen, in future studies~~,~~ ~~if~~ whether this phylotype contributes to the nitrogen fixation ~~that we saw in summer and fall.~~in this region.

UCYN-B was not detected by qPCR except ~~for~~at one station. This result is consistent with previous knowledge. UCYN-B becomes abundant with increasing temperature, similar to *Trichodesmium* (Moisander et al., 2010), and is rarely observed in the temperate region (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012; Bentzon-Tilia et al., 2015). Furthermore, UCYN-B abundance is low in shallow nitracline regions (Shiozaki et al., 2014a,c). The nitracline depth in this region (≤60 m) was shallower than that of >100-m

depths of regions where UCYN-B is abundant (Shiozaki et al., 2014a). Therefore, although UCYN-B might also have been delivered from subtropical region, it could not survive in the shallower nitracline region.

In nitrate-rich water ~~in~~ during winter and spring, Cluster III diazotrophs were dominant at most of the stations~~, excluding terrestrially derived sequences~~. Furthermore, from early summer to fall, *nifH* sequences of Cluster III diazotrophs were recovered by the clone library analysis in samples from all cruises (except KK-13-1 Jan)~~ and were the major diazotrophs in samples collected during the KT-12-27 cruise~~. Because UCYN-A, *Trichodesmium*, and γ-24774A11 were scarce during winter and spring, Cluster III diazotrophs were likely to be major diazotrophs at these times.

~~It is rare for Cluster III diazotrophs to be dominant in the surface diazotrophic community. Because anaerobic diazotrophs are~~ Cluster III diazotrophs are putative anaerobes (Hamersley et al., 2011; Farnelid et al., 2013; Bentzon-Tilia et al., 2014~~b~~), and hence, they are usually dominant in the diazotrophic community of~~recovered from~~ oxygen-depleted waters (Hamersley et al., 2011; Farnelid et al., 2013) or~~and~~ marine sediments (Bertics et al., 2013). In this study, dissolved oxygen was not depleted (>3.16 ml L^{-1}) in the upper winter maximum mixed layer depth in this region (~200 m; Shiozaki et al., 2014b) (Fig. ~~S6~~S7). Therefore, ~~t~~, ~~and hence, here~~it is ~~a~~ possib~~i~~l~~ity~~e that the Cluster III diazotrophs that we detected in~~at~~ the

surface layer ~~might be~~were derived from resuspensions of ~~from~~ coastal marine sediments in which anoxic conditions may prevail because of organic matter decomposition. The Cluster III activity was likely strongly suppressed in the water column because of the high oxygen concentration.

Many *nifH* sequences recovered by the clone library analysis were similar to terrestrially derived sequences. These results agree with previous data collected in coastal regions, where terrestrially derived *nifH* sequences were also found (Rees et al., 2009; Mulholland et al., 2012; Blais et al., 2012). ~~The 2011 Tohoku-oki tsunami substantially altered the benthic environment of the region (Seike et al., 2013; Urabe et al., 2013). Such environmental change might be related to detection of Cluster III diazotrophs at the surface.~~ We obtained a *Leptolyngbya*-like *nifH* gene during the KT-13-2 Jan cruise. The organism has been ~~is~~ found on beaches or coastal land areas (Brito et al. 2012), ~~and is~~ but ~~rarely detected~~not in the open ocean. Because nitrogen fixation was not ~~observed~~ detected during the KT-13-2 Jan cruise, the ~~bacteria~~ organism must have been inactivated after being flushed out from the coastal region.

~~4.~~5. CONCLUSION ~~AND REMARKS ON THE IMPACT OF THE TSUNAMI~~

This study demonstrated that nitrogen fixation ~~did indeed occur~~can and does proceed at high

rates, depending on the season, in the temperate region of the northwestern North Pacific, although we failed to detect nitrogen fixation in DIN-replete cold waters. *nifH* sequences were omnipresent and recovered throughout the~~a~~ year, displaying a marked seasonality in their composition. UCYN-A was a major diazotroph during summer, whereas *Trichodesmium* was abundant ~~in~~during fall, despite low temperatures. It has been suggested that *Trichodesmium* was laterally transported f~~or~~rom the adjacent subtropical region, which displays high temperatures. The Cluster III diazotrophs were abundant in surface waters ~~in~~during winter, which was ascribed to their delivery from the anoxic sediments via bottom resuspension. The failure to detect nitrogen fixation when Cluster III was abundant implied that the activity of this diazotroph group was strongly suppressed in oxic water columns.

~~We found that UCYN-A and the γ-proteobacterial phylotype γ-24774A11 were generally recovered during the period when nitrogen fixation rates were high, raising the possibility that these phylotypes play key roles in the diazotrophy of our study region. This study was conducted in coastal regions heavily damaged by the 2011 Tohoku earthquake-induced tsunami. The tsunami substantially changed the coastal geography and benthic environment, and impacted on benthic ecosystems (Seike et al., 2013; Urabe et al., 2013). Given that nitrogen fixation in a temperate estuary could be an important source of new nitrogen for adjacent coastal waters, and that such fixation is influenced by the condition of the benthic~~

~~environment (Bentson-Tilia et al., 2014b), the tsunami-induced alteration in and the following recovery of benthic ecosystems could affect nitrogen fixation and the distribution of the diazotroph community in the coastal waters. In future studies, continuous monitoring of nitrogen fixation and recovery of the benthic environment would produce a unique dataset that could be used to explore possible links between benthic environmental conditions and pelagic diazotrophy in temperate coastal regions.~~

Author Contributions

T.S., T.N., and K.F. designed the experiment and T.S. collected the samples at sea. T.S. determined nitrogen fixation and nutrient concentrations and analyzed satellite datasets. T.S. and M.I. conducted the genetic analyses. T.S. prepared the manuscript with contributions from all co-authors.

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751 Table 1. Summary of recovered *nifH* sequences belonging to *Trichodesmium* (Tri), UCYN-A

752 (UA), Leptolyngbya (Lep), α-proteobacteria (α-Pro), β-proteobacteria (β-Pro),

753 γ-proteobacteria (γ-Pro), δ-proteobacteria (δ-Pro), and Cluster III (CIII)

Cruise	Station	No. of clones	Cyanobacteria			α-Pro	β-Pro	γ-Pro	δ-Pro	CIII
			Tri	UA	Lep					
KT-12-20	OT4	12		9		3				
	ON1	5		2						3
	ON5	8		8						
	ON7	7		1		6				
Total		**32**	**0**	**20**	**0**	**9**	**0**	**0**	**0**	**3**
KT-12-27	OT4	7	1							6
	ON1	9							4(2)	5(5)
	ON5	6						1		5
	ON7	13	6	1		5(5)		1(1)		
Total		**35**	**7**	**1**	**0**	**5(5)**	**0**	**2(1)**	**4(2)**	**16(5)**
KT-13-2	OT4	11			10			1		
	ON1	1								1
	ON5	14				5(5)			2(2)	7
Total		**26**	**0**	**0**	**10**	**5(5)**	**0**	**1**	**2(2)**	**8**
KK-13-1	OT4	10		2		8(8)				
	ON1	15		3				2	10(10)	
	ON5	11		4		7(7)				
	ON8	1					1			
Total		**37**	**0**	**9**	**0**	**15(15)**	**1**	**2**	**10(10)**	**0**
KK-13-6	OT4	7							4(4)	1
	ON5	11		11						
	ON7	10		2		1		7		
Total		**28**	**0**	**13**	**0**	**1**	**0**	**7**	**4(4)**	**1**
KS-14-2	OT4	10							1(1)	9
	ON1	13				3(3)	3	1(1)	3(3)	
	ON5	15				2(2)				9
Total		**38**	**0**	**0**	**0**	**5(5)**	**3**	**1(1)**	**4(4)**	**18**

754 Numbers in parentheses indicate the number of sequences with >97% similarity at the amino

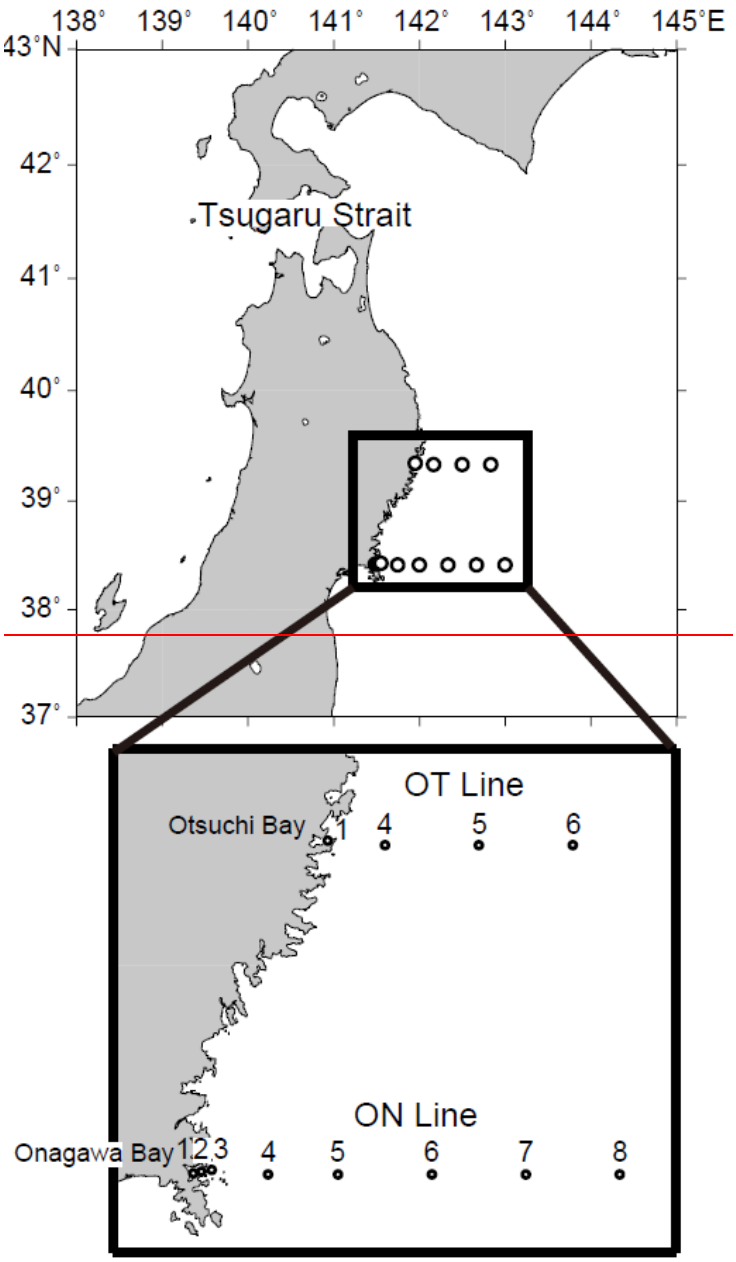
755 acid level to terrestrial diazotroph sequences.

Table 2 Pearson's correlation matrix of N_2 fixation rates and water properties at the surface (n=73).

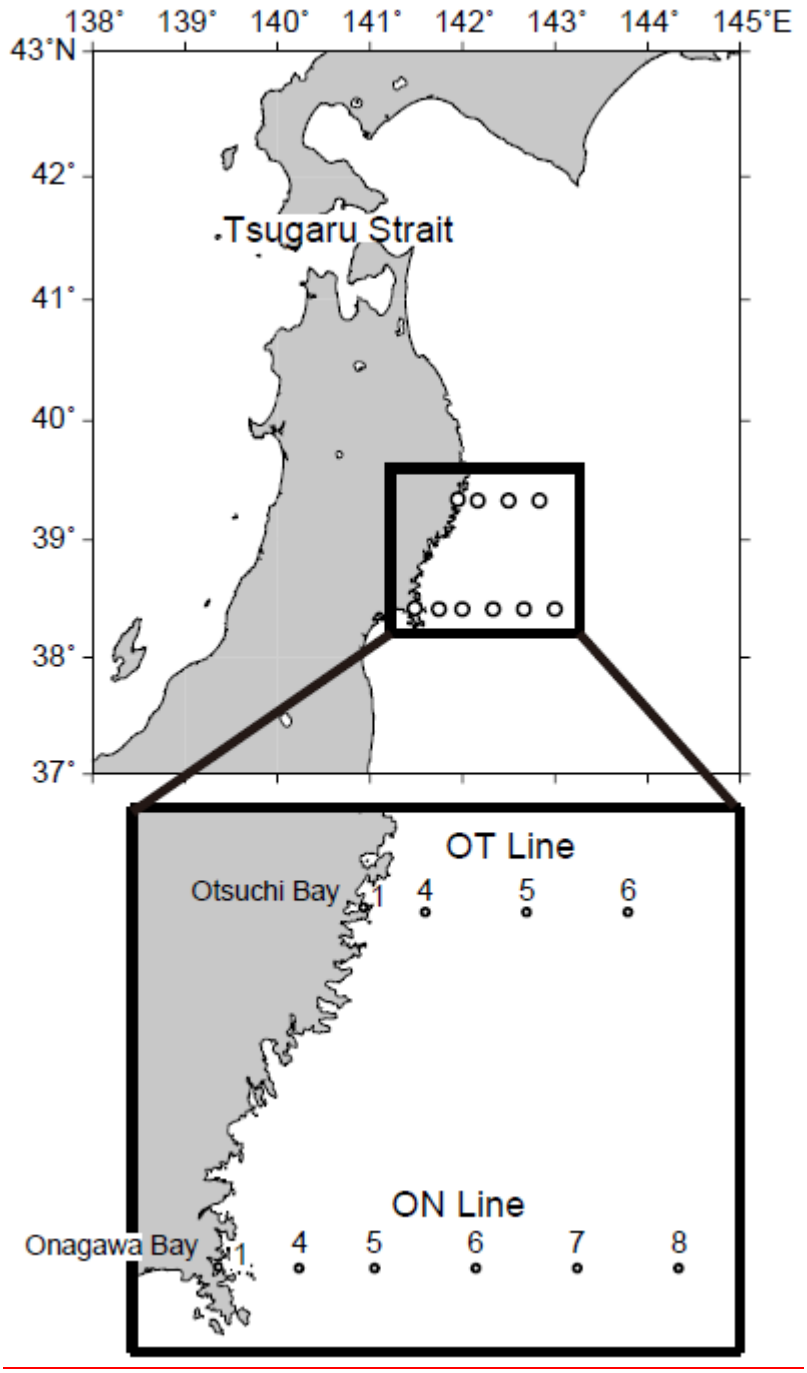
	Temperature	Nitrate	Ammonium	Phosphate	N/P ratio	N_2 fixation
Temperature	1					
Nitrate	-0.722**	1				
Ammonium	-0.038	0.440**	1			
Phosphate	-0.863**	0.867**	0.135	1		
N/P ratio	-0.125	0.216	0.171	0.009	1	
N_2 fixation	0.435**	-0.325**	-0.124	-0.335**	-0.130	1

*$p < 0.05$, **$p < 0.01$

N/P ratio denotes the ratio of (nitrate + nitrite + ammonium) to phosphate


138°
139°
140°
141°
142°
143°
144°
145°E
43°N
42°
41°
40°
39°
38°
37°
Tsugaru Strait
OT Line
Otsuchi Bay
1
4
5
6
ON Line
Onagawa Bay
1
2
3
4
5
6
7
8

761



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763

764 Fig. 1. Sampling locations in the northwestern North Pacific Ocean.

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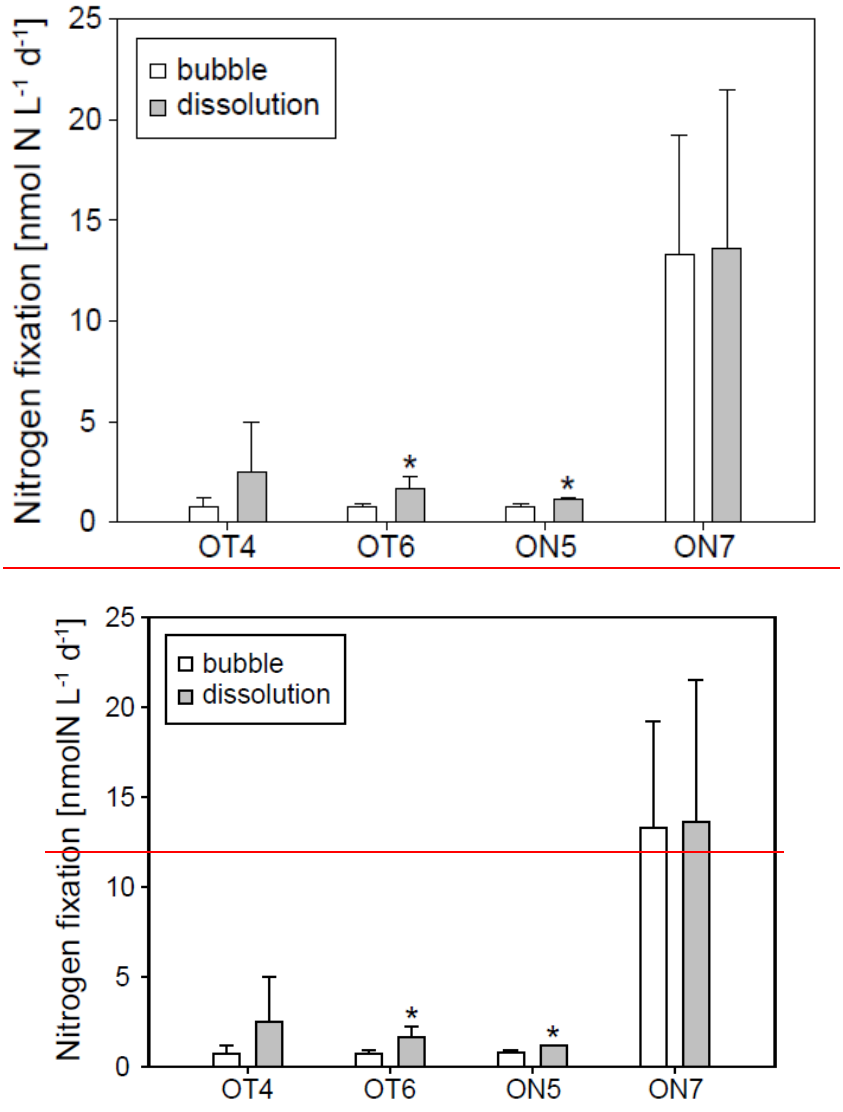
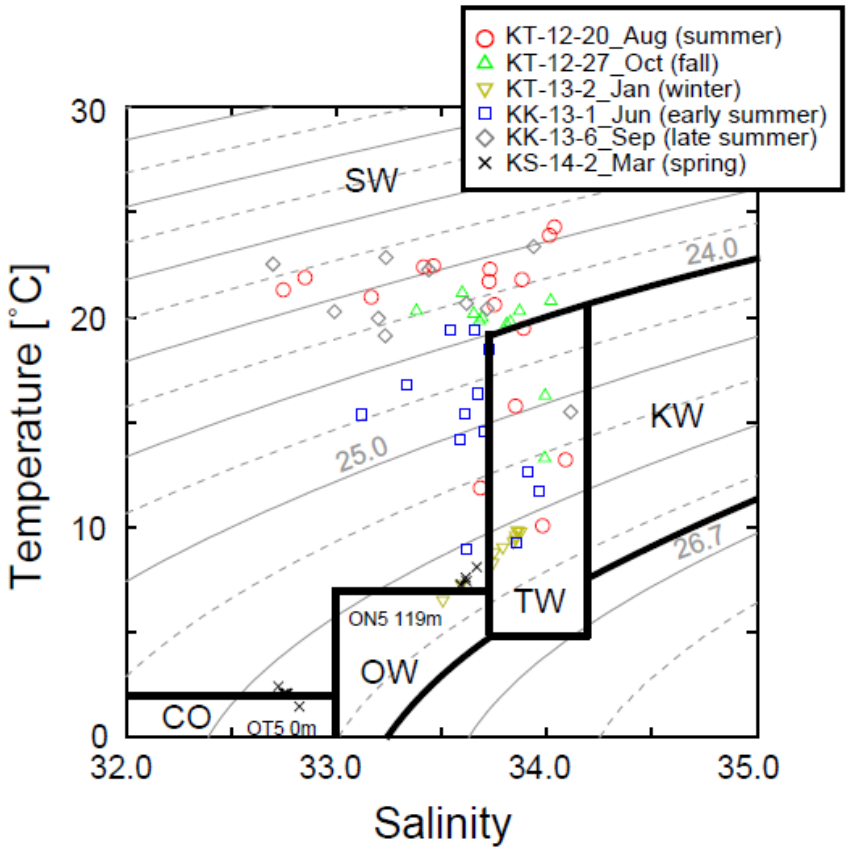


Fig.2. Nitrogen fixation rates estimated simultaneously by the $^{15}N_2$ gas bubble and dissolution methods during the KK-13-6_Sep cruise. An asterisk indicates a significant difference between the two methods (p <0.05).


KT-12-20_Aug (summer)
KT-12-27_Oct (fall)
KT-13-2_Jan (winter)
KK-13-1_Jun (early summer)
KK-13-6_Sep (late summer)
KS-14-2_Mar (spring)
SW
KW
TW
OW
CO
ON5 119m
OT5 0m
24.0
25.0
26.7
Temperature [°C]
Salinity
0
10
20
30
32.0
33.0
34.0
35.0

774

775

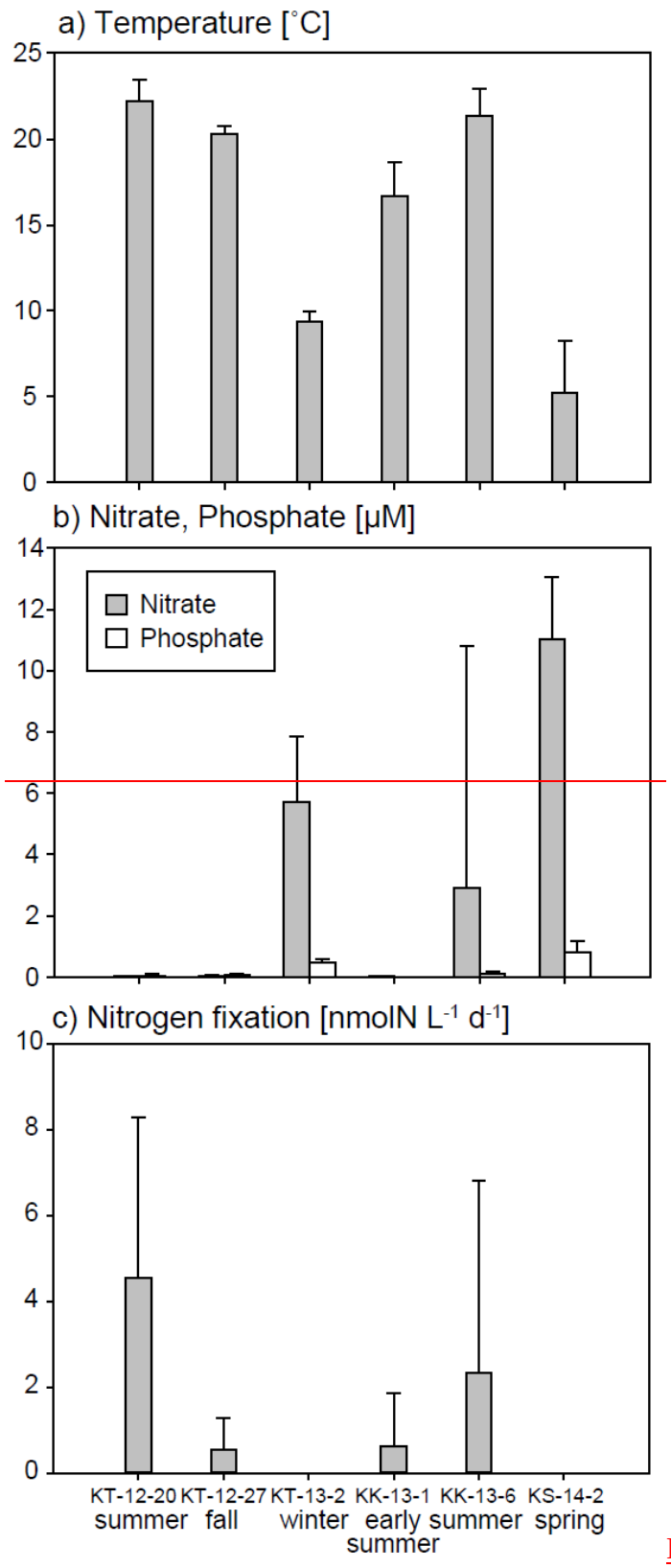
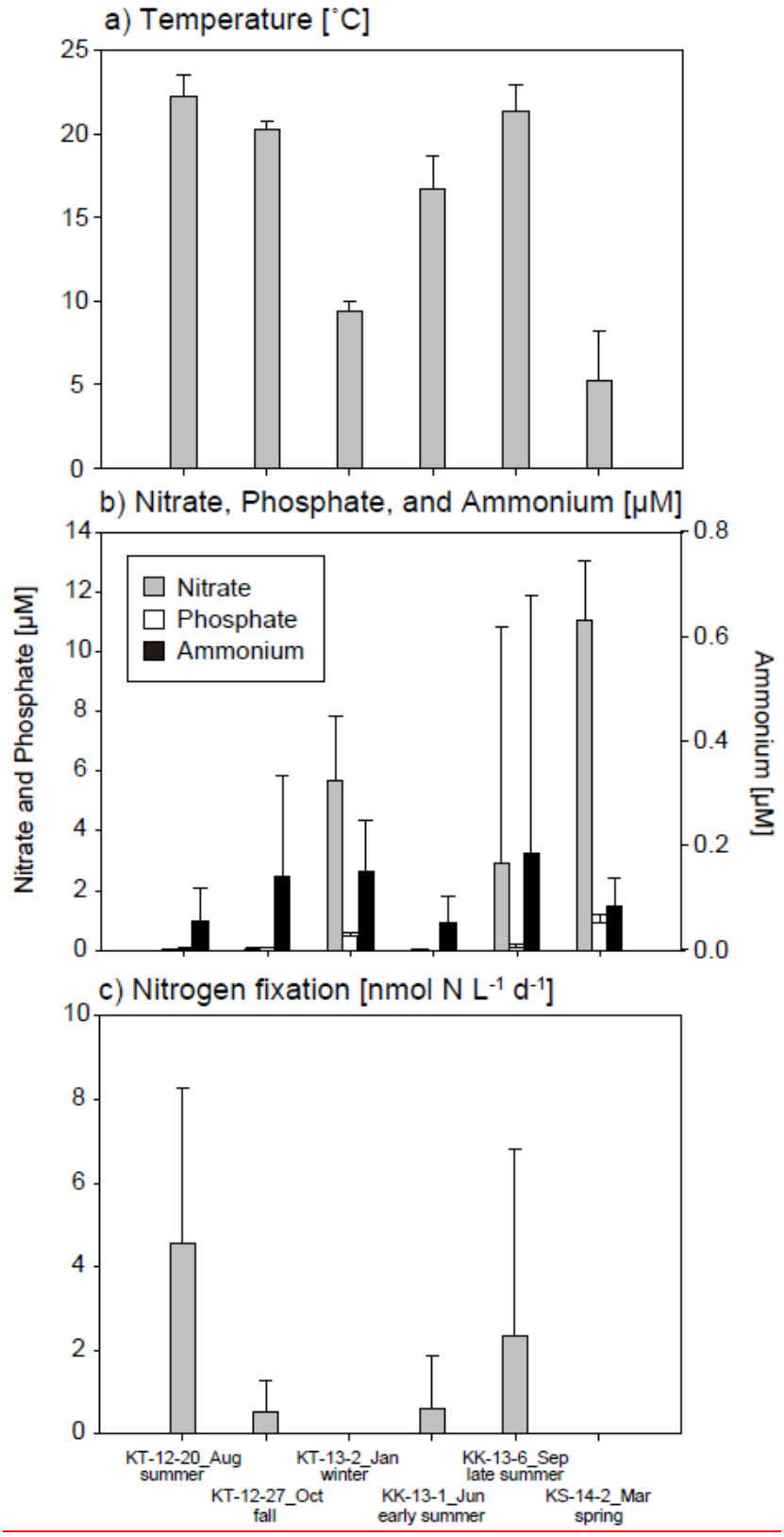


Fig. 3. Temperature-salinity diagram at each sampling point. The water classification was defined by Hanawa and Mitsudera (1986). SW, KW, TW, OW, and CO denote the surface layer water system, Kuroshio water

system, Tsugaru Warm Current water system, Oyashio water system, and Coastal Oyashio water system, respectively.

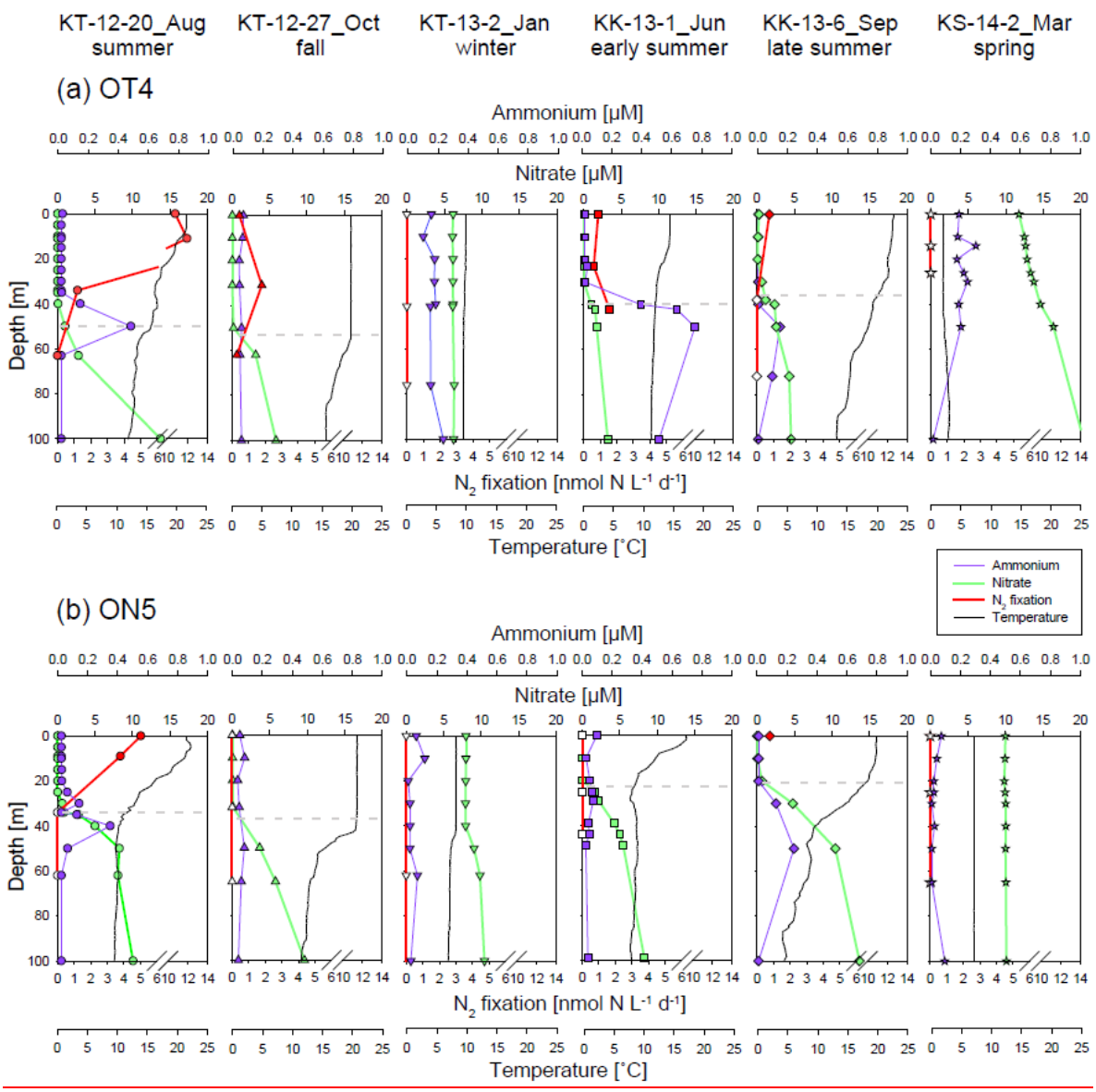


782

783 Fig. ~~3~~4. Average (a) temperature [ºC], (b) nitrate, ~~and~~ phosphate, and ammonium ~~levels~~

784 concentrations [µM], and (c) nitrogen fixation [nmol N L^{-1} d^{-1}] at the surface ~~for~~ during each

785 cruise.



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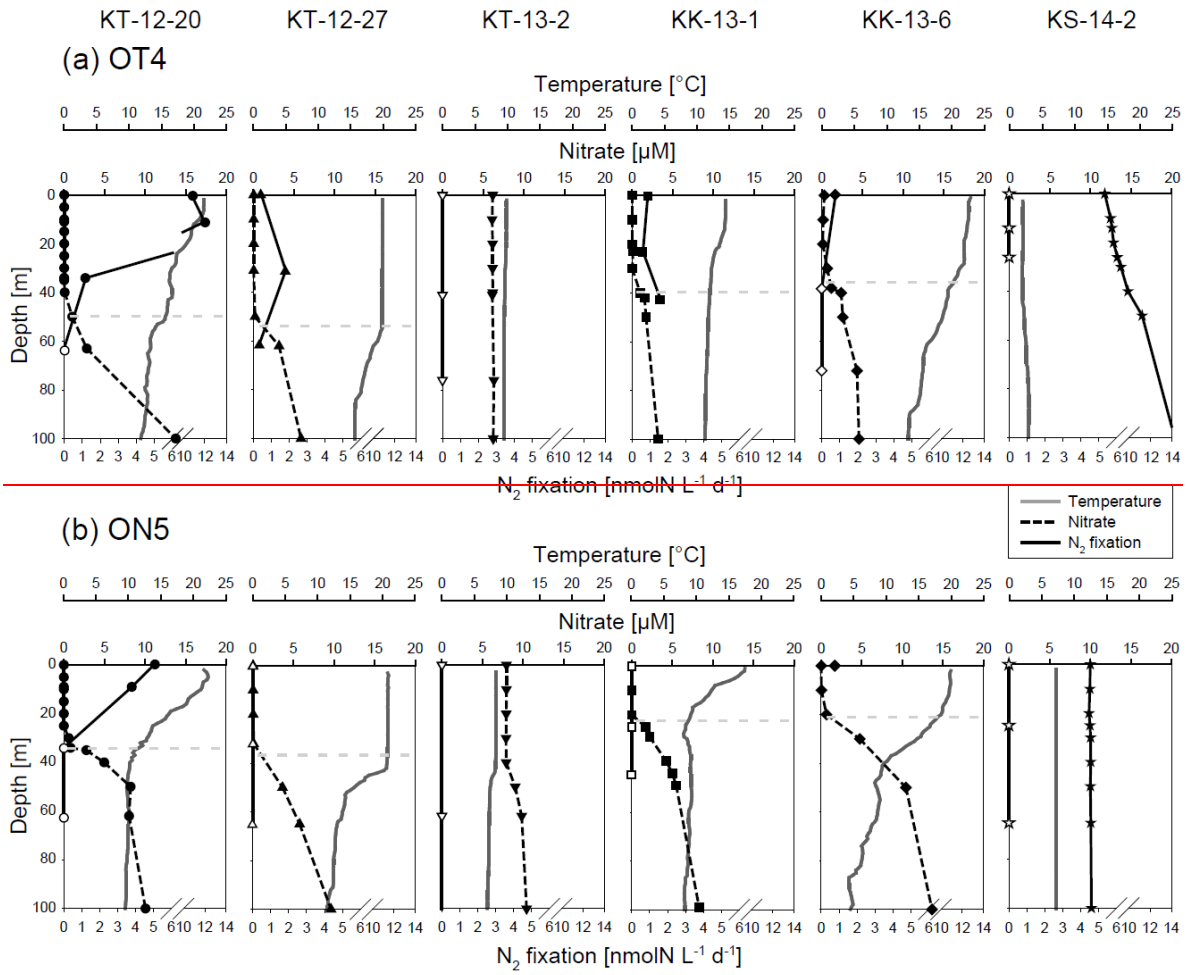

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788

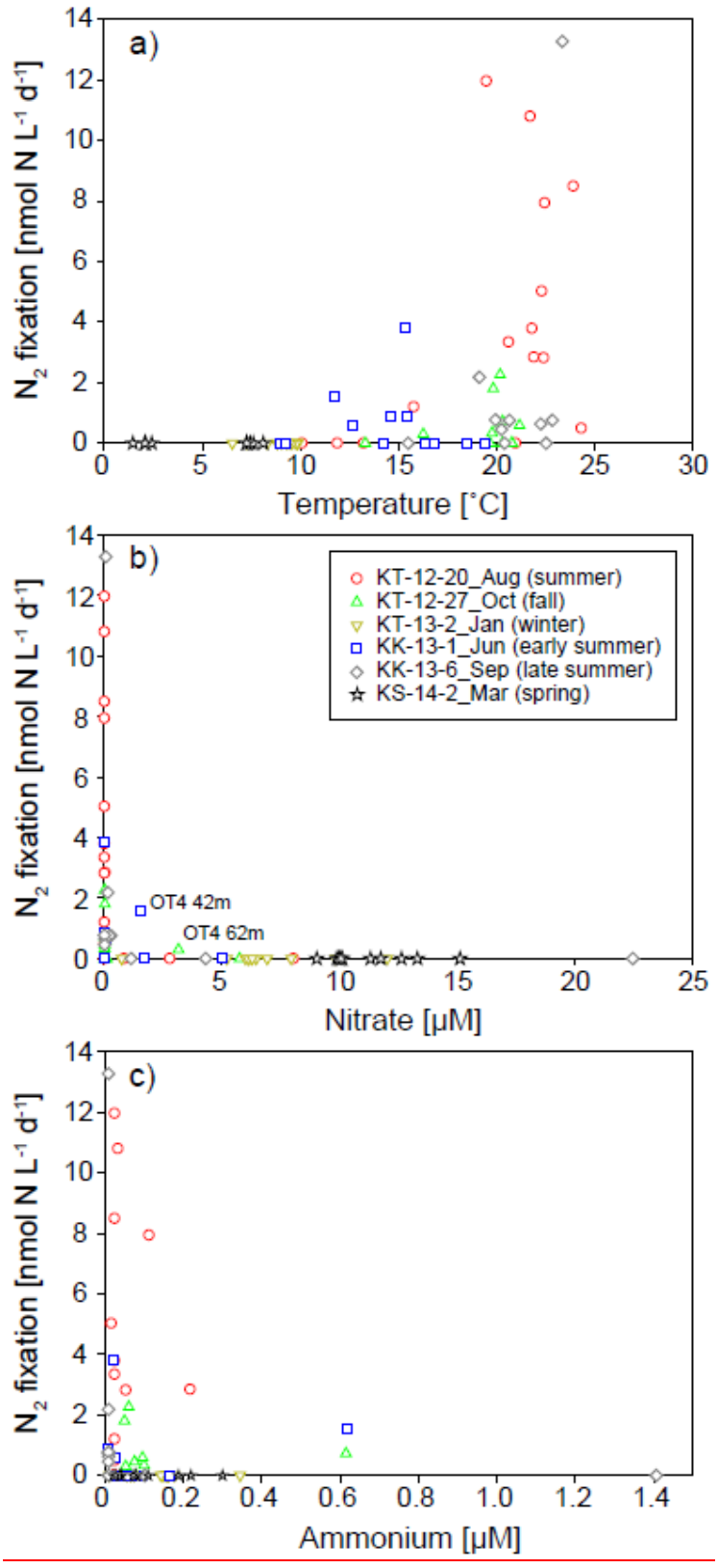
789 Fig. ~~4~~5. Time-series variations in the vertical profiles of temperature [ºC] (black),

790 ~~temperature [ºC]~~ammonium (purple) and~~,~~ nitrate (green) concentration [μM], and nitrogen

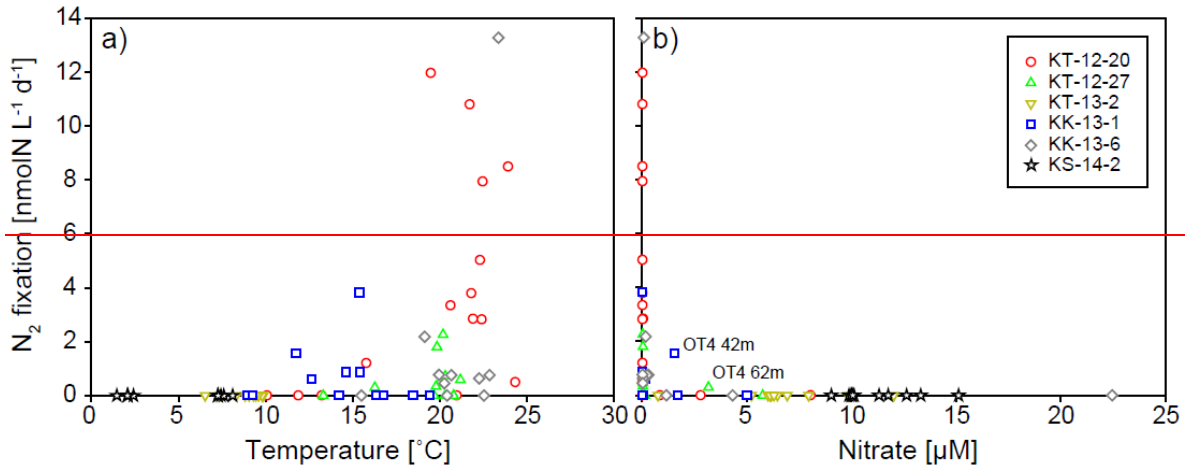
791 fixation (red) [nmol N L^{-1} d^{-1}] at Stns (a) OT4 and (b) ON5. Open symbols indicate that

792 nitrogen fixation was not detected. The horizontal dashed line indicates the nitracline depth.

793 The strait lines of temperature and nitrate were ascribable to strong mixing.


a)
b)
c)
N_2 fixation [nmol N L^{-1} d^{-1}]
Temperature [˚C]
Nitrate [µM]
Ammonium [µM]
KT-12-20_Aug (summer)
KT-12-27_Oct (fall)
KT-13-2_Jan (winter)
KK-13-1_Jun (early summer)
KK-13-6_Sep (late summer)
KS-14-2_Mar (spring)
OT4 42m
OT4 62m

794



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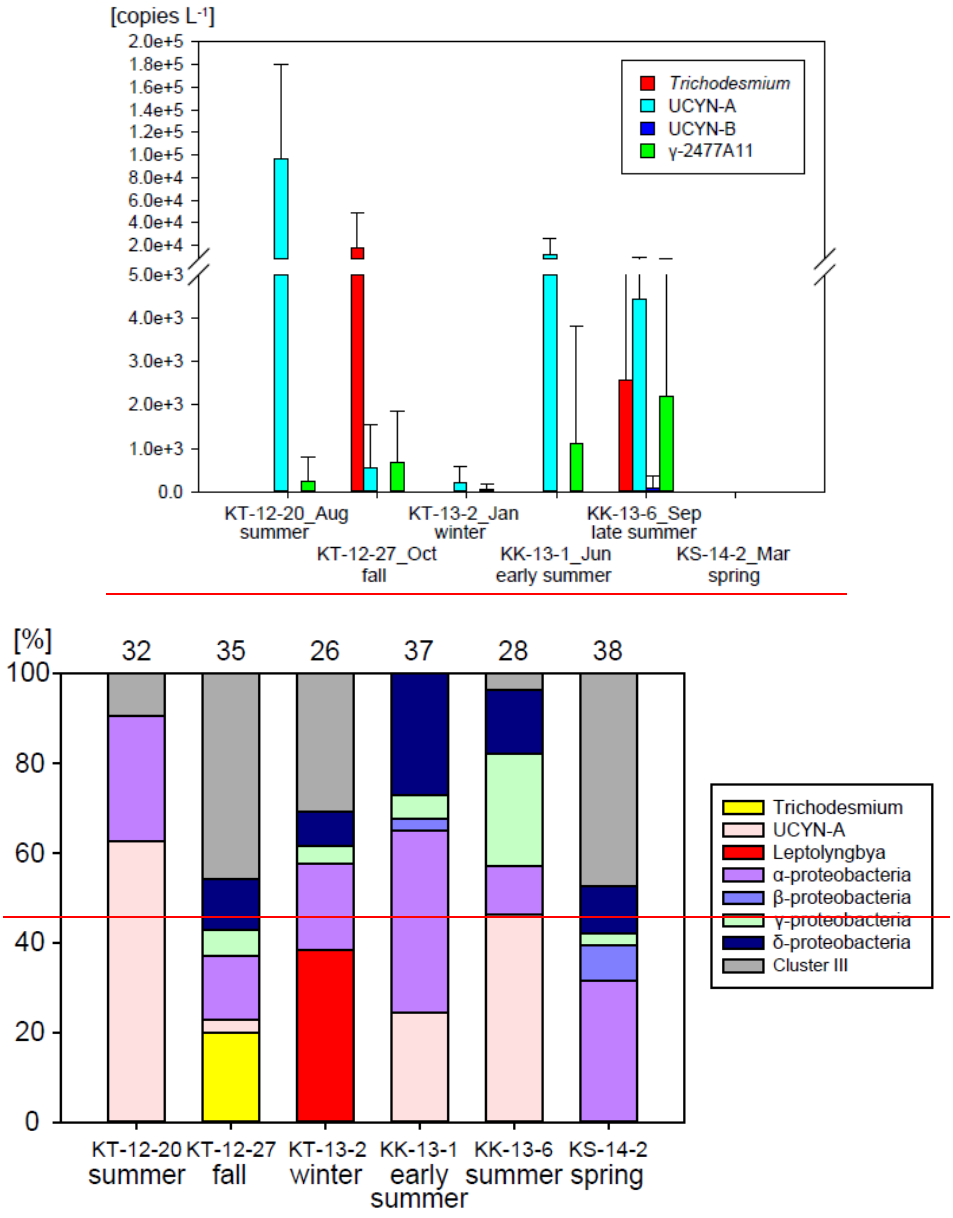
796

797 Fig. ~~5~~6. Relationship between nitrogen fixation [nmol N L^{-1} d^{-1}] and (a) temperature [ºC]

798 ~~and,~~ (b) nitrate [µM], and (c) ammonium ~~concentration~~ [µM] for all six cruises.

799

800



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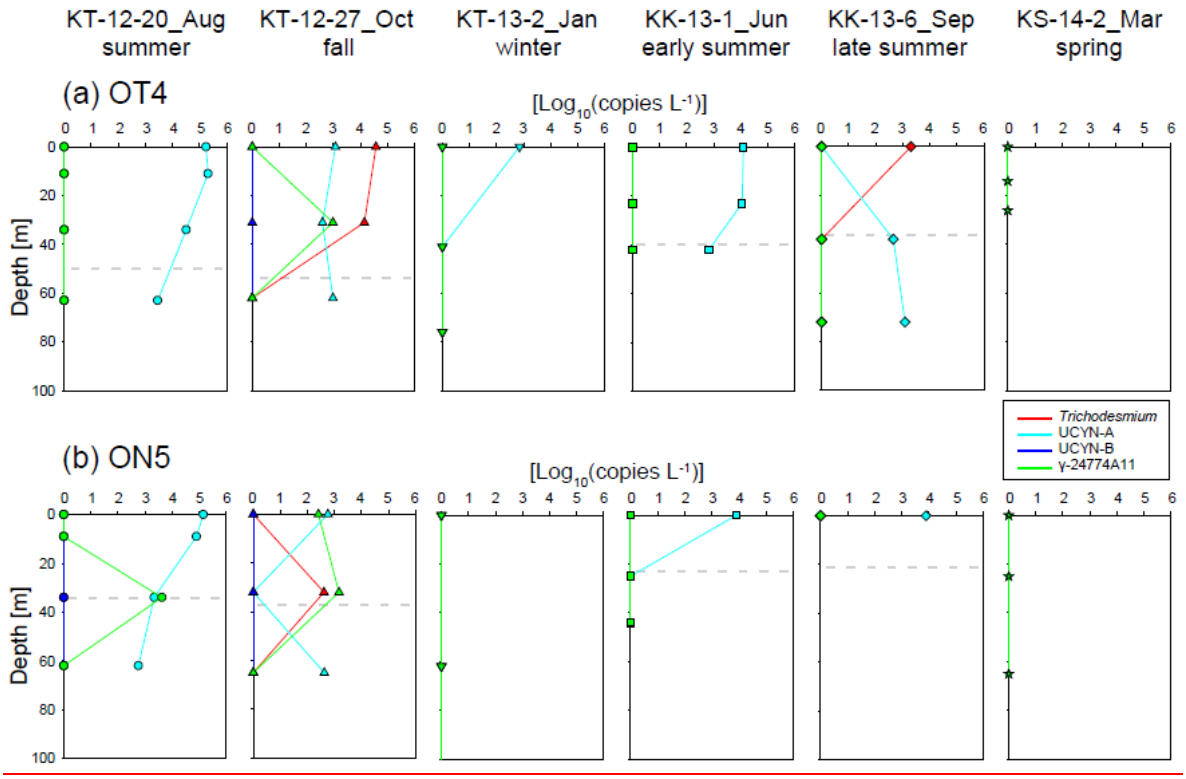
803 Fig. ~~6~~7. Average abundances of *Trichodesmium* (red), UCYN-A (light blue), UCYN-B

804 (blue), and γ-24774A11 (green) [copies L^{-1}] at the surface during each cruise.

805



806

807

808 Fig. 8. Time-series variations in the vertical profiles of *Trichodesmium* (red), UCYN-A

809 (light blue), UCYN-B (blue), and γ-24774A11 (green) [copies L^{-1}] at Stns. (a) OT4 and (b)

810 ON5. The horizontal dashed line indicates the nitracline depth.~~Relative abundance of each~~

811 ~~*nifH* group for each cruise. The number on each bar corresponds to the number of~~

812 ~~recovered *nifH* sequences.~~