

1 **Referee #2**

2  
3 *General comment:*

4 *Nitrogen fixation data is rare in the temperate marine ecosystems, and the authors clarified*  
5 *the accuracy of their rate measurement data. Therefore, this paper can provide significant*  
6 *contribution to our understanding of nitrogen fixation in the ocean. After the revision, the*  
7 *authors added quantitative PCR (qPCR) data of the three major phylotypes, which provided*  
8 *more useful molecular information and compensated the limitation of their sequencing results.*  
9 *Hence, they can explain (discuss) the seasonal and spatial variations of nitrogen fixation in a*  
10 *more convincing way. However, there are still some problems and questions needed to be*  
11 *considered, especially the influences of environmental factors to nitrogen fixation.*

12  
13 *Specific comments:*

14 *P.4, L.72: the methods of Pearson's correlation matrix were missing. At least, the authors*  
15 *should explain what the "surface water" meant (Table 2). Did they average the surface data*  
16 *of different stations for each cruise or input each data point during calculation? It seems that*  
17 *the error bars of the averaged nitrogen fixation rate and nutrient data were very large.*  
18 *Therefore, it is not appropriated if they used averaged data when they were calculating the*  
19 *correlation matrix. The author can simply display the data used for calculation in a table in*  
20 *supplementary information. Also, why didn't the author use the data of other water layers*  
21 *during calculation? It seems that nitrogen fixation was also significant in other water layers.*

22  
23 We have added the description about statistical analysis (L134-140). We wrote incorrectly  
24 the title of the Table 2. Although we wrote that "at the surface", we analyzed the data  
25 obtained from the entire water column. Furthermore, since data of phosphate concentration  
26 and N/P ratio during KT-12-20\_Aug was miscalculated in the previous manuscript, we  
27 have revised the data. This correction did not affect our major conclusion. We have added  
28 the data we used in supplementary information (Table S1).

29  
30 *P.13, L.222: the unit of nitrogen fixation rate should be uniform throughout the paper. The*  
31 *author used "L" or "l" in different places of the paper.*

32  
33 We have corrected to "L" throughout the manuscript.

34  
35 *P.14, L.252: according to Table 2, correlation between nitrate, phosphate and temperature*  
36 *was strong, and all this factors had significant correlation with nitrogen fixation rate.*

37 *However, it is not necessary that all these factors influenced nitrogen fixation directly. For*  
38 *examples, the negative correlation of nitrate (phosphate) with nitrogen fixation rate can be*  
39 *due to their negative correlations with temperature. Therefore, this issue is needed to be*  
40 *considered during discussion. Especially, both DIN concentration (ammonium should only*  
41 *contribute very small portion) and N:P ratio did not show significant negative correlation*  
42 *with nitrogen fixation rate.*

43

44 Since nitrogen fixation in the DIN-rich region has been highlighted in recent studies  
45 (L53-58), we discussed relationship between nitrate and nitrogen fixation (L342-372).  
46 Nitrogen fixation was negatively related with phosphate as with nitrate, and positively  
47 with temperature (Table 2). As the reviewer mentioned, correlation between nitrate,  
48 phosphate, and temperature was significant, and thus it is not necessary that all these  
49 factors influenced nitrogen fixation directly. Rather, one or more the factors that varied  
50 with nitrate could synergistically influence nitrogen fixation. We have added these  
51 statements in L376-381.

52

53 *P.18, L.308: It is suggested not to use one page to discuss the two rate measurement methods*  
54 *in the beginning of discussion, as this study was not focusing on this issue.*  
55 *Moreover, if gas dissolution method is better in this study, further discussion of these two*  
56 *methods is not helpful to the objective of this study.*

57

58 We agree. In the revised manuscript, the discussion of these two methods has been  
59 rewritten more briefly (L195-197).

60

61 *P.22, L388: Correlations of the qPCR result and nitrogen fixation rate and environmental*  
62 *factor are suggested to be analyzed. The authors tried to discuss numbers of different*  
63 *phylotypes with nitrogen fixation rate and environmental factors. Conducting environmental*  
64 *correlation analysis will support their points and provide clear picture. In order to estimate*  
65 *gene copies of different nifH phylotypes accurately, the authors can use qPCR of 16S rRNA to*  
66 *normalize the data of nifH.*

67

68 As we mentioned in L409-421 and L436-444 , *Trichodesmium* and UCYN-B would be  
69 delivered by the Tsugaru Warm Current. The delivered organisms do not necessarily adapt  
70 the environment in a place where they arrive. Therefore, the relationship between their  
71 abundance and environmental variables could not explain their distribution, but rather  
72 confuse the interpretation of data. Hence, we have not added the correlation matrix.

73 We used *nifH* qPCR analysis to examine seasonal variation in targeted diazotrophs.  
74 Normalization using 16SrRNA indicates the ratio of diazotrophs to bacteria, and thus is a  
75 bit far from our objectives.

76

77 *P.24, L.415: “disappearing during in spring” seems grammatically incorrect to me.*

78

79 We have corrected to “disappeared in spring”.

80

81 *p.26, L.452: as the author did not do qPCR of cluster III, it is inappropriate to imply that*  
82 *cluster III was increased in abundance with “suspension of sediment”. The abundance of*  
83 *cluster III might not be changing a lot throughout different seasons, and their higher relative*  
84 *abundance in cold condition may be simply due to repression of the cyanobacterial*  
85 *diazotrophs. Therefore, without qPCR data of cluster III, the author should not strengthen the*  
86 *importance of cluster III in cold conditions. Also, the nitrogen fixation of marine cluster III is*  
87 *still not well confirmed and this study was based on DNA works, so, it is not helpful and*  
88 *convince to mention too much about cluster III in discussion and conclusion. Besides that,*  
89 *relatively abundant cluster III was also reported in Arctic (Farnelid et al 2001).*

90

91 In the revised manuscript, we have deleted the related sentences that Cluster III  
92 diazotrophs were derived from resuspension of sediment. Further, we have not mentioned  
93 seasonal variation in abundance of Cluster III. We have just mentioned that Cluster III  
94 diazotrophs were recovered almost throughout a year and the Cluster III activity was likely  
95 suppressed in the water column because of the high oxygen concentration. (L445-455)

96

97 *S3,Phylogenetic tree: the Trichodesmium should not be clustering with cluster I*  
98 *proteobacteria. The tree was not stable, and the reason may be due to insufficient sequences*  
99 *of cyanobacteria. The author can consider adding more reference sequences of cyanobacteria,*  
100 *which should make the tree more stable.*

101

102 In figure S3 of the previous manuscript, cluster of cyanobacteria was separated from that  
103 of proteobacteria, and *Trichodesmium* was in the cluster of cyanobacteria. To avoid such  
104 misunderstanding, we have separated cyanobacterial sequences from the original  
105 phylogenetic tree of Cluster I (Fig. S3 and S4).

106

107

108

109 **Referee #3**

110

111 *The paper has been substantially improved by revision. Especially, I appreciated the addition*  
112 *by the authors of the qPCR analysis. However, on one point we continue to disagree. As all of*  
113 *the reviewers mentioned, the number of the recovered nifH sequence was not enough. The*  
114 *authors did not change this point. It is still difficult to discuss about the dominance or*  
115 *seasonal variation from the clone library analysis, even if the results was similar to those of*  
116 *qPCR.*

117

118 *I suggest that the authors discuss about the abundance and variation of four groups based on*  
119 *the results of qPCR. The result of clone library experiments shows only the existence of the*  
120 *other diazotroph (Leptolyngbya,  $\delta$ -Proteobacteria, and Cluster III) if the number of the*  
121 *sequences will not be changed.*

122

123 As the reviewer suggested, the result of clone library analysis have been shown only the  
124 existence of the other diazotroph in the revised manuscript. Please see the following  
125 individual responses.

126

127 *Line 315-317: I suggest adding the cruise KK-13-6 and referring to figure S5.*

128

129 Since this part is pointed out by the reviewer #2 not to fit with the study objectives, we  
130 have deleted it from the revised manuscript.

131

132 *Line 399-402: The result of clone library analysis cannot say “seasonal trend”.*

133

134 We have deleted this sentence and changed as follows. (L394-397)

135 “Therefore, the diazotrophs targeted by the qPCR analysis were likely important for  
136 nitrogen fixation in this study region. In the discussion below, we mainly discuss possible  
137 factors responsible for seasonal variation in the diazotrophs targeted by the qPCR  
138 analysis.”

139

140 *Line 452-453: The result of clone library analysis cannot say “dominant”.*

141

142 We have changed the sentence as follows. (L445-446)

143 “In nitrate-rich water during winter and spring, Cluster III diazotrophs were detected at

144 most of the stations.”

145

146 *Line 455-456: The number of the recovered sequence is not enough to say “major*  
147 *diazotroph”.*

148

149 We have not used the word “major diazotroph” in the revised manuscript. We have  
150 changed the sentence as follows. (L448)

151 “Therefore, Cluster III diazotrophs likely presented throughout a year.”

152

153 *Line 472-474: It is difficult to say that the organism was inactivated by the flushed out from*  
154 *the coastal region. There is no data which shows the organism condition (active or inactive)*  
155 *before the flushed out.*

156

157 We agree. We have changed the sentence as follows. (L461-462)

158 “Because nitrogen fixation was not detected during the KT-13-2\_Jan cruise, the organism  
159 was considered not to perform nitrogen fixation.”

160

161 *Fig. 7 the number of copies should be shown with log scale.*

162

163 We have changed it with log scale.

1 **Nitrogen fixation and the diazotroph community in the**  
2 **temperate coastal region of the northwestern North Pacific**

3  
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10  
11 **Abstract**

12 Nitrogen fixation in temperate oceans is a potentially important, but poorly understood  
13 process that may influence the marine nitrogen budget. This study determined seasonal  
14 variations in nitrogen fixation and the diazotroph community within the euphotic zone in the  
15 temperate coastal region of the northwestern North Pacific. Nitrogen fixation as high as  
16 13.6 nmol N L<sup>-1</sup> d<sup>-1</sup> was measured from early summer to fall when the surface temperature  
17 exceeded 14.2° C (but was lower than 24.3° C) and the surface nitrate concentration was low  
18 (≤0.30 μM), although we also detected nitrogen fixation in subsurface layers (42–62 m)

19 where nitrate concentrations were high ( $>1 \mu\text{M}$ ). Clone library analysis results indicated  
20 that *nifH* gene sequences were omnipresent throughout the investigation period. During the  
21 period when nitrogen fixation was detected (early summer to fall), the genes affiliated with  
22 UCYN-A, *Trichodesmium*, and  $\gamma$ -proteobacterial phylotype  $\gamma$ -24774A11 were frequently  
23 recovered. In contrast, when nitrogen fixation was undetectable (winter to spring), many  
24 sequences affiliated with Cluster III diazotrophs (putative anaerobic bacteria) were recovered.  
25 Quantitative PCR analysis revealed that UCYN-A was relatively abundant from early to late  
26 summer compared with *Trichodesmium* and  $\gamma$ -24774A11, whereas *Trichodesmium* abundance  
27 was the highest among the three groups during fall.

28

## 29 **1. Introduction**

30 The amount of bioavailable nitrogen introduced into the global ocean via nitrogen fixation is  
31 considered to be roughly balanced at the large spatiotemporal scale by nitrogen loss through  
32 denitrification, as indicated by the sedimentary nitrogen isotope record during the Holocene  
33 epoch (Brandes and Devol, 2002; Deutsch et al., 2004). However, rate measurement data  
34 have revealed that denitrification far exceeds nitrogen fixation (Codispoti, 2007). This  
35 discrepancy in the nitrogen balance has raised the possibility that the current estimate of  
36 marine nitrogen fixation, which is primarily based on data collected in tropical and

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

48 The temperate coastal ocean is one of the regions where nitrogen fixation rates have been  
49 understudied and potentially underestimated. Conventionally, nitrogen fixation in temperate  
50 oceans has been assumed to be low because of the relatively low temperatures (<~20°C),  
51 which generally inhibit the growth of large cyanobacterial diazotrophs (Breitbarth et al.,  
52 2007), and development of high dissolved inorganic nitrogen (DIN) concentrations (>1 µM).  
53 High DIN concentrations are generally regarded to inhibit nitrogen fixation (Falkowski,  
54 1983), especially during mixing periods. However, recent studies have indicated that



55 nitrogen fixation, presumably mediated by unicellular cyanobacteria and heterotrophic  
56 bacteria, is detectable even in the relatively cold ( $<10^{\circ}\text{C}$ ) and DIN-rich waters ( $>1\ \mu\text{M}$ ) of the  
57 Atlantic coast (Mulholland et al., 2012) and the Baltic Sea estuaries (Bentzon-Tilia et al.,  
58 2015). These results highlight the necessity of re-evaluating the extent, variation, and  
59 control mechanisms of nitrogen fixation in temperate oceans, with recognition of the  
60 widespread occurrence of diverse diazotrophic microbes.

61 This study examined the seasonal variation in nitrogen fixation in the temperate inside bays  
62 and open ocean located in the interfrontal zone of the northwestern North Pacific. In this  
63 region, physical, chemical, and biological properties vary widely between seasons (Shiozaki  
64 et al., 2014b) due to the confluence of three currents: the Kuroshio (warm current), the  
65 Tsugaru Warm Current, and Oyashio (cold current). Data on nitrogen fixation rates in the  
66 temperate Pacific are limited (Needoba et al., 2007), and to the best of our knowledge, the  
67 present study is the first to examine diazotrophy during all seasons in the temperate ocean.

68 This study was conducted as part of a project to monitor the dynamics of the coastal  
69 ecosystem and the recovery thereof after the 2011 Tohoku-oki tsunami, which struck the  
70 region on 11 March 2011.

71

## 72 **2. Materials and Methods**

73 The experiments were conducted during six cruises in the temperate coastal region of the  
74 western North Pacific. These cruises covered a full seasonal cycle, including spring  
75 (KS-14-2\_Mar, 14–19 March 2014), early summer (KK-13-1\_Jun, 24–29 June 2013),  
76 summer (KT-12-20\_Aug, 7–12 August 2012), late summer (KK-13-6\_Sep, 14–21 September  
77 2013), fall (KT-12-27\_Oct, 15–22 October 2012), and winter (KT-13-2\_Jan, 19–25 January  
78 2013). Sampling stations were located along the transect lines OT (39°20'N,  
79 141°56'–142°50'E) and ON (38°25'N, 141°29'–142°20'E). Eight stations were located  
80 offshore (OT4–6, ON4–8), while two stations were deployed in the Otsuchi (OT1) and  
81 Onagawa (ON1) bays (Fig. 1). Just before the KK-13-6\_Sep cruise, Typhoon Man-yi  
82 passed from southwest to northeast in the study area (Fig. S1).

83 Temperature, salinity, and dissolved oxygen profiles of regions near the bottom floor were  
84 measured using a SBE 911-plus conductivity-temperature-pressure (CTD) system (Sea-bird  
85 Electronics, Bellevue, WA, USA). Water samples were collected in an acid-cleaned bucket  
86 and Niskin-X bottles. At offshore stations, samples for nutrient analysis were collected  
87 from 7–15 different depths in the upper 200 m, while at shallower (<200 m) bay stations,  
88 samples were collected from 4–9 different depths in the entire water column, except at Stn.  
89 OT1 where only surface water samples were collected. Samples for DNA analysis and  
90 incubation experiments were collected from the surface at almost every station, and from

91 depths corresponding to 10% and 1% of the surface light intensities at Stns. OT4 and ON5.

92 Light attenuation was determined using a submersible PAR sensor.

### 93 **2.1. Nutrients**

94 Samples for nutrient analysis were stored in 10-mL polyethylene tubes and kept frozen until

95 onshore analyses. Nitrate, nitrite, ammonium, and phosphate concentrations were

96 determined using an AACSII auto-analyzer (Bran+Luebbe, Norderstedt, Germany). The

97 detection limits of nitrate, nitrite, ammonium, and phosphate ranged from 0.01–0.04  $\mu\text{M}$ ,

98 0.01–0.02  $\mu\text{M}$ , 0.01–0.03  $\mu\text{M}$ , and 0.01–0.02  $\mu\text{M}$ , respectively. The nitracline was defined

99 as the depth where nitrate concentrations increased above 1  $\mu\text{M}$ .

### 100 **2.2. Nitrogen fixation activity and mannitol enrichment experiment**

101 Nitrogen fixation was determined by the  $^{15}\text{N}_2$  gas bubble method (hereafter, the bubble

102 method; Montoya et al., 1996). Samples for incubation were collected in duplicate

103 acid-cleaned 2-L polycarbonate (PC) bottles. The time-zero samples (n=1) were

104 immediately filtered onto precombusted GF/F filters. Two milliliters of  $^{15}\text{N}_2$  gas [SI

105 Science Co. Japan, for this gas, contaminations of nitrate, nitrite, and ammonium were

106 determined to be low (< nM level), indicating that the overestimation of nitrogen fixation

107 rates due to the uptake of  $^{15}\text{N}$ -labeled contaminants (Dabundo et al. 2014) was minimal

108 (Shiozaki et al., unpublished data)] were injected directly into the incubation bottles through

109 a septum using a gastight syringe. The tracer-added samples were covered with  
110 neutral-density screens to adjust the light level and incubated for 24 h in an on-deck incubator  
111 filled with flowing surface seawater. After the incubation, the samples were filtered onto  
112 precombusted GF/F filters. The isotopic analyses were performed as described previously  
113 (Shiozaki et al., 2009). The rate of nitrogen fixation was calculated using the equations of  
114 Montoya et al. (1996).

115 To examine the possibility of underestimation of nitrogen fixation as determined by the  
116 bubble method (Mohr et al., 2010; Großkopf et al., 2012), we compared the nitrogen fixation  
117 rates determined using the  $^{15}\text{N}_2$  gas dissolution method (hereafter, the dissolution method;  
118 Mohr et al., 2010) with those determined using the bubble method (see above) during the  
119 KK-13-6\_Sep and KS-14-2\_Mar cruises. For the dissolution method,  $^{15}\text{N}_2$ -enriched  
120 seawater was prepared according to Mohr et al. (2010) and Großkopf et al. (2012). Briefly,  
121 filtered seawater was degassed using a Sterapore membrane unit (20M1500A: Mitsubishi  
122 Rayon Co., Ltd., Tokyo, Japan) at a flow rate of  $\sim 500 \text{ mL min}^{-1}$  (recirculation period, 10 min).

123 Degassed seawater was stored in 1-L Tedlar bags without headspaces and ~~10 mL~~  $^{15}\text{N}_2$  gas was  
124 added at a ratio of 10 ml  $^{15}\text{N}_2$  per 1L seawater. After complete dissolution, the  
125  $^{15}\text{N}_2$ -enriched seawater was added to seawater samples contained in 2-L PC bottles, which  
126 were incubated and used for isotopic analyses as described above. The  $^{15}\text{N}_2$ -enriched

127 seawater was prepared at each station, and was added to the incubation bottles within 1 h  
128 after preparation. The nitrogen fixation rate was calculated according to Mohr et al. (2010).  
129 For this comparison, triplicate samples were used for both the dissolution and bubble  
130 methods.

131 To examine if sugar addition affected nitrogen fixation rates (Bonnet et al., 2013; Rahav et al.,  
132 2013; Moisander et al., 2011), we determined nitrogen fixation rates (the  $^{15}\text{N}_2$  gas bubble  
133 method, see above) for surface seawater samples (stations ON4 and OT6 during the  
134 KS-14-2\_Mar cruise) with and without addition of mannitol (final conc.  $0.8\ \mu\text{M}$ ) ( $n=3$ ).

### 135 **2.3. Statistical analysis**

136 Pearson's correlation coefficient was used to examine the relationships between nitrogen  
137 fixation activities and environmental variables including temperature, nitrate, ammonium,  
138 phosphate, and the ratio of nitrate+nitrite+ammonium to phosphate (N/P ratio) in the entire  
139 water column (the data used for the calculation were shown in Table S1). When the nutrient  
140 concentration was below the detection limit, the value of the detection limit was used for the  
141 analysis. When nitrogen fixation was not detected, the value was assumed to be zero.

### 142 **2.3.2.4. DNA analysis**

#### 143 2.3.1.2.4.1. \_\_\_\_\_ DNA extraction, sequencing, and phylogenetic analysis

144 Samples (0.38–1 L) for DNA analysis were filtered through 0.2- $\mu\text{m}$ -pore-sized Nuclepore

145 filters and stored in a deep freezer (-80° C) until onshore analysis. Total DNA was extracted  
146 using a ChargeSwitch Forensic DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) with  
147 slight modification of the manufacturer's protocol (Shiozaki et al., 2014a). Partial *nifH*  
148 fragments were amplified using a nested PCR strategy (Zehr and Turner, 2001) from samples  
149 collected from surface water at Stns. OT4, ON1, ON5, and ON7 during the KT-12-20\_Aug  
150 and KT-12-27\_Oct cruises, at Stns. OT4, ON1, and ON5 during the KT-13-2\_Jan and  
151 KS-14-2\_Mar cruises, at Stns. OT4, ON1, ON5, and ON8 during the KK-13-1\_Jun cruise,  
152 and at Stns OT4, ON5, ON7 during the KK-13-6\_Sep cruise (Table 1). PCR reagents were  
153 applied as described by Shiozaki et al. (2014a). The first and second PCRs were run using  
154 the same cycling conditions: 95° C for 30 s followed by 30 cycles of 98° C for 10 s, 52° C for  
155 30 s, and 72° C for 30 s; followed by a final extension at 72° C for 7 min. Sterile distilled  
156 water was used as the negative control. After PCR analysis, we confirmed there was no  
157 band in agarose gel of electrophoresis from the negative control. The PCR products were  
158 cloned and sequenced according to Shiozaki et al. (2014a). The present study obtained 197  
159 *nifH* sequences in total. The *nifH* sequences were translated into amino acid sequences and  
160 searched against the protein database of the National Center for Biotechnology Information  
161 using the BLASTp algorithm. Clones with 100% amino acid sequence similarity were  
162 defined as the same operational taxonomic unit (OTU) using the CD-HIT suite (Huang et al.,

163 2010). The amino acid sequences were aligned using multiple sequence comparisons by the  
164 log-expectation (MUSCLE) module in the MEGA5 package (Tamura et al., 2011). A  
165 phylogenetic tree was constructed using the maximum likelihood method employing the  
166 Dayhoff matrix-based mode, and 1,000 bootstrap replicates were run. The obtained  
167 sequences were assigned to bacterial groups based on known sequences included in a cluster  
168 within the phylogenetic tree (Zehr et al., 2003a). The sequences from this study were  
169 deposited in the DNA Data Bank of Japan (DDBJ) as accession numbers LC013480 to  
170 LC013676.

171 | 2.3.2.2.4.2. Quantitative PCR (qPCR) analysis

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

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

188

### 189 **3. RESULTS**

#### 190 **3.1. Comparison of the bubble method and the dissolution method**

191 Nitrogen fixation rates determined by the bubble and dissolution methods were compared  
192 during the KK-13-6\_Sep and KS-14-2\_Mar cruises (Fig. 2). Both methods failed to detect  
193 nitrogen fixation in samples collected during the KS-14-2 cruise. During the KK-13-6\_Sep  
194 cruise, the nitrogen fixation rates determined by the dissolution method were significantly  
195 higher (1.5–2.2 fold) than those determined by the bubble method at Stns. OT6 and ON5 ( $p$   
196  $<0.05$ ). At Stns. OT4 and ON7, the nitrogen fixation rates determined by the two methods  
197 did not differ significantly. The following nitrogen fixation results were obtained by the  
198 bubble method; we sought to standardize values among all cruises and to compare them with



199 previous results. Hence, the levels could be underestimates.

### 200 **3.2. Seasonal variations in nitrogen fixation rates**

201 According to the temperature-salinity (TS) diagram proposed by Hanawa and Mitsudera  
202 (1987), both the offshore and bay waters collected during this investigation primarily  
203 belonged to either the surface layer water system (SW) or the Tsugaru Warm Current water  
204 system (TW) (Fig. 3), with the exception of waters collected from the 1% light depth (119 m)  
205 at Stn. ON5 during the KT-13-2\_Jan cruise and those collected at the surface of OT5 during  
206 the KS-14-2\_Mar cruise, which were classified as belonging to the Oyashio water system  
207 (OW) and the Coastal Oyashio water system (CO), respectively. These water classifications  
208 based on the TS diagram were generally consistent with the geostrophic current field of the  
209 investigated region (Fig.S1). Based on these results, it was considered that surface waters  
210 collected during the same cruise in a particular season generally belonged to the same water  
211 system that was prevalent in the investigated region at the time of our sampling.

212 Sea surface temperatures (SST) (range, 1.5 to 24.3° C) (Figs. 4a and S1) and surface nitrate  
213 and phosphate concentrations determined during each cruise were averaged to emphasize the  
214 seasonal variability of these parameters (Fig. 4b). In general, surface nitrate and phosphate  
215 concentrations were low ( $\leq 0.07 \mu\text{M}$  and  $\leq 0.20 \mu\text{M}$ , respectively) in the warmer seawaters  
216 (14.2–24.3° C) sampled in early summer (KK-13-1\_Jun), summer (KT-12-20\_Aug), and fall

217 (KT-12-27\_Oct), whereas they were relatively high ( $\geq 0.75 \mu\text{M}$  and  $\geq 0.28 \mu\text{M}$ , respectively)  
218 in the colder seawaters ( $1.5\text{--}9.8^\circ \text{C}$ ) sampled during winter (KT-13-2\_Jan), and spring  
219 (KS-14-2\_Mar). During the KK-13-6\_Sep cruise (late summer), the nitrate concentrations  
220 were relatively high and variable (mean  $\pm$  SD;  $2.92 \pm 7.90 \mu\text{M}$ ). This was because the  
221 highest nitrate concentration ( $22.6 \mu\text{M}$ ) was determined at the near-shore Stn. OT1 (Fig. S2).  
222 Similar to nitrate, surface phosphate concentrations tended to be high during winter  
223 (KT-13-2\_Jan) and spring (KS-14-2\_Mar), while they were low during the warmer seasons.  
224 The seasonal variation pattern of the average ammonium concentration at the surface differed  
225 from those of nitrate and phosphate concentrations (Fig. 4b), characterized by low  
226 concentrations ( $\leq \sim 1 \mu\text{M}$ ) throughout the year. The high variation in surface ammonium  
227 concentration during the KK-13-6\_Sep cruises were due to relatively high ammonium  
228 concentrations at Stn. OT1 ( $1.41 \mu\text{M}$ ) (Fig. S2).  
229 Nitrogen fixation was detected in the surface waters of most samples collected during the  
230 four cruises conducted in early summer (KK-13-1\_Jun), summer (KT-12-20\_Aug), late  
231 summer (KK-13-6\_Sep), and fall (KT-12-27\_Oct), and varied in the range of  $0.33\text{--}13.6 \text{ nmol}$   
232  $\text{N L}^{-1} \text{ d}^{-1}$  (Figs. 4c and S2). Relatively high nitrogen fixation rates were determined for  
233 samples collected during the KT-12-20\_Aug cruise, although the highest value was obtained  
234 at Stn. ON7 during the KK-13-6\_Sep cruise. Nitrogen fixation was not detected in seawater

235 samples collected during the winter and spring cruises, even after addition of mannitol  
236 (KS-14-2\_Mar). Furthermore, nitrogen fixation was not detected in DIN-replete water at  
237 Stn. OT1 in late summer (KK-13-6\_Sep).

238 The rates of nitrogen fixation in samples collected at different depths (0–119 m) were  
239 examined at Stns. OT4 and ON5 (Fig. 5). Nitrogen fixation was detectable only during the  
240 four cruises conducted in early summer (KK-13-1\_Jun), summer (KT-12-20\_Aug), late  
241 summer (KK-13-6\_Sep), and fall (KT-12-27\_Oct), the same seasons during which surface  
242 nitrogen fixation was observed (Fig. 4). Nitrogen fixation rates tended to be higher at the  
243 surface than in the deeper layers during summer (KT-12-20\_Aug) and late summer  
244 (KK-13-6\_Sep (at Stn. OT4)), whereas this vertical trend was less evident during fall  
245 (KT-12-27\_Oct) and early summer (KK-13-1\_Jun). At Stn. OT4, nitrogen fixation was  
246 detectable even in deeper layers below the nitracline, where nitrate concentrations were  
247 relatively high (KT-12-27\_Oct, depth = 62 m; KK-13-1\_Jun, depth = 42 m). In this layer,  
248 the ammonium concentrations were 0.05  $\mu\text{M}$  (KT-12-27\_Oct) and 0.62  $\mu\text{M}$  (KK-13-1\_Jun).  
249 The nitrogen fixation rate below the nitracline ( $1.56 \text{ nmol N L}^{-1} \text{ d}^{-1}$ ) was higher than that at  
250 the surface ( $0.87 \text{ nmol N L}^{-1} \text{ d}^{-1}$ ) during the KK-13-1\_Jun cruise. The maximum  
251 depth-integrated nitrogen fixation ( $294 \text{ } \mu\text{mol N m}^{-2} \text{ d}^{-1}$ ) was observed at Stn. OT4 during  
252 summer (KT-12-20\_Aug).

253 **3.3. Relationship between nitrogen fixation rates and environmental**  
254 **variables**

255 Nitrogen fixation rates tended to increase with temperature ( $p < 0.01$ ) (Fig. 6a and Table 2).

256 Nitrogen fixation was detected only when seawater temperatures exceeded 11.7° C, with

257 higher rates ( $>6 \text{ nmol N L}^{-1} \text{ d}^{-1}$ ) noted in waters warmer than 19.5° C. However, there were

258 exceptions to this general relationship between the nitrogen fixation rate and temperature.

259 For example, from the data collected during the KK-13-1\_Jun cruise the nitrogen fixation

260 rate was highest at 15.4° C, while it was low (undetectable) at higher temperatures.

261 Nitrogen fixation rates were negatively correlated with nitrate and phosphate concentrations

262 ( $p < 0.01$ ) (Table 2). There was no significant correlation between nitrogen fixation rates

263 and ammonium concentration ( $p > 0.05$ ). We also found no significant correlation between

264 nitrogen fixation rates and the ratio of total inorganic nitrogen (nitrate + nitrite + ammonium)

265 to phosphate (Table 2). A plot of the nitrogen fixation against nitrate concentrations

266 indicated that nitrogen fixation was generally detectable only when nitrate was depleted (Fig.

267 6b), except that relatively high nitrogen fixation rates were determined in the subsurface layer

268 of Stn. OT4 (KT-12-27\_Oct and KK-13-1\_Jun). Active nitrogen fixation tended to occur at

269 low ammonium concentration  $\leq \sim 0.1 \text{ }\mu\text{M}$ . However, seasonal variation in ammonium

270 concentration was small and no statistically significant relationship with nitrogen fixation

271 was observed (Fig. 6c).

272

### 273 **3.4. Seasonal variation in the diazotroph community**

#### 274 3.4.1. Diazotroph community

275 PCR reagents have been suggested to be a potential source of *nifH* genes during analysis of  
276 the diazotroph community (Zehr et al., 2003b). Although we confirmed the absence of any  
277 bands from the negative control in agarose gel electrophoresis, sequences with similarity  
278 (>97%) at the amino acid level to contaminants in PCR reagents were recovered from  
279 samples obtained during the KK-13-6\_Sep and KS-14-2\_Mar cruises (10 clones in total).  
280 We did not include these sequences in our data analysis.

281 The *nifH* gene was recovered from all samples that we collected during this study across  
282 different stations and seasons (Table 1). Sixty-one OTUs were grouped from 187 *nifH*  
283 clones, based on 100% amino acid sequence similarity. The OTUs were assigned to  
284 cyanobacteria,  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -proteobacteria, and Cluster III diazotrophs (Zehr et al., 2003a)  
285 (Figs. S3, ~~and~~ S4, and S5).

286 The detected cyanobacterial sequences belonged to *Trichodesmium*, UCYN-A, and  
287 *Leptolyngbya*. The *nifH* sequences of UCYN-B, UCYN-C, and *Richelia intracellularis*  
288 were not recovered. The *nifH* sequence of *Trichodesmium* was recovered only during the

289 KT-12-27\_Oct cruise (Table 1). UCYN-A was generally observed from early summer to  
290 fall, while *nifH* of *Leptolyngbya* was detected during winter. During the KS-14-2\_Mar  
291 cruise, all recovered sequences were derived from heterotrophic bacteria, and were  
292 dominated by Cluster III diazotrophs at Stns. OT4 and ON5. The Cluster III diazotroph  
293 *nifH* sequences were recovered on all cruises except the KK-13-1\_Jun cruise. Note that 58  
294 out of 187 sequences displayed >97% similarity, at the amino acid level, to terrestrial  
295 diazotroph sequences derived from soil, mudflats, and lakes (Fig. S3, ~~and S4,~~ and S5).  
296 These sequences were mainly affiliated with  $\alpha$ - and  $\delta$ -proteobacterial diazotrophs, with 29 of  
297 39  $\alpha$ -proteobacterial sequences and 22 of 24  $\delta$ -proteobacterial sequences being similar to  
298 terrestrial diazotroph sequences.

### 299 3.4.2. Diazotrophs abundances

300 The *nifH* sequence of *Trichodesmium* was detected by qPCR assay during the KT-12-27\_Oct  
301 and KK-13-6\_Sep cruises (Fig. 7 and 8). During these two cruises, the abundance of  
302 *Trichodesmium* ranged from below the detection limit to  $8.7 \times 10^4$  copies L<sup>-1</sup> at all depths.  
303 *Trichodesmium* abundance at the surface was higher than those of UCYN-A, UCYN-B, and  
304  $\gamma$ -24774A11 at most stations during the KT-12-27\_Oct cruise (Fig. 7 and ~~S5~~S6). UCYN-A  
305 was detected on all cruises except for the KS-14-2\_Mar cruise (Fig. 7 and 8). The  
306 maximum abundance of UCYN-A generally occurred at the surface except at Stn. OT4

307 during the KK-13-6\_Sep cruise where the peak ( $1.2 \times 10^3$  copies L<sup>-1</sup>) was observed at 72 m  
308 (Fig. 8). The abundance of UCYN-A varied from below the detection limit to  $2.6 \times 10^5$   
309 copies L<sup>-1</sup> at all depths. At the surface, UCYN-A was the most abundant among the four  
310 groups at most of the stations investigated during the KT-12-20\_Aug, KT-13-2\_Jan,  
311 KK-13-1\_Jun, and KK-13-6\_Sep cruises (Fig. 7 and ~~S5S6~~). UCYN-B was detected only at  
312 Stn. ON7 during the KK-13-6\_Sep cruise (Fig. 7, 8, and ~~S5S6~~).  $\gamma$ -24774A11 was detected  
313 during all cruises except for the KS-14-2\_Mar cruise (Fig. 7 and 8). The abundance of  
314  $\gamma$ -24774A11 ranged from below the detection limit to  $1.8 \times 10^4$  copies L<sup>-1</sup>, with a tendency of  
315 a subsurface peak at both stations (Fig. 8).

316

## 317 4. DISCUSSION

### 318 ~~4.1. Differences in nitrogen fixation rates between the bubble method and~~ 319 ~~the dissolution method~~

320 ~~The present study revealed that a significant difference between the bubble and the~~  
321 ~~dissolution methods was not always present. Großkopf et al. (2012) indicated that the~~  
322 ~~difference was smaller when *Trichodesmium* dominated in the diazotroph community than~~  
323 ~~when unicellular cyanobacteria and  $\gamma$  proteobacteria dominated presumably because~~  
324 ~~*Trichodesmium* can float to the top of the bottle and directly use the added <sup>15</sup>N<sub>2</sub> in the bubble~~

325 ~~method. Interestingly, *Trichodesmium* abundance was higher than or similar to those of~~  
326 ~~UCYN-A, UCYN-B, and  $\gamma$ -24774A11 at Stns. OT4 and ON7, at which there was no~~  
327 ~~significant difference detected between the two methods. On the other hand,~~  
328 ~~*Trichodesmium* was not detected and UCYN-A was the most abundant among the four groups~~  
329 ~~at Stns. OT6 and ON5, at which nitrogen fixation determined by the dissolution method was~~  
330 ~~significantly higher than that by the bubble method. These results were consistent with the~~  
331 ~~report by Großkopf et al. (2012). The larger variations in nitrogen fixation at Stns. OT4 and~~  
332 ~~ON7 than at Stns. OT6 and ON5 were probably due to the heterogeneity of *Trichodesmium*~~  
333 ~~abundance (Carpenter et al., 2004). Although nitrogen fixation rates determined by the~~  
334 ~~bubble method in the present study were underestimated on all cruises, the level of~~  
335 ~~underestimation was relatively small during the KT-12-27\_Oct cruise when *Trichodesmium*~~  
336 ~~was dominant at most of the stations.~~

#### 337 **4.2.4.1. Seasonal variations in nitrogen fixation rates in the temperate coastal**

##### 338 **ocean**

339 Nitrogen fixation rates were measurable mainly from early summer to fall when nitrate was  
340 generally depleted in sample seawaters, although there were some exceptions. Our  
341 estimates of the nitrogen fixation rates (0.33–13.6 nmol N  $\mu\text{L}^{-1}$  d<sup>-1</sup>) were significantly ( $p$   
342 <0.05) higher than the corresponding values previously reported in the temperate region of



343 the eastern North Pacific (0.15–0.31 nmol N  $\mu\text{L}^{-1} \text{d}^{-1}$ ; Needoba et al., 2007) and the  
344 oligotrophic region of the western and central North Pacific (0.17–3.62 nmol N  $\mu\text{L}^{-1} \text{d}^{-1}$ ;  
345 Shiozaki et al., 2010), whereas they were comparable to those determined in the Kuroshio  
346 (0.54–28 nmol N  $\mu\text{L}^{-1} \text{d}^{-1}$ ; Shiozaki et al., 2010) and the western Atlantic coastal regions  
347 (1.3–49.8 nmol N  $\mu\text{L}^{-1} \text{d}^{-1}$ ; Mulholland et al., 2012). Higher nitrogen fixation rates have  
348 been determined in other temperate oceans, including the western English Channel  
349 ( $18.9 \pm 0.01$  and  $20.0$  nmol N  $\mu\text{L}^{-1} \text{d}^{-1}$ ; Rees et al., 2009) and the Baltic Sea estuaries (47–83  
350 nmol N  $\mu\text{L}^{-1} \text{d}^{-1}$ ; Bentzon-Tilia et al., 2015).

351 In our study, spatiotemporal variability in nitrogen fixation rates appeared to be partly related  
352 to the Tsugaru Warm Current path. This current, which flows from the north (after passage  
353 through the Tsugaru Strait) to the study region (Fig. S1), may carry active diazotrophs and  
354 therefore enhance nitrogen fixation in our study region. This is supported by the fact that  
355 nitrogen fixation rates during individual cruises tended to be higher at Stn. OT4 than at Stn.  
356 ON5. These stations were located up- and down-stream of the Tsugaru Warm Current,  
357 respectively. In addition, variations in nitrogen fixation rates among stations and seasons  
358 might also be related to the extent of vertical mixing in the Tsugaru Warm Current. It has  
359 been suggested that vertical mixing may introduce iron-rich subsurface water to the surface  
360 of the Tsugaru Strait (Saitoh et al., 2008). Such input of iron may enhance nitrogen fixation

361 rates. Consistent with this notion, our results showed that the nitrogen fixation rate was  
362 relatively high at Stn. OT4, where the nitracline was relatively deep.

363 Blais et al. (2012) proposed that nitrogen fixation can proceed even in nutrient-replete waters,  
364 if large amounts of iron and organic materials are available for consumption by bacterial  
365 diazotrophs. In the present study, this possibility was examined by conducting mannitol  
366 addition experiments using surface seawaters collected during spring. These waters,  
367 belonging to the Oyashio Current system (Nishioka et al., 2007, 2011; Shiozaki et al., 2014b),  
368 were considered to be rich in iron during spring, as indicated by a previous study (iron conc.,  
369 0.79–8.46 nM; Nishioka et al. 2007). Despite potentially high iron concentrations, our  
370 results showed that nitrogen fixation was undetectable even after the mannitol addition,  
371 suggesting that, contrary to the Blais et al. proposition, diazotrophs remained inactive under  
372 our experimental settings.

373 Our data showed that nitrogen fixation rates were below the detection limit during winter,  
374 spring, and late summer (KK-13-6\_Sep), when nitrate concentrations were high. These  
375 results were consistent with the results of previous studies in the Pacific Ocean, which  
376 indicated that nitrogen fixation rates were low or undetectable in DIN-replete waters  
377 (Shiozaki et al., 2010). In contrast, Mulholland et al. (2012) reported that, in temperate  
378 regions of the Atlantic Ocean, nitrogen fixation rates were high even in DIN-replete ( $>1 \mu\text{M}$ )

379 and cold (<10°C) surface seawaters. Their study was conducted downstream of the Gulf  
380 Stream, where diazotrophs could be delivered from subtropical oceans where DIN is depleted.  
381 Previous studies have suggested that cyanobacterial diazotrophs can travel over long  
382 distances (>1,000 km) in currents, without losing their capacity for N<sub>2</sub> fixation (Shiozaki et  
383 al., 2013), and that activity is not lost immediately even after mixing with DIN-replete  
384 seawaters (Holl and Montoya, 2005; Dekaezemacker and Bonnet, 2011). In our region,  
385 because the Tsugaru Warm Current flows from north to south, diazotrophs entrained by the  
386 current have little chance of meeting high-DIN water at the surface. DIN-replete water  
387 during summer was observed at the inside bay station OT1 (Fig. S2). Concomitantly,  
388 | low-salinity surface waters spread offshore along the OT transect line (Fig. [S6S7](#)), suggesting  
389 that anomalously high DIN concentrations were likely attributable to terrestrial surface  
390 discharge enhanced by Typhoon Man-yi, which passed over the region immediately before  
391 the cruise. Subramaniam et al. (2008) reported that nitrogen fixation rates near the Amazon  
392 River estuary, with low salinity and high nitrate levels, were fairly low. Their results are  
393 consistent with ours. Ammonium inhibits nitrogen fixation, especially when ammonium  
394 concentrations exceed 1 μM, as demonstrated by *Trichodesmium* (Mulholland et al. 2001).  
395 In our study, no negative relationship between nitrogen fixation and ammonium  
396 concentration was found. This can likely be explained by relatively low ammonium

397 concentrations ( $\leq \sim 1 \mu\text{M}$ ) throughout the year and across the investigated region. Nitrogen  
398 fixation was also negatively correlated with phosphate as with nitrate, and positively  
399 correlated with temperature (Table 2). Since correlation between nitrate, phosphate, and  
400 temperature was significant, all these factors would not necessarily influence nitrogen  
401 fixation directly. Rather, one or more the factors that varied with nitrate could  
402 synergistically influence nitrogen fixation.

403

#### 404 **4.3.4.2. Seasonal variation in the diazotroph community in the temperate** 405 **coastal ocean**

406 The qPCR analysis demonstrated that the target groups were quantifiable even at stations at  
407 which their sequences were not recovered by the clone library analysis, suggesting that the  
408 number of clones was not sufficient to capture the diazotroph community structure on each  
409 cruise. Despite this limitation, the sequences more frequently recovered in the clone library  
410 generally corresponded to the most abundant group revealed by the qPCR analysis. For  
411 example, UCYN-A was frequently recovered in the library during the KT-12-20\_Aug,  
412 KK-13-1\_Jun, and KK-13-6\_Sep cruises; for these samples, the qPCR results showed that  
413 UCYN-A was the most abundant group among the four examined. Similarly, qPCR data  
414 indicated that *Trichodesmium* was the most abundant group during fall, when this group was

415 frequently recovered in the library (during the KT-12-27\_Oct cruise). Therefore, the  
416 diazotrophs targeted by the qPCR analysis were likely important for nitrogen fixation in this  
417 study region. ~~This consistency in the general results obtained by the clone library and qPCR~~  
418 ~~suggests that both of these approaches captured a similar seasonal trend in community~~  
419 ~~composition changes for at least the major diazotroph groups.~~ In the discussion below, we  
420 mainly discuss possible factors responsible for seasonal variation in the diazotrophs targeted  
421 by the qPCR analysis. ~~community by focusing on the major diazotroph groups.~~

422 UCYN-A was detected by qPCR in all seasons except spring (KS-14-2\_Mar), suggesting that  
423 this group of diazotrophs could be important agents of nitrogen fixation in this region.  
424 Especially from early to late summer, the abundance of UCYN-A was generally higher than  
425 that of *Trichodesmium*, UCYN-B, and  $\gamma$ -24774A11. UCYN-A has been widely detected in  
426 temperate regions, and is considered to be one of the major diazotrophs of these locations  
427 (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012; Bentzon-Tilia et al., 2015).  
428 UCYN-A is known to be most abundant in relatively warm waters around  $\sim 20^\circ$  C (Needoba  
429 et al., 2007; Moisander et al., 2010). UCYN-A was detected by qPCR even during winter at  
430 some stations, yet, was not observed during spring. This could be because UCYN-A  
431 abundance decreased from fall to winter with decreasing temperatures, eventually  
432 disappearing-disappeared during in spring.

433 *Trichodesmium* was detected from late summer to fall by qPCR analysis, when water  
434 temperatures ranged from 19.1 to 23.4° C at the surface. Given that the optimal growth  
435 temperature for *Trichodesmium* has been reported to be high (24–30° C) (Breitbarth et al.,  
436 2007), *Trichodesmium* detected in the investigated region likely existed under suboptimum  
437 conditions. The relatively high abundance of *Trichodesmium* observed during fall, despite  
438 the suboptimal temperature conditions, might indicate that *Trichodesmium* was transported  
439 from the adjacent subtropical region where seawater temperatures were high (>24° C). In  
440 the western North Pacific subtropical region, *Trichodesmium* is abundant from July to  
441 September (Marumo and Nagasawa, 1976; Chen et al., 2008). *Trichodesmium* that  
442 flourished in the subtropical region during summer could be transported by the Tsugaru  
443 Warm Current, displaying peak abundance during fall in the investigated region. This could  
444 support the above discussion that waters containing active nitrogen fixation were delivered to  
445 this region by the Tsugaru Warm Current.

446 We observed  $\gamma$ -24774A11 by qPCR analysis during all cruises except for the KS-14-2\_Mar  
447 cruise. This phylotype has not been reported previously in other temperate oceans  
448 (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012). The *nifH* sequence of  
449  $\gamma$ -24774A11 was similar to that of *Pseudomonas stutzeri* (94% similarity at the amino acid  
450 level), which was observed in waters including temperate regions (Bentzon-Tilia et al., 2015).

451 Bentzon-Tilia et al. (2015) reported that *P. stutzeri*-like *nifH* genes (99% similarity at the  
452 nucleotide level) were the most abundant sequences among their samples collected from the  
453 temperate Baltic Sea estuary. In the present study, we recovered *P. stutzeri*-like *nifH* genes  
454 (>97% similarity at the amino acid level) from Stn. OT4 during the KT-13-2\_Jan cruise by  
455 the clone library analysis. However,  $\gamma$ -24774A11 was not detected on that occasion by  
456 qPCR analysis, suggesting that  $\gamma$ -24774A11 was not quantified as *P. stutzeri* and that *P.*  
457 *stutzeri* ~~was~~could not be a major diazotroph in this study region. The ecology of  
458  $\gamma$ -24774A11 is still fairly unknown. It remains to be seen, in future studies whether this  
459 phylotype contributes to the nitrogen fixation in this region.

460 UCYN-B was not detected by qPCR except at one station. This result is consistent with  
461 previous knowledge. UCYN-B becomes abundant with increasing temperature, similar to  
462 *Trichodesmium* (Moisander et al., 2010), and is rarely observed in the temperate region  
463 (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012; Bentzon-Tilia et al., 2015).  
464 Furthermore, UCYN-B abundance is low in shallow nitracline regions (Shiozaki et al.,  
465 2014a,c). The nitracline depth in this region ( $\leq 60$  m) was shallower than that of >100-m  
466 depths of regions where UCYN-B is abundant (Shiozaki et al., 2014a). Therefore, although  
467 UCYN-B might also have been delivered from subtropical region, it could not survive in the  
468 shallower nitracline region.

469 In nitrate-rich water during winter and spring, Cluster III diazotrophs were ~~dominant~~ detected  
470 at most of the stations. Furthermore, from early summer to fall, *nifH* sequences of Cluster  
471 III diazotrophs were recovered by the clone library analysis in samples from all cruises  
472 (except KK-13-1\_Jan). Therefore, Cluster III diazotrophs likely presented throughout a year.  
473 ~~Because UCYN-A, Trichodesmium, and γ-24774A11 were scarce during winter and spring,~~  
474 ~~Cluster III diazotrophs were likely to be major diazotrophs at these times.~~

475 Cluster III diazotrophs are putative anaerobes (Hamersley et al., 2011; Farnelid et al., 2013;  
476 Bentzon-Tilia et al., 2014), and hence, they are usually dominant in the diazotrophic  
477 community of oxygen-depleted waters (Hamersley et al., 2011; Farnelid et al., 2013) or  
478 marine sediments (Bertics et al., 2013). In this study, dissolved oxygen was not depleted  
479 ( $>3.16 \text{ ml L}^{-1}$ ) in the upper winter maximum mixed layer depth in this region (~200 m;  
480 Shiozaki et al., 2014b) (Fig. [S7S8](#)). Therefore, ~~it is possible that the Cluster III diazotrophs~~  
481 ~~that we detected in the surface layer were derived from resuspensions of coastal marine~~  
482 ~~sediments in which anoxic conditions may prevail because of organic matter decomposition.~~  
483 ~~The~~ Cluster III activity was likely strongly suppressed in the water column because of the  
484 high oxygen concentration.

485 Many *nifH* sequences recovered by the clone library analysis were similar to terrestrially  
486 derived sequences. These results agree with previous data collected in coastal regions,



487 where terrestrially derived *nifH* sequences were also found (Rees et al., 2009; Mulholland et  
488 al., 2012; Blais et al., 2012). We obtained a *Leptolyngbya*-like *nifH* gene during the  
489 KT-13-2\_Jan cruise. The organism has been found on beaches or coastal land areas (Brito  
490 et al. 2012), but not in the open ocean. Because nitrogen fixation was not detected during  
491 the KT-13-2\_Jan cruise, the organism was considered not to perform nitrogen fixation~~must~~  
492 have been inactivated after being flushed out from the coastal region.

493

## 494 5. CONCLUSION

495 This study demonstrated that nitrogen fixation can and does proceed at high rates, depending  
496 on the season, in the temperate region of the northwestern North Pacific, although we failed  
497 to detect nitrogen fixation in DIN-replete cold waters. *nifH* sequences were omnipresent  
498 and recovered throughout the year, displaying a marked seasonality in their composition.  
499 UCYN-A was a major diazotroph during summer, whereas *Trichodesmium* was abundant  
500 during fall, despite low temperatures. It has been suggested that *Trichodesmium* was  
501 laterally transported from the adjacent subtropical region, which displays high temperatures.

502 Although tThe Cluster III diazotrophs were recovered almost throughout a year, they were  
503 considered to be inactivated in oxic water columns.  
504 ~~abundant in surface waters during winter, which was ascribed to their delivery from the~~

505 ~~anoxic sediments via bottom resuspension. The failure to detect nitrogen fixation when~~  
506 ~~Cluster III was abundant implied that the activity of this diazotroph group was strongly~~  
507 ~~suppressed in oxic water columns.~~

508

## 509 **Author Contributions**

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

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681

682 Table 1. Summary of recovered *nifH* sequences belonging to *Trichodesmium* (Tri), UCYN-A  
683 (UA), *Leptolyngbya* (Lep),  $\alpha$ -proteobacteria ( $\alpha$ -Pro),  $\beta$ -proteobacteria ( $\beta$ -Pro),  
684  $\gamma$ -proteobacteria ( $\gamma$ -Pro),  $\delta$ -proteobacteria ( $\delta$ -Pro), and Cluster III (CIII)

Cruise	Station	No. of clones	Cyanobacteria			$\alpha$ -Pro	$\beta$ -Pro	$\gamma$ -Pro	$\delta$ -Pro	CIII
			Tri	UA	Lep					
KT-12-20 <u>Aug</u> <u>summer</u>	OT4	12		9		3				
	ON1	5		2					3	
	ON5	8		8						
	ON7	7		1		6				
<b>Total</b>		<b>32</b>	<b>0</b>	<b>20</b>	<b>0</b>	<b>9</b>	<b>0</b>	<b>0</b>	<b>3</b>	
KT-12-27 <u>Oct</u> <u>fall</u>	OT4	7	1						6	
	ON1	9						4(2)	5(5)	
	ON5	6					1		5	
	ON7	13	6	1		5(5)		1(1)		
<b>Total</b>		<b>35</b>	<b>7</b>	<b>1</b>	<b>0</b>	<b>5(5)</b>	<b>0</b>	<b>2(1)</b>	<b>4(2)</b>	
KT-13-2 <u>Jan</u> <u>winter</u>	OT4	11			10		1			
	ON1	1							1	
	ON5	14				5(5)		2(2)	7	
<b>Total</b>		<b>26</b>	<b>0</b>	<b>0</b>	<b>10</b>	<b>5(5)</b>	<b>0</b>	<b>1</b>	<b>2(2)</b>	
KK-13-1 <u>Jun</u> <u>early summer</u>	OT4	10		2		8(8)				
	ON1	15		3			2	10(10)		
	ON5	11		4		7(7)				
	ON8	1					1			
<b>Total</b>		<b>37</b>	<b>0</b>	<b>9</b>	<b>0</b>	<b>15(15)</b>	<b>1</b>	<b>2</b>	<b>10(10)</b>	
KK-13-6 <u>Sep</u> <u>late summer</u>	OT4	7						4(4)	1	
	ON5	11		11						
	ON7	10		2		1		7		
<b>Total</b>		<b>28</b>	<b>0</b>	<b>13</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>7</b>	<b>4(4)</b>	
KS-14-2 <u>Mar</u> <u>spring</u>	OT4	10						1(1)	9	
	ON1	13				3(3)	3	1(1)	3(3)	
	ON5	15				2(2)			9	
<b>Total</b>		<b>38</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>5(5)</b>	<b>3</b>	<b>1(1)</b>	<b>4(4)</b>	

685 Numbers in parentheses indicate the number of sequences with >97% similarity at the amino  
686 acid level to terrestrial diazotroph sequences.

687 Table 2 Pearson's correlation matrix of N<sub>2</sub> fixation rates and water properties in the entire  
 688 water column (n=73). at the-

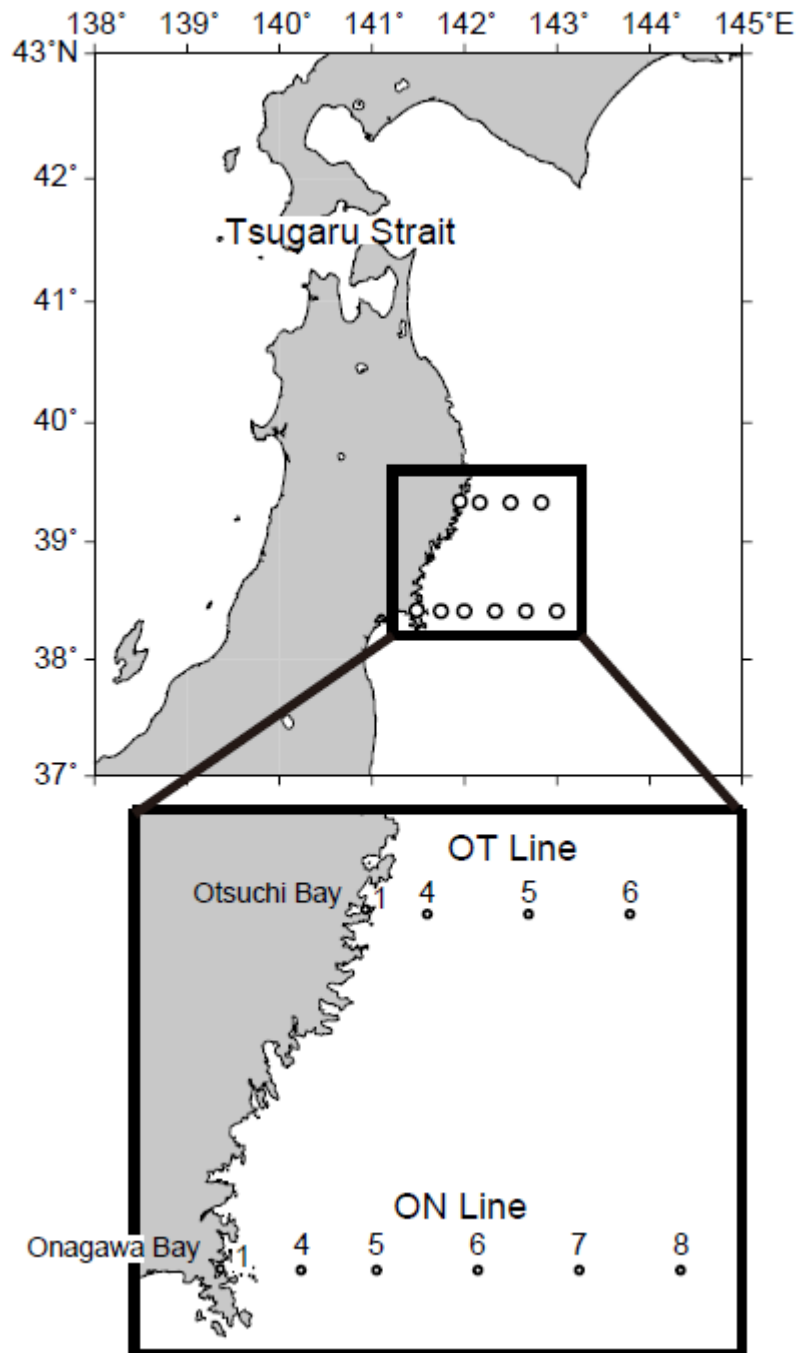
	Temperature	Nitrate	Ammonium	Phosphate	N/P ratio	N <sub>2</sub> fixation
Temperature	1					
Nitrate	-0.722**	1				
Ammonium	-0.036	0.439**	1			
Phosphate	-0.8063**	0.868817**	0.435119	1		
N/P ratio	-0.425266*	0.216722**	0.171751**	0.009349**	1	
N <sub>2</sub> fixation	0.435**	-0.325**	-0.122	-0.335351**	-0.430219	1

689 surface (n=73).

690 \* $p < 0.05$ , \*\* $p < 0.01$

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

692

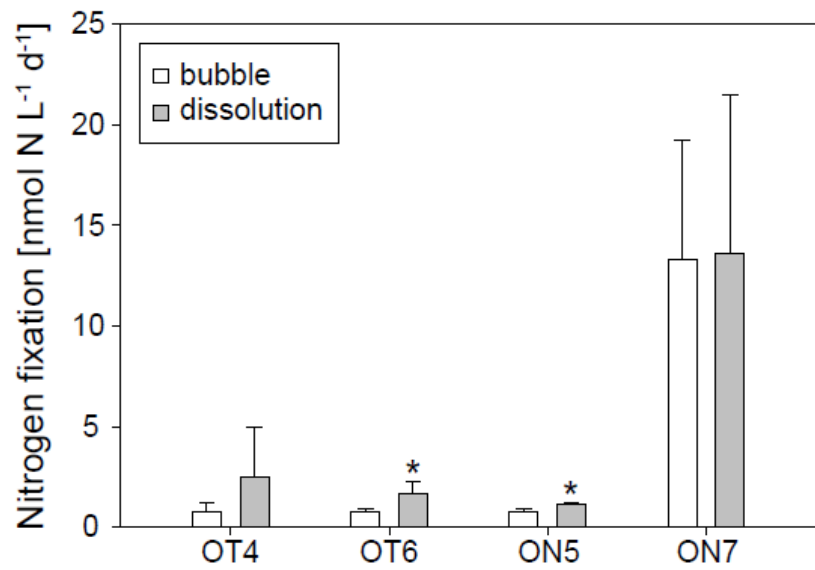


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695 Fig. 1. Sampling locations in the northwestern North Pacific Ocean.

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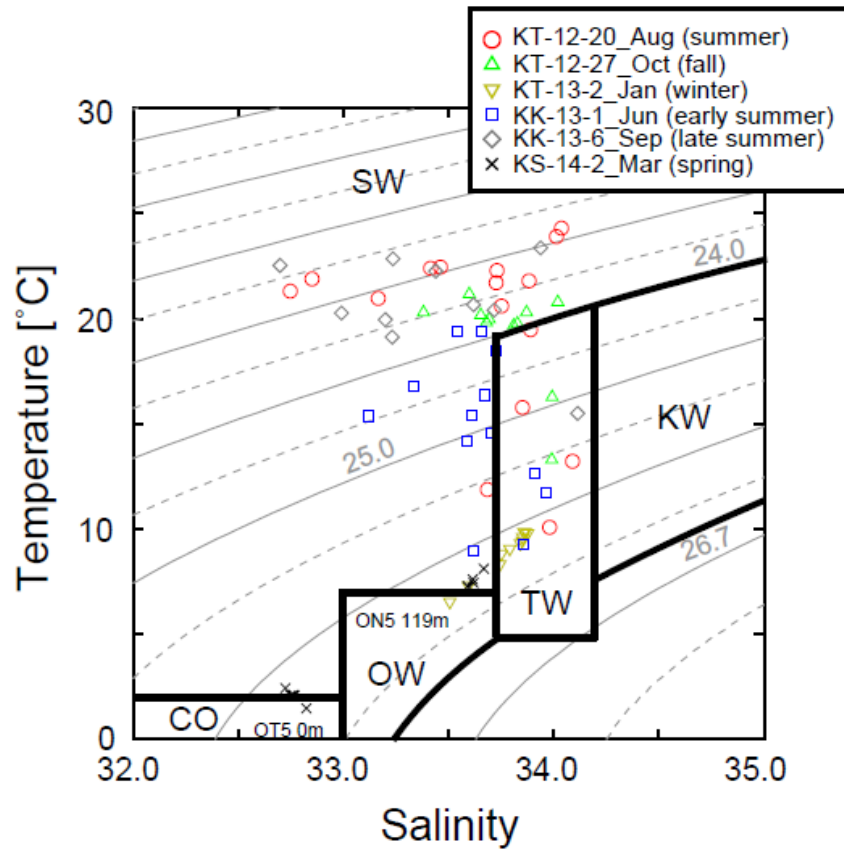
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699 Fig.2. Nitrogen fixation rates estimated simultaneously by the <sup>15</sup>N<sub>2</sub> gas bubble and  
700 dissolution methods during the KK-13-6\_Sep cruise. An asterisk indicates a significant  
701 difference between the two methods ( $p < 0.05$ ).

702



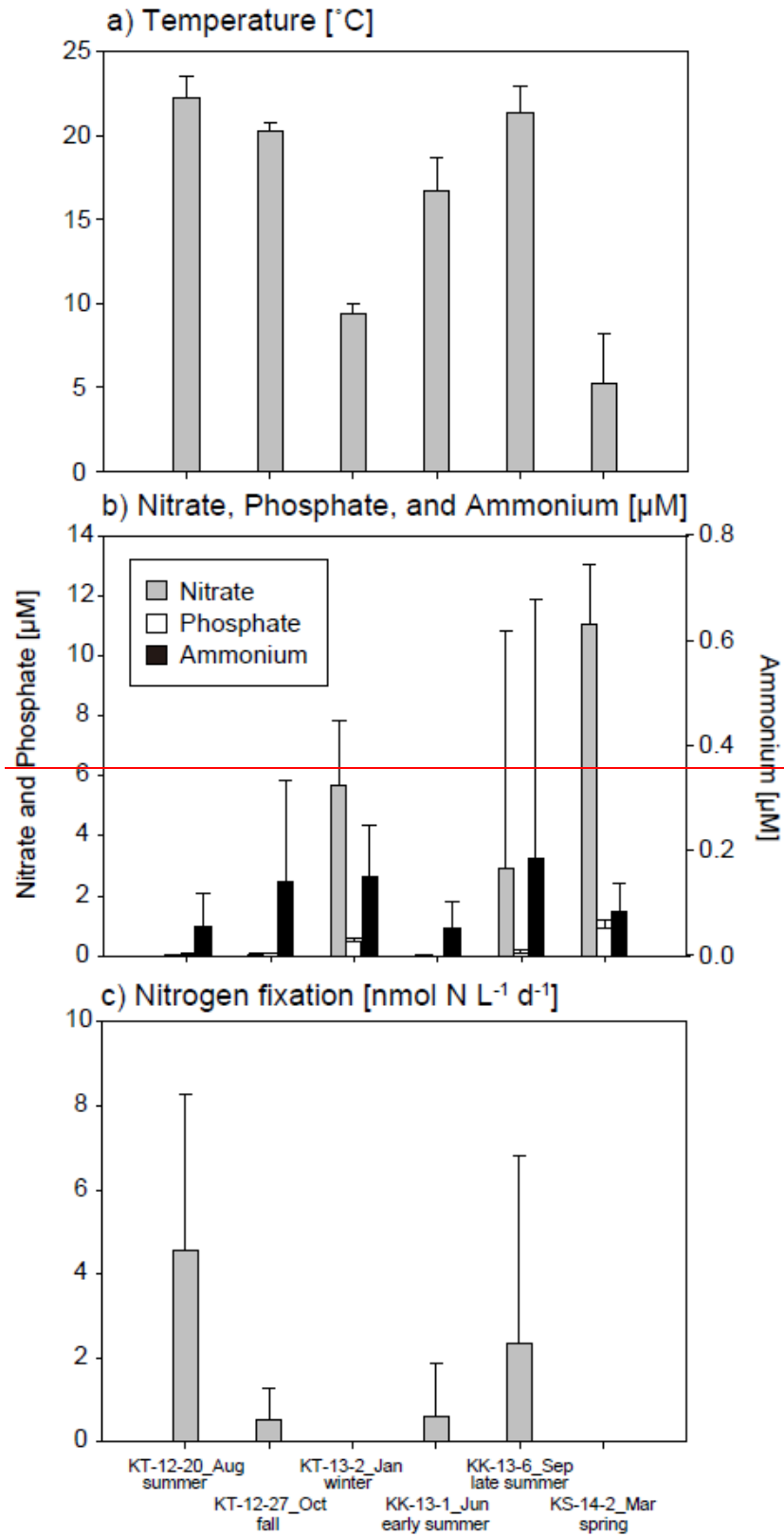


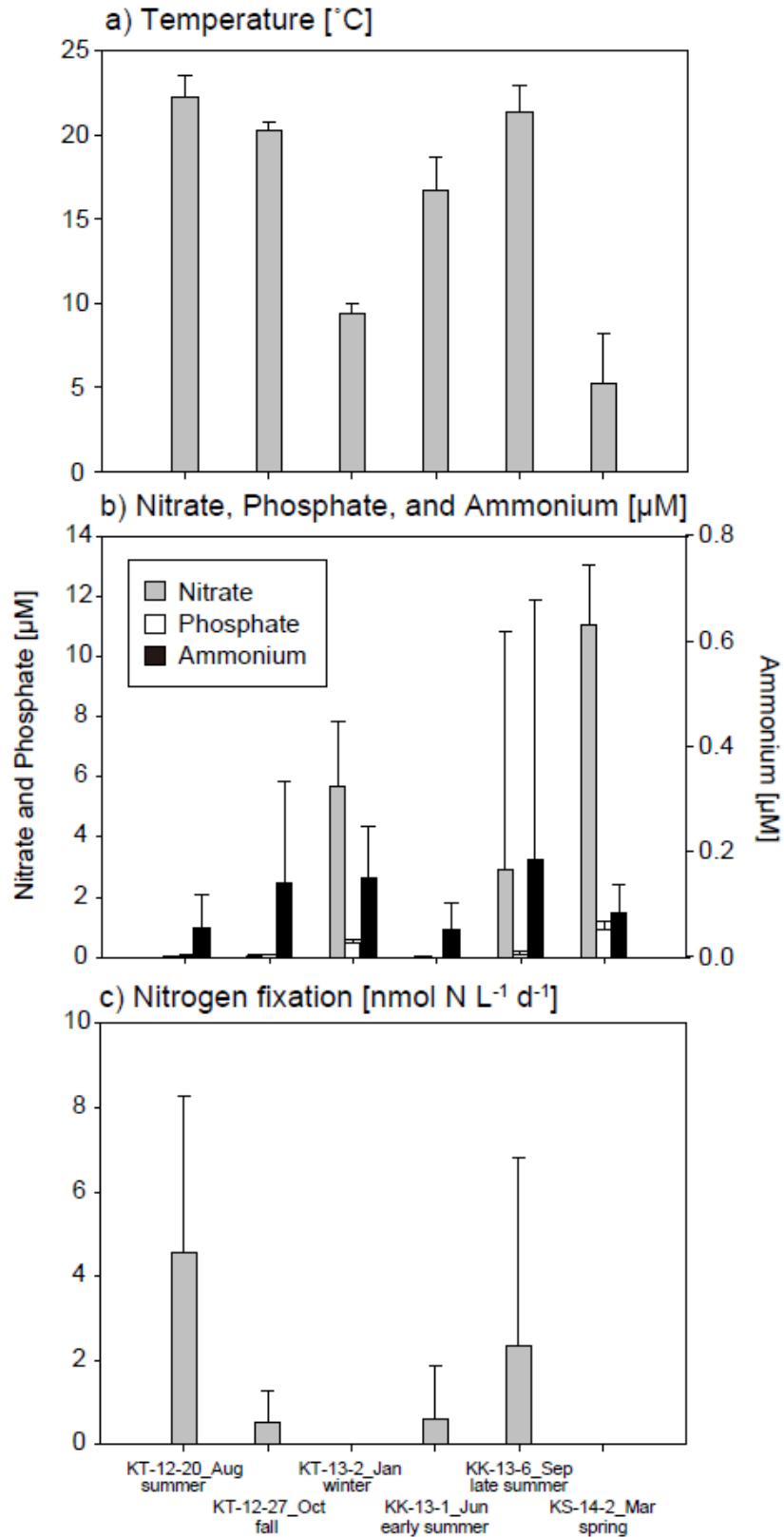
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705 Fig. 3. Temperature-salinity diagram at each sampling point. The water classification was  
 706 defined by Hanawa and Mitsudera (1986). SW, KW, TW, OW, and CO denote the surface  
 707 layer water system, Kuroshio water system, Tsugaru Warm Current water system, Oyashio  
 708 water system, and Coastal Oyashio water system, respectively.

709

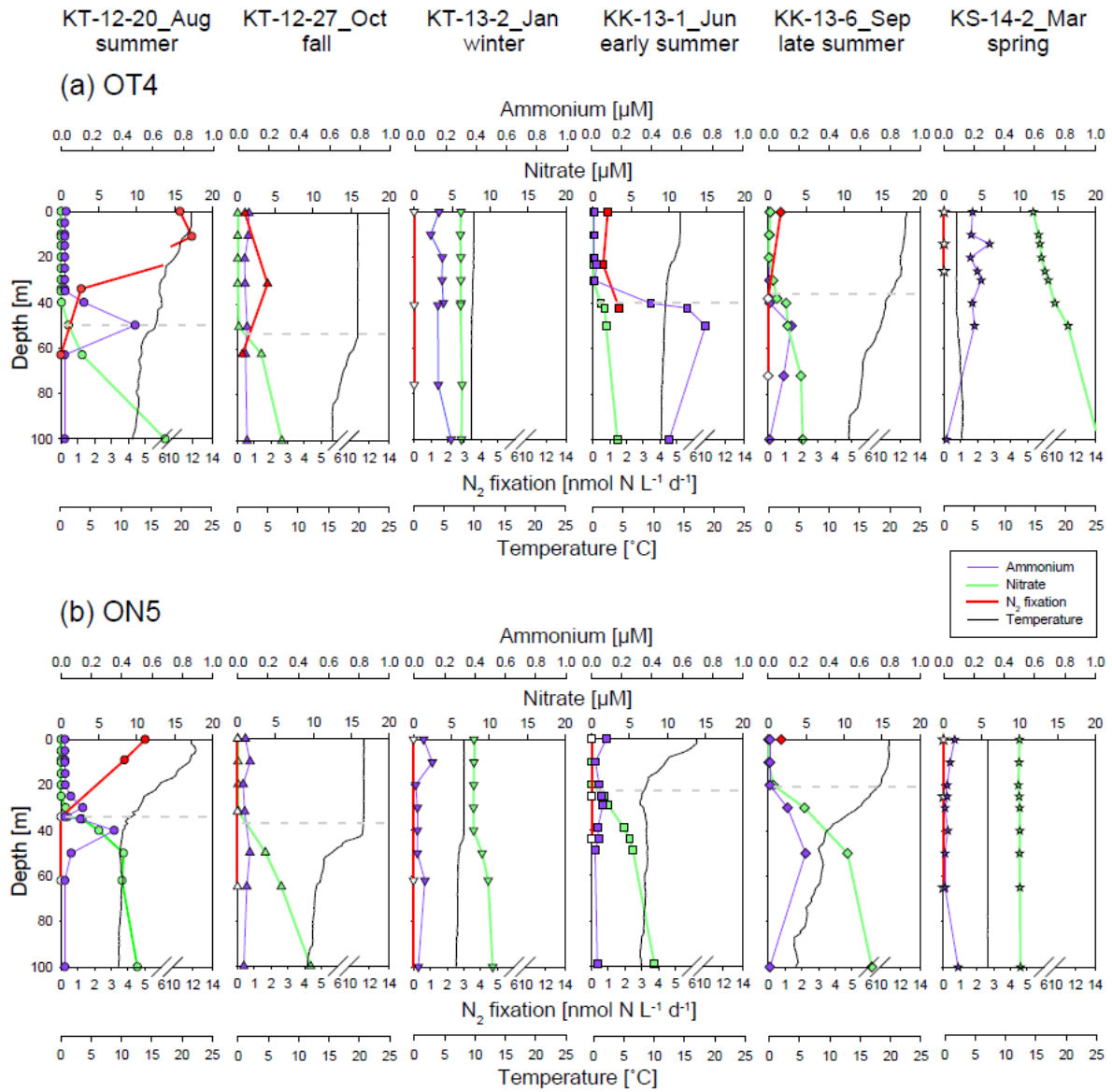




711

712 Fig. 4. Average (a) temperature [°C], (b) nitrate, phosphate, and ammonium concentrations

713 [μM], and (c) nitrogen fixation [nmol N L<sup>-1</sup> d<sup>-1</sup>] at the surface during each cruise.



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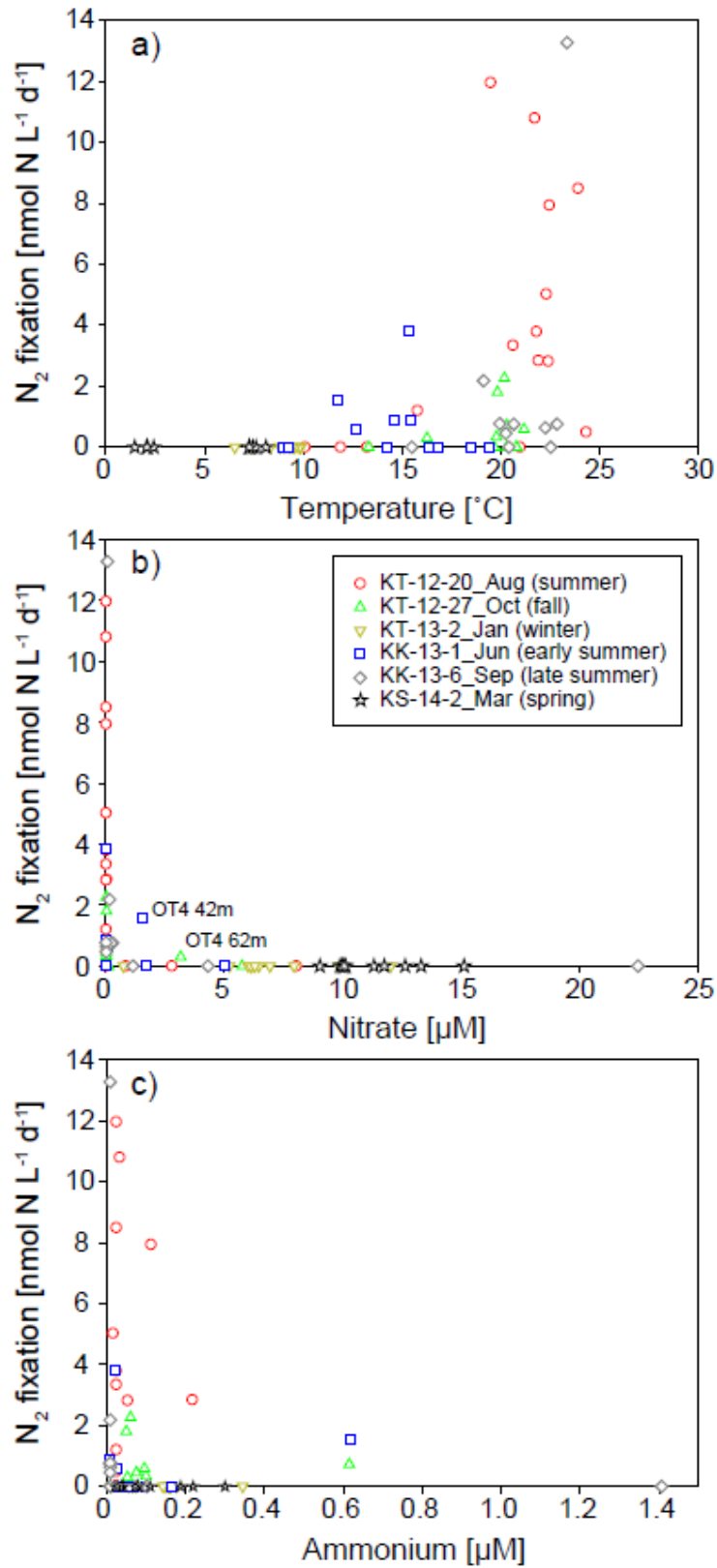
716 Fig. 5. Time-series variations in the vertical profiles of temperature [°C] (black),

717 ammonium (purple) and nitrate (green) concentration [µM], and nitrogen fixation (red) [nmol

718 N L<sup>-1</sup> d<sup>-1</sup>] at Stns (a) OT4 and (b) ON5. Open symbols indicate that nitrogen fixation was

719 not detected. The horizontal dashed line indicates the nitracline depth. The straight lines of

720 temperature and nitrate were ascribable to strong mixing.



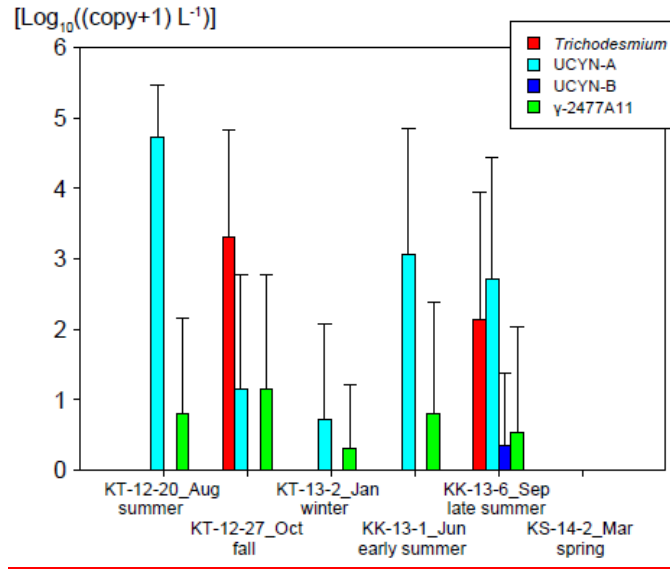
721

722 Fig. 6. Relationship between nitrogen fixation [ $\text{nmol N L}^{-1} \text{d}^{-1}$ ] and (a) temperature [ $^{\circ}\text{C}$ ], (b)

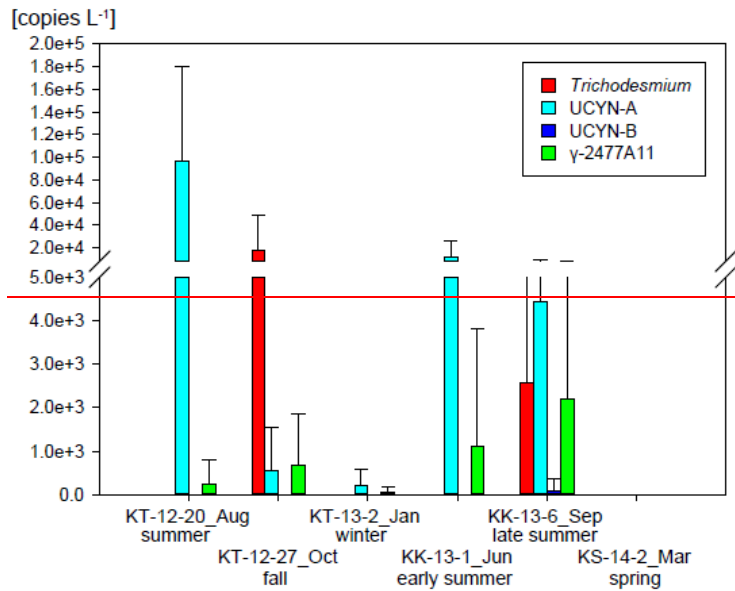
723 nitrate [ $\mu\text{M}$ ], and (c) ammonium [ $\mu\text{M}$ ] for all six cruises.

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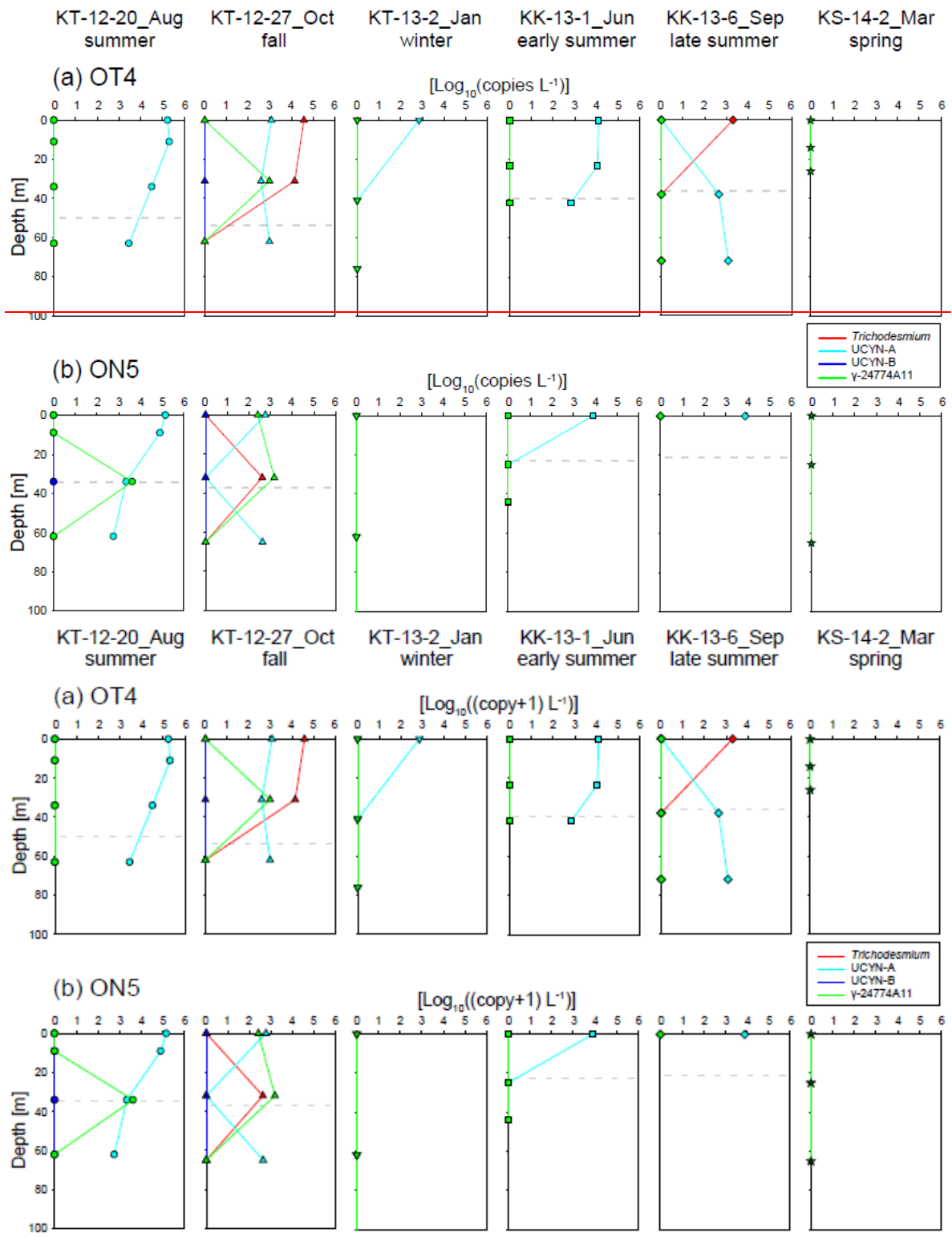
728 Fig. 7. Average abundances of *Trichodesmium* (red), UCYN-A (light blue), UCYN-B

729 (blue), and  $\gamma$ -24774A11 (green)  $[\text{Log}_{10}(\text{copies} + 1) \text{ L}^{-1}]$  at the surface during each

730 cruise. When the target *nifH* gene was not detected, the copy number was assumed to be

731 zero.

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735

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

737 (light blue), UCYN-B (blue), and  $\gamma$ -24774A11 (green) [ $\text{Log}_{10}(\text{copy}+1) \text{ies L}^{-1}$ ] at Stns. (a)

738 OT4 and (b) ON5. The horizontal dashed line indicates the nitracline depth.