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 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 14.2°C (but was lower than 24.3°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, rate measurement data 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

37 subtropical oceans where large cyanobacterial diazotrophs (e.g., *Trichodesmium* spp. and
38 *Richelia intracellularis*) are considered to be primarily 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°C) and DIN-rich waters (>1 μM) 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 along two inshore-offshore
62 transects in the interfrontal zone of the northwestern North Pacific. In this temperate region,
63 physical, chemical, and biological properties vary widely between seasons (Shiozaki et al.,
64 2014b) due to the confluence of three currents: the Kuroshio (warm current), the Tsugaru
65 Warm Current, and Oyashio (cold current). Data on nitrogen fixation rates in the temperate
66 Pacific are limited (Needoba et al., 2007), and to the best of our knowledge, the present study
67 is the first to examine diazotrophy during all seasons in the temperate ocean. This study
68 was conducted as part of a project to monitor the dynamics of the coastal ecosystem and the
69 recovery thereof after the 2011 Tohoku-oki tsunami, which struck the region on 11 March
70 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

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 acrylic 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. 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-L polycarbonate (PC) bottles. 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

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 $^{15}N_2$ 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, $^{15}N_2$-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 ~500 mL min^{-1} (recirculation period, 10 min).
123 Degassed seawater was stored in 1-L Tedlar bags without headspaces and $^{15}N_2$ gas was added
124 at a ratio of 10 ml $^{15}N_2$ per 1L seawater. After complete dissolution, the $^{15}N_2$-enriched
125 seawater was added to seawater samples contained in 2-L PC bottles, which were incubated
126 and used for isotopic analyses as described above. The $^{15}N_2$-enriched seawater was

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

To examine if sugar addition affected nitrogen fixation rates (Bonnet et al., 2013; Rahav et al., 2013; Moisander et al., 2011), we determined nitrogen fixation rates (the bubble method, see above) for surface seawater samples (stations ON4 and OT6 during the KS-14-2_Mar cruise) with and without addition of mannitol (final conc. 0.8 μM) (n=3).

2.3. Statistical analysis

Pearson's correlation coefficient was used to examine the relationships between nitrogen fixation activities and environmental variables including temperature, nitrate, ammonium, phosphate, and the ratio of nitrate + nitrite + ammonium to phosphate (N/P ratio) in the entire water column (the data used for the calculation are shown in Table S1). When the nutrient concentration was below the detection limit, the value of the detection limit was used for the analysis. When nitrogen fixation was undetectable, the value was assumed to be zero.

2.4. DNA analysis

2.4.1. DNA extraction, sequencing, and phylogenetic 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 (-80°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: 95°C for 30 s followed by 30 cycles of 98°C for 10 s, 52°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 7 min. Sterile distilled water was used as the negative control. After PCR analysis, we confirmed that the negative control showed no bands in the gel. 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 1000 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.

2.4.2. 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 during 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; Moisander et al., 2014). 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

181 Applied Science, Germany). The qPCR assays were run in triplicate reactions. Linear
182 regression r^2 values for the standard curves were >0.99 for all reactions. The efficiency of
183 the qPCR assays ranged from 90.9 to 98.4%, with an average of 95.1%. As the negative
184 control, sterile distilled water was used, from which no amplification signals were detected.
185 The detection limit was 75 copies L^{-1}.

186 ## 3. RESULTS

187 ### 3.1. Comparison of the bubble method and the dissolution method

188 Nitrogen fixation rates determined by the bubble and dissolution methods were compared
189 during the KK-13-6_Sep and KS-14-2_Mar cruises (Fig. 2). Both methods failed to
190 detect nitrogen fixation in samples collected during the KS-14-2 cruise. During the
191 KK-13-6_Sep cruise, the nitrogen fixation rates determined by the dissolution method
192 were significantly higher (1.5–2.2 fold) than those determined by the bubble method at
193 Stns. OT6 and ON5 (p <0.05). At Stns. OT4 and ON7, the nitrogen fixation rates
194 determined by the two methods did not differ significantly. Thus, the bubble method
195 may have significantly underestimated the nitrogen fixation rates in some, if not all, of
196 the samples that we analyzed. Although the nitrogen fixation rates reported in the rest
197 of this paper are those obtained using the bubble method, which was used as the standard
198 protocol during all cruises, the possibility that some of these rates could be

199 underestimated must be kept in mind.

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 mostly belonged
203 mostly to either the surface layer water system (SW) or the Tsugaru Warm Current water
204 system (TW) (Fig. 3). Exceptions included the waters collected from the 1% light depth (119
205 m) at Stn. ON5 during the KT-13-2_Jan cruise (classified as the Oyashio water system (OW))
206 and those collected at the surface of OT5 during the KS-14-2_Mar cruise (classified as the
207 Coastal Oyashio water system (CO)). These water classifications based on the TS diagram
208 were generally consistent with the geostrophic current field of the investigated region
209 (Fig.S1). Based on these results, it was assumed that surface waters collected during the
210 same cruise in a particular season generally belonged to the same water system that was
211 prevalent in the investigated region at the time of our sampling.

212 Sea surface temperatures (SSTs) (range, 1.5–24.3°C) (Figs. 4a and S1) and surface nitrate and
213 phosphate concentrations determined during each cruise were averaged to indicate the
214 seasonal variability of these parameters (Fig. 4b). In general, surface nitrate and phosphate
215 concentrations were low (≤0.07 μM and ≤0.20 μM, respectively) in the warmer seawaters
216 (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 during winter (KT-13-2_Jan), and spring (KS-14-2_Mar). During the KK-13-6_Sep cruise (late summer), the nitrate concentrations were relatively high and variable (mean ± SD; 2.92 ± 7.90 μM). This was because 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. By contrast, surface ammonium concentrations were generally low (≤ ~1 μM) throughout the year (Fig. 4b), except for the high ammonium concentration determined at Stn. OT1 (1.41 μM) during the KK-13-6_Sep cruise (Fig. S2).

During the four cruises conducted in early summer (KK-13-1_Jun), summer (KT-12-20_Aug), late summer (KK-13-6_Sep), and fall (KT-12-27_Oct), nitrogen fixation was measurable in most of the samples collected from surface waters: the nitrogen fixation rates varied in the range of 0.33–13.6 nmol N L^{-1} d^{-1} (Figs. 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 Stn. ON7 during the KK-13-6_Sep cruise. Nitrogen fixation was below the detection limit in seawater samples collected during the winter and spring cruises. For those samples, nitrogen fixation was undetectable even after the addition of mannitol

235 (KS-14-2_Mar). Also, nitrogen fixation was undetectable in a DIN-replete water collected at
236 Stn. OT1 in late summer (KK-13-6_Sep).
237 Nitrogen fixation rates were determined for samples collected from different depths (0–119
238 m) at Stns. OT4 and ON5 (Fig. 5). Nitrogen fixation was detected in surface and deeper
239 layers during four cruises conducted in early summer (KK-13-1_Jun), summer
240 (KT-12-20_Aug), late summer (KK-13-6_Sep), and fall (KT-12-27_Oct) (Fig. 4). Nitrogen
241 fixation rates tended to be higher at the surface than in the deeper layers during summer
242 (KT-12-20_Aug) and late summer (KK-13-6_Sep (at Stn. OT4)), whereas this vertical trend
243 was less evident during fall (KT-12-27_Oct) and early summer (KK-13-1_Jun). At Stn.
244 OT4, nitrogen fixation was detected even in the layers below the nitracline (KT-12-27_Oct,
245 depth = 62 m; KK-13-1_Jun, depth = 42 m). During KK-13-1_Jun cruise, the nitrogen
246 fixation rate determined at the depth of 42 m (1.56 nmol N L^{-1} d^{-1}) was 1.8 fold higher than
247 the corresponding rate at the surface (0.87 nmol N L^{-1} d^{-1}). The concentrations of nitrate and
248 ammonium in these layers varied in the range of <0.02–22.5 μM and <0.01–1.41 μM,
249 respectively. The maximum depth-integrated nitrogen fixation (294 μmol N m^{-2} d^{-1}) was
250 found at Stn. OT4 during summer (KT-12-20_Aug).

251 **3.3. Relationship between nitrogen fixation rates and environmental**
252 **variables**

253 Nitrogen fixation rates tended to increase with temperature ($p < 0.01$) (Fig. 6a and Table 2).
254 Nitrogen fixation was detected only when seawater temperatures exceeded 11.7° C, with
255 higher rates (>6 nmol N L^{-1} d^{-1}) noted in waters warmer than 19.5° C. However, there were
256 exceptions to this general relationship between the nitrogen fixation rate and temperature.
257 For example, from the data collected during the KK-13-1_Jun cruise the nitrogen fixation
258 rate was highest at 15.4° C, while it was low (below the detection limit) at higher
259 temperatures.
260 Nitrogen fixation rates were negatively correlated with nitrate and phosphate concentrations
261 ($p < 0.01$) (Table 2), whereas they were not significantly correlated with ammonium
262 concentrations ($p > 0.05$) (Table 2). We also found no significant correlation between
263 nitrogen fixation rates and the ratio of total inorganic nitrogen (nitrate + nitrite + ammonium)
264 to phosphate (Table 2). Nitrogen fixation was generally detectable only when nitrate was
265 depleted (Fig. 6b), except that relatively high nitrogen fixation rates were determined in the
266 subsurface layer of Stn. OT4 (KT-12-27_Oct and KK-13-1_Jun). High nitrogen fixation
267 rates tended to be detected when ammonium concentrations were low (≤ ~0.1 μM), although
268 there was no statistically significant relationship between nitrogen fixation rates and
269 ammonium concentrations.

270 **3.4. Seasonal variation in the diazotroph community**

271 3.4.1. Diazotroph community

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

278 The *nifH* gene was recovered from all the samples that we collected during this study across
279 different stations and seasons (Table 1). Sixty-one OTUs were grouped from 187 *nifH*
280 clones, based on 100% amino acid sequence similarity. The OTUs were assigned to
281 cyanobacteria, α-, β-, γ-, and δ-proteobacteria, and Cluster III diazotrophs (Zehr et al., 2003a)
282 (Figs. S3, S4, and S5).

283 The recovered cyanobacterial sequences belonged to *Trichodesmium*, UCYN-A, and
284 *Leptolyngbya*. The *nifH* sequences of UCYN-B, UCYN-C, and *Richelia intracellularis*
285 were not recovered. The *nifH* sequence of *Trichodesmium* was recovered only during the
286 KT-12-27_Oct cruise (Table 1). UCYN-A was generally recovered from early summer to
287 fall, while *nifH of Leptolyngbya* was recovered during winter. The present study detected
288 the sequences of γ-24774A11 during the KT-12-27_Oct and KK-13-6_Sep cruises. This

289 heterotrophic bacterial phylotype is considered to significantly contribute to nitrogen fixation
290 in a wide range of oceanic environments (Moisander et al., 2014). During the KS-14-2_Mar
291 cruise, all of the sequences that we recovered were derived from heterotrophic bacteria, and
292 were dominated by Cluster III diazotrophs at Stns. OT4 and ON5. The Cluster III
293 diazotroph *nifH* sequences were recovered during all cruises except for the KK-13-1_Jun
294 cruise. Note that 58 out of 187 sequences displayed >97% similarity, at the amino acid level,
295 to terrestrial diazotroph sequences derived from soil, mudflats, and lakes (Fig. S3, S4, and
296 S5). These sequences were mainly affiliated with α- and δ-proteobacterial diazotrophs, with
297 29 of 39 α-proteobacterial sequences and 22 of 24 δ-proteobacterial sequences being similar
298 to 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 8.7×10^4 copies L^{-1} at all depths.
303 *Trichodesmium* abundance at the surface was higher than those of UCYN-A, UCYN-B, and
304 γ-24774A11 at most stations during the KT-12-27_Oct cruise (Fig. 7 and 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 (1.2×10^3 copies L^{-1}) was observed at 72 m
308 (Fig. 8). The abundance of UCYN-A varied from below the detection limit to 2.6×10^5
309 copies L^{-1} 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 S6). UCYN-B was detected only at
312 Stn. ON7 during the KK-13-6_Sep cruise (Fig. 7, 8, and S6). γ-24774A11 was detected
313 during all cruises except for the KS-14-2_Mar cruise (Fig. 7 and 8). The abundance of
314 γ-24774A11 ranged from below the detection limit to 1.8×10^4 copies L^{-1}, with a tendency of
315 a subsurface peak at both stations (Fig. 8).
316
317 ## 4. DISCUSSION
318 ### 4.1. Seasonal variations in nitrogen fixation rates in the temperate coastal
319 ocean
320 Nitrogen fixation rates were measurable mainly from early summer to fall when nitrate was
321 generally depleted in sample seawaters, although there were some exceptions. Our
322 estimates of the nitrogen fixation rates (0.33–13.6 nmol N L^{-1} d^{-1}) were significantly (p
323 <0.05) higher than the corresponding values previously reported in the temperate region of
324 the eastern North Pacific (0.15–0.31 nmol N L^{-1} d^{-1}; Needoba et al., 2007) and the

325 oligotrophic region of the western and central North Pacific (0.17–3.62 nmol N L^{-1} d^{-1};
326 Shiozaki et al., 2010), whereas they were comparable to those determined in the Kuroshio
327 (0.54–28 nmol N L^{-1} d^{-1}; Shiozaki et al., 2010) and the western Atlantic coastal regions
328 (1.3–49.8 nmol N L^{-1} d^{-1}; Mulholland et al., 2012). Higher nitrogen fixation rates have been
329 determined in other temperate oceans, including the western English Channel (18.9±0.01 and
330 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};
331 Bentzon-Tilia et al., 2015).
332 In our study, spatiotemporal variability in nitrogen fixation rates appeared to be partly related
333 to the Tsugaru Warm Current path. This current, which flows from the north (after passage
334 through the Tsugaru Strait) to the study region (Fig. S1), may carry active diazotrophs and
335 therefore enhance nitrogen fixation in our study region. This is supported by the fact that
336 nitrogen fixation rates during individual cruises tended to be higher at Stn. OT4 than at Stn.
337 ON5. These stations were located up- and down-stream of the Tsugaru Warm Current,
338 respectively. In addition, variations in nitrogen fixation rates among stations and seasons
339 might also be related to the extent of vertical mixing in the Tsugaru Warm Current. It has
340 been suggested that vertical mixing may introduce iron-rich subsurface water to the surface
341 of the Tsugaru Strait (Saitoh et al., 2008). Such input of iron may enhance nitrogen fixation
342 rates. Consistent with this notion, our results showed that the nitrogen fixation rate was

343 relatively high at Stn. OT4, where the nitracline was relatively deep.

344 Blais et al. (2012) proposed that nitrogen fixation can proceed even in nutrient-replete waters,
345 if large amounts of iron and organic materials are available for consumption by bacterial
346 diazotrophs. In the present study, this possibility was examined by conducting mannitol
347 addition experiments using surface seawaters collected during spring. These waters,
348 belonging to the Oyashio Current system (Nishioka et al., 2007, 2011; Shiozaki et al., 2014b),
349 were considered to be rich in iron during spring, as indicated by a previous study (iron conc.,
350 0.79–8.46 nM; Nishioka et al. 2007). Despite potentially high iron concentrations, our
351 results showed that nitrogen fixation was undetectable even after the mannitol addition,
352 suggesting that, contrary to the Blais et al. proposition, diazotrophs remained inactive under
353 our experimental settings.

354 Our data showed that nitrogen fixation rates were below the detection limit during winter,
355 spring, and late summer (KK-13-6_Sep), when nitrate concentrations were high. These
356 results were consistent with the results of previous studies in the Pacific Ocean, which
357 indicated that nitrogen fixation rates were low or undetectable in DIN-replete waters
358 (Shiozaki et al., 2010). In contrast, Mulholland et al. (2012) reported that, in temperate
359 regions of the Atlantic Ocean, nitrogen fixation rates were high even in DIN-replete (>1 μM)
360 and cold (<10ºC) surface seawaters. Their study was conducted downstream of the Gulf

361 Stream, where diazotrophs could be delivered from subtropical oceans where DIN is depleted.
362 Previous studies have suggested that cyanobacterial diazotrophs can travel over long
363 distances (>1,000 km) in currents, without losing their capacity for N_2 fixation (Shiozaki et
364 al., 2013), and that activity is not lost immediately even after mixing with DIN-replete
365 seawaters (Holl and Montoya, 2005; Dekaezemacker and Bonnet, 2011). In our region,
366 because the Tsugaru Warm Current flows from north to south, diazotrophs entrained by the
367 current have little chance of meeting high-DIN water at the surface. DIN-replete water
368 during summer was observed at the inside bay station OT1 (Fig. S2). Concomitantly,
369 low-salinity surface waters spread offshore along the OT transect line (Fig. S7), suggesting
370 that anomalously high DIN concentrations were likely attributable to terrestrial surface
371 discharge enhanced by Typhoon Man-yi, which passed over the region immediately before
372 the cruise. Subramaniam et al. (2008) reported that nitrogen fixation rates near the Amazon
373 River estuary, with low salinity and high nitrate levels, were fairly low. Their results are
374 consistent with ours. Ammonium inhibits nitrogen fixation, especially when ammonium
375 concentrations exceed 1 μM, as demonstrated for *Trichodesmium* (Mulholland et al. 2001).
376 In our study, ammonium concentrations were generally low (≤ ~1 μM) throughout the
377 investigation, and no negative relationship between nitrogen fixation and ammonium
378 concentration was found. Our data showing that nitrogen fixation rates were negatively

379 correlated with nitrate concentrations (Table 2) are consistent with the general notion that
380 nitrogen fixation rates are generally low in nitrate replete waters (Falkowski, 1983). Our data
381 also showed nitrogen fixation rates tended to increase with increasing temperature and with
382 decreasing phosphate concentrations (Table 2). Because temperature and phosphate
383 concentrations were correlated with nitrate concentrations, these factors would not
384 necessarily influence nitrogen fixation directly. Rather, one or more factors that varied with
385 nitrate could synergistically influence nitrogen fixation.
386

387 **4.2. Seasonal variation in the diazotroph community in the temperate**
388 **coastal ocean**

389 The qPCR analysis demonstrated that the target groups were quantifiable even at stations at
390 which their sequences were not recovered by the clone library analysis, suggesting that the
391 number of clones was not sufficient to capture the diazotroph community structure on each
392 cruise. Despite this limitation, the sequences more frequently recovered in the clone library
393 generally corresponded to the most abundant group revealed by the qPCR analysis. For
394 example, UCYN-A was frequently recovered in the library during the KT-12-20_Aug,
395 KK-13-1_Jun, and KK-13-6_Sep cruises; for these samples, the qPCR results showed that
396 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). Therefore, the diazotrophs targeted by the qPCR analysis were likely important for nitrogen fixation in this study region. In the discussion below, we mainly discuss possible factors responsible for seasonal variation in the diazotrophs targeted by the qPCR analysis.

UCYN-A was detected in all seasons except spring (KS-14-2_Mar), suggesting that this group of diazotrophs could be important agents of nitrogen fixation in this region. 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 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). In our study, UCYN-A was detected during winter at some stations. It appears that UCYN-A abundance decreased with decreasing temperature from fall to winter, and then became undetectable in spring.

Trichodesmium was detected from late summer to fall, 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 (>24º 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 detected γ-24774A11 during all cruises except for the KS-14-2_Mar cruise. γ-24774A11 is considered to be one of the most important heterotrophic diazotrophs in the tropical and subtropical oligotrophic ocean (Moisander et al., 2014). However, the γ-24774A11 sequence has not been detected previously in other temperate oceans (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012). The γ-24774A11 sequence was similar (94% similarity at the amino acid level) to the *nifH* sequence of *Pseudomonas stutzeri*, which has been reported to be present in temperate estuaries (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

433 abundant sequences among their samples collected from the Baltic Sea estuary. In the
434 present study, we recovered *P. stutzeri*-like *nifH* genes (>97% similarity at the amino acid
435 level) only at Stn. OT4 during the KT-13-2_Jan cruise by the clone library analysis, and
436 γ-24774A11 was not detected on that occasion by qPCR analysis probably due to the
437 difference in the sequence between γ-24774A11 and *P. stutzeri*. The ecology of γ-24774A11
438 is still fairly unknown. It remains to be seen, in future studies whether this phylotype
439 contributes to the nitrogen fixation in this region.
440 UCYN-B was not detected except at one station. This result is consistent with previous
441 knowledge. UCYN-B becomes abundant with increasing temperature, similar to
442 *Trichodesmium* (Moisander et al., 2010), and is rarely observed in the temperate region
443 (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012; Bentzon-Tilia et al., 2015).
444 Furthermore, UCYN-B abundance is low in shallow nitracline regions (Shiozaki et al.,
445 2014a,c). The nitracline depth in this region (≤60 m) was shallower than that of >100-m
446 depths of regions where UCYN-B is abundant (Shiozaki et al., 2014a). Therefore, although
447 UCYN-B might also have been delivered from subtropical region, it could not survive in the
448 shallower nitracline region.
449 In nitrate-rich water during winter and spring, Cluster III diazotrophs were detected at most
450 of the stations. 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). Therefore, Cluster III diazotrophs appeared to be present throughout the investigation period. Cluster III diazotrophs are putative anaerobes (Hamersley et al., 2011; Farnelid et al., 2013; Bentzon-Tilia et al., 2014), and hence, they are usually dominant in the diazotrophic community of oxygen-depleted waters (Hamersley et al., 2011; Farnelid et al., 2013) or 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. S8). Therefore, 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). We obtained a *Leptolyngbya*-like *nifH* gene during the KT-13-2_Jan cruise. The organism has been found on beaches or coastal land areas (Brito et al. 2012), but not in the open ocean. Because nitrogen fixation was not detected during the KT-13-2_Jan cruise, the organism was considered not to perform nitrogen fixation.

5. CONCLUSION

This study demonstrated that nitrogen fixation can and does proceed at high rates, depending on the season, in the temperate coastal 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 year, displaying a marked seasonality in their composition. UCYN-A was a major diazotroph during summer, whereas *Trichodesmium* was abundant during fall, despite low temperatures. It has been suggested that *Trichodesmium* was laterally transported from the adjacent subtropical region, which displays high temperatures. Although the Cluster III diazotrophs were recovered almost throughout a year, they were considered to be inactivated in oxic water columns.

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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References

Bentzon-Tilia, M., Farnelid, H., Jürgens, K. and Riemann, L.: Cultivation and isolation of N_2-fixing bacteria from suboxic waters in the Baltic Sea, FEMS Microbiol. Ecol., 88, 358-371, 2014.

Bentzon-Tilia, M., Traving, S.J., Mantikci, M., Knudsen-Leerbeck, H., Hansen, J. L. S., Markager, S., and Riemann, L.: Significant N_2 fixation by heterotrophs, photoheterotrophs and heterocystous cyanobacteria in two temperate estuaries, ISME J., 9, 273-285, 2015.

Bertics ,V. J., Löscher, C. R., Salonen, I., Dale, A. W., Gier, J., Schmitz, R. A., and Treude, T.: Occurrence of benthic microbial nitrogen fixation coupled to sulfate reduction in the seasonally hypoxic Eckernförde Bay, Baltic Sea, Biogeosciences, 10, 1243-1258, 2013.

Blais, M., Tremblay, J.-E., Jungblut, A. D., Gagnon, J., Martin, J., Thaler, M., and Lovejoy, C.: Nitrogen fixation and identification of potential diazotrophs in the Canadian Arctic, Glob. Biogeochem. Cycles 26, GB3022, doi:10.1029/2011GB004096, 2012.

Bonnet, S., Dekaezemacker, J., Turk-Kubo, K. A., Moutin, T., Hamersley, R. M., Grosso, O., Zehr, J. P., and Capone, D. G.: Aphotic N_2 fixation in the Eastern Tropical South Pacific Ocean, PLoS one 8(12), e81265, doi:10.1371/journal.pone.0081265, 2013.

Brandes, J. A., and Devol, A. H.: A global marine-fixed nitrogen isotopic budget: Implications for Holocene nitrogen cycling, Glob. Biogeochem. Cycles. 16, 4, 1120,

518 doi:10.1029/2001GB001856, 2002.

519 Breitbarth, E., Oschlies, A., and LaRoche, J: Physiological constraints on the global

520 distribution of *Trichodesmium* –effect of temperature on diazotrophy, Biogeosciences, 4,

521 53-61, 2007.

522 Brito, A., Ramos, V., Seabra, R., Santos, A., Santos, C. L., Lopo, M., Ferreira, S., Martins, A.,

523 Mota, R., Frazão, B., Martins, R., Vasconcelos, V., and Tamagnini, P.: Culture-dependent

524 characterization of cyanobacterial diversity in the intertidal zones of the Portuguese coast: A

525 polyphasic study, Syst. Appl. Microbiol. 35, 110-119, 2012.

526 Capone, D. G., Zehr, J. P., Paerl, H. W., Bergman, B., and Carpenter, E. J.: Trichodesmium, a

527 globally significant marine cyanobacterium, Science, 276, 1221-1229, 1997.

528 Carpenter, E. J., Subramaniam, A., Capone, D. G.: Biomass and primary productivity of the

529 cyanobacterium *Trichodesmium* spp. in the tropical N Atlantic ocean, Deep-Sea Res., 51,

530 173-203, 2004.

531 Church, M. J., Mahaffey, C., Letelier, R. M., Lukas, R., Zehr, J. P., and Karl, D. M.: Physical

532 forcing of nitrogen fixation and diazotroph community structure in the North Pacific

533 subtropical gyre, Glob. Biogeochem. Cycles., 23, GB2020, doi:10.1029/2008GB003418,

534 2009.

535 Chen, Y.-L. L., Chen, H.-Y., Tuo, S.-H., and Ohki, K.: Seasonal dynamics of new production

536 from *Trichodesmium* N_2 fixation and nitrate uptake in the upstream Kuroshio and South
537 China Sea basin, Limnol. Oceanogr., 53(5), 1705-1721, 2008.
538 Codispoti, L. A.: An oceanic fixed nitrogen sink exceeding 400 TgN a^{-1} vs the concept of
539 homeostasis in the fixed-nitrogen inventory, Biogeosciences, 4, 233-253, 2007.
540 Dabundo, R., Lehmann, M. F., Treibergs, L., Tobias, C. R., Altabet, M. A., Moisander, P. H.,
541 and Granger, J.: The contamination of commercial $^{15}N_2$ gas stocks with ^{15}N-labeled nitrate
542 and ammonium and consequences for nitrogen fixation measurements, PLoS ONE, 9(10),
543 e110335, doi:10.1371/journal.pone.0110335, 2014.
544 Dekaezemacker, J., and Bonnet, S.: Sensitivity of N_2 fixation to combined nitrogen forms
545 (NO_3^- and NH_4^+) in two strains of the marine diazotroph *Crocosphaera watsonii*
546 (Cyanobacteria), Mar. Ecol. Progr. Ser., 438, 33-46, 2011.
547 Deutsch, C., Sigman, D. M., Thunell, R. C., Meckler, A. N., and Haug, G. H.: Isotopic
548 constraints on glacial/interglacial changes in the oceanic nitrogen budget, Glob. Biogeochem.
549 Cycles., 18, GB4012, doi:10.1029/2003GB002189, 2004.
550 Falkowski, P.G.: Enzymology of nitrogen assimilation, in: Carpenter, J., Capone, D. G. (eds)
551 Nitrogen in the marine environment, Academic Press, New York, 839-868, 1983.
552 Farnelid, H., Bentzon-Tilia, M., Andersson, A. F., Bertilsson, S., Jost, G., Labrenz, M.,
553 Jürgens, K., and Riemann, L.: Active nitrogen-fixing heterotrophic bacteria at and below the

554 chemocline of the central Baltic Sea, ISME J., 7, 1413-1423, 2013.

555 Großkopf, T., Mohr, W., Baustian, T., Schunck, H., Gill, D., Kuypers, M. M. M., Lavik, G.,

556 Schmitz, R. A., Wallace, D. W. R., and LaRoche, J.: Doubling of marine dinitrogen-fixation

557 rates based on direct measurements, Nature, 488, 361-364, 2012.

558 Halm, H., Lam, P., Ferdelman, T. G., Lavik, G., Dittmar, T., LaRoche, J., D'Hondt, S., and

559 Kuypers, M.M.M.: Heterotrophic organisms dominate nitrogen fixation in the South Pacific

560 Gyre, ISME J., 6, 1238-1249, 2012.

561 Hamersley, M. R., Turk, K. A., Leinweber, A., Gruber, N., Zehr, J. P., Gunderson, T., and

562 Capone, D. G.: Nitrogen fixation within the water column associated with two hypoxic basins

563 in the Southern California Bight, Aquat. Microbial. Ecol., 63, 193-205, 2011.

564 Hanawa, K., and Mitsudera, H.: Variation of water system distribution in the Sanriku coastal

565 area, J. Oceanogr. Soc. Jap., 42, 435-446, 1987.

566 Holl, C. M., and Montoya, J. P.: Interactions between nitrate uptake and nitrogen fixation in

567 continuous cultures of the marine diazotroph *Trichodesmium* (Cyanobacteria), J. Phycol., 41,

568 1178-1183, 2005.

569 Huang, Y., Niu, B. F., Gao, Y., Fu, L. M., and Li, W.Z.: CD-HIT Suite: a web server for

570 clustering and comparing biological sequences, Bioinformatics, 26, 680-682, 2010.

571 Marumo, R., and Nagasawa S.: Seasonal variation of the standing crop of a pelagic

572 blue-green alga, *Trichodesmium* in the Kuroshio water, Bull. Plankton Soc. Japan, 23(1),
573 19-25, 1976 (in Japanese with English abstract).
574 Moisander, P. H., Beinart, R. A., Hewson, I., White, A. E., Johnson, K. S., Carlson, C. A.,
575 Montoya, J. P., and Zehr, J. P.: Unicellular cyanobacterial distributions broaden the oceanic
576 N_2 fixation domain, Science, 327, 1512-1514, 2010.
577 Moisander, P.H., Zhang, R., Boyle, E.A., Hewson, I., Montoya, J.P., Zehr, J.P.: Analogous
578 nutrient limitations in unicellular diazotrophs and *Prochlorococcus* in the South Pacific
579 Ocean, ISME J., 6, 733-744, 2011.
580 Moisander, P. H., Serros, T., Paerl, R. W., Beinart, R. A., and Zehr, J.P.:
581 Gammaproteobacterial diazotrophs and *nifH* gene expression in surface waters of the South
582 Pacific Ocean, ISME J., 8, 1962-1973, 2014.
583 Mohr, W., Großkopf, T., Wallace, D. W. R., and LaRoche, J.: Methodological
584 underestimation of oceanic nitrogen fixation rates, PLoS ONE, 5(9), e12583,
585 doi:10.1371/journal.pone.0012583, 2010.
586 Montoya, J. P., Voss, M., Kähler, P., and Capone, D. G.: A simple, high-precision,
587 high-sensitivity tracer assay for N_2 fixation, Appl. Environ. Microbiol., 62(3), 986-993, 1996.
588 Mulholland, M. R., Ohki, K., and Capone, D. G.: Nutrient controls on nitrogen uptake and
589 metabolism by natural populations and cultures of *Trichodesmium* (Cyanobacteria), J.

590 Phycol., 37, 1001-1009, 2001.

591 Mulholland, M. R., Bernhardt, P. W., Blanco-Garcia, J. L., Mannino, A., Hyde, K.,

592 Mondragon, E., Turk, K., Moisander, P. H., and Zehr, J. P.: Rates of dinitrogen fixation and

593 the abundance of diazotrophs in North American coastal waters between Cape Hatteras and

594 Georges Bank, Limnol. Oceanogr., 57(4), 1067-1083, 2012.

595 Needoba, J. A., Foster, R. A., Sakamoto, C., Zehr, J. P, and Johnson, K. S.: Nitrogen fixation

596 by unicellular diazotrophic cyanobacteria in the temperate oligotrophic North Pacific Ocean,

597 Limnol. Oceanogr., 52(4), 1317-1327, 2007.

598 Nishioka, J., Ono, T., Saito, H., Nakatsuka, T., Takeda, S., Yoshimura, T., Suzuki, K., Kuma,

599 K., Nakabayashi, S., Tsumune, D., Mitsudera, H., Johnson, W. K., and Tsuda, A.: Iron supply

600 to the western subarctic Pacific: Importance of iron export from the Sea of Okhotsk, J.

601 Geophys. Res., 112, C10012, doi:10.1029/2006JC004055, 2007.

602 Nishioka, J., Ono, T., Saito, H., Sakaoka, K., and Yoshimura, T.: Oceanic iron supply

603 mechanisms which support the spring diatom bloom in the Oyashio region, western subarctic

604 Pacific, J. Geophys. Res., 116, C02021, doi:10.1029/2010JC006321, 2011.

605 Rahav. E., Bar-Zeev, E., Ohayon, S., Elifantz, H., Belkin, N., Herut, B., Mulholland, M. R.,

606 and Berman-Frank, I.: Dinitrogen fixation in aphotic oxygenated marine environment, Front.

607 Microbiol., 4, 277, doi:10.3389/fmicb.2013.00227, 2013.

608 Riemann, L., Farnelid, H., and Steward, G. F.: Nitrogenase genes in non-cyanobacterial
609 plankton: prevalence, diversity and regulation in marine waters, Aquat. Microbial Ecol. 61,
610 235-247, 2010.
611 Rees, A. P., Gilbert, J. A., and Kelly-Gerreyn, B. A.: Nitrogen fixation in the western English
612 Channel (NE Atlantic Ocean), Mar. Ecol. Progr. Ser., 374, 7-12, 2009.
613 Saitoh, Y., Kuma, K., Isoda, Y., Kuroda, H., Matsuura, H., Wagawa, T., Takana, H.,
614 Kobayashi, N., Nagao, S., and Nakatsuka, T.: Processes influencing iron distribution in the
615 coastal waters of the Tsugaru Strait, Japan, J. Oceanogr., 64, 815-830, 2008.
616 Shiozaki, T., Furuya, K., Kodama, T., and Takeda, S.: Contribution of N_2 fixation to new
617 production in the western North Pacific Ocean along 155ºE, Mar. Ecol. Progr. Ser. 377,
618 19-32, 2009.
619 Shiozaki, T., Furuya, K., Kodama, T., Kitajima, S., Takeda, S., Takemura, T., and Kanda, J.:
620 New estimation of N_2 fixation in the western and central Pacific Ocean and its marginal seas,
621 Global. Biogeochem. Cycles., 24, GB1015, doi:10.1029/2009GB003620, 2010.
622 Shiozaki, T., Kodama, T., Kitajima, S., Sato, M., and Furuya, K.: Advective transport of
623 diazotrophs and importance of their nitrogen fixation on new and primary production in the
624 western Pacific warm pool, Limnol. Oceanogr., 58(1), 49-60, 2013.
625 Shiozaki. T., Ijichi, M., Kodama, T., Takeda, S., and Furuya, K.: Heterotrophic bacteria are

626 major nitrogen fixers in the euphotic zone of the Indian Ocean, Glob. Biogeochem. Cycles,
627 28, doi:10.1002/2014GB004886, 2014a.
628 Shiozaki, T., Ito S.-I., Takahashi, K., Saito, H., Nagata, T., and Furuya, K.: Regional
629 variability of factors controlling the onset timing and magnitude of spring algal blooms in the
630 northwestern North Pacific, J. Geophys. Res., 119, 1-13, doi:10.1002/2013JC009187, 2014b.
631 Shiozaki, T., Chen, Y.-L. L., Lin, Y.-H., Taniuchi, Y., Sheu, D.-S., Furuya, K., and Chen,
632 H.-Y.: Seasonal variations of unicellular diazotroph groups A and B, and *Trichodesmium* in
633 the northern South China Sea and neighboring upstream Kuroshio Current, Cont. Shelf Res.,
634 80, 20-31, 2014c.
635 Subramaniam, A., Yager, P. L., Carpenter, E. J., Mahaffey, C., Björkman, K., Cooley, S.,
636 Kustka, A. B., Montoya, J. P., Sañudo-Wilhelmy, S. A., Shipe, R., and Capone, D. G.:
637 Amazon River enhances diazotrophy and carbon sequestration in the tropical North Atlantic
638 Ocean, Proc. Natl. Acad. Aci. U.S.A., 108, 2184-2189, 2008.
639 Tamura, K., Peterson, N., Peterson, G., Stecher, M., Nei, M., and Kumar, S.: MEGA5:
640 Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance,
641 and maximum parsimony methods, Mol. Biol. Evol., 28, 2731-2739, 2011.
642 Zehr, J. P., and Turner, P. J.: Nitrogen fixation: nitrogenase genes and gene expression, Meth.
643 Microbiol., 30, 271-285, 2001.

644 Zehr, J. P., Jenkins, B. D., Short, S. M., and Steward, G. F.: Nitrogenase gene diversity and
645 microbial community structure: a cross-system comparison, Environ. Micorbiol., 5(7),
646 539-554, 2003a.
647 Zehr, J. P., Crumbliss, L. L., Church, M. J., Omoregie, E. O., Jenkins, B. D.: Nitrogenase
648 genes in PCR and RT-PCR reagents: implications for studies of diversity of functional genes,
649 BioTechniques, 35, 996-1005, 2003b.
650 Zehr, J. P.: Nitrogen fixation by marine cyanobacteria, Trends Microbiol. 19, 162-173, 2011.
651

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

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

654 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_Aug	OT4	12		9		3				
summer	ON1	5		2						3
	ON5	8		8						
	ON7	7		1		6				
Total		**32**	**0**	**20**	**0**	**9**	**0**	**0**	**0**	**3**
KT-12-27_Oct	OT4	7	1							6
fall	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_Jan	OT4	11			10			1		
winter	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_Jun	OT4	10		2		8(8)				
early summer	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_Sep	OT4	7							4(4)	1
late summer	ON5	11		11						
	ON7	10		2		1		7		
Total		**28**	**0**	**13**	**0**	**1**	**0**	**7**	**4(4)**	**1**
KS-14-2_Mar	OT4	10							1(1)	9
spring	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**

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

656 acid level to terrestrial diazotroph sequences.

657 Table 2 Pearson's correlation matrix of N_2 fixation rates and water properties in the entire

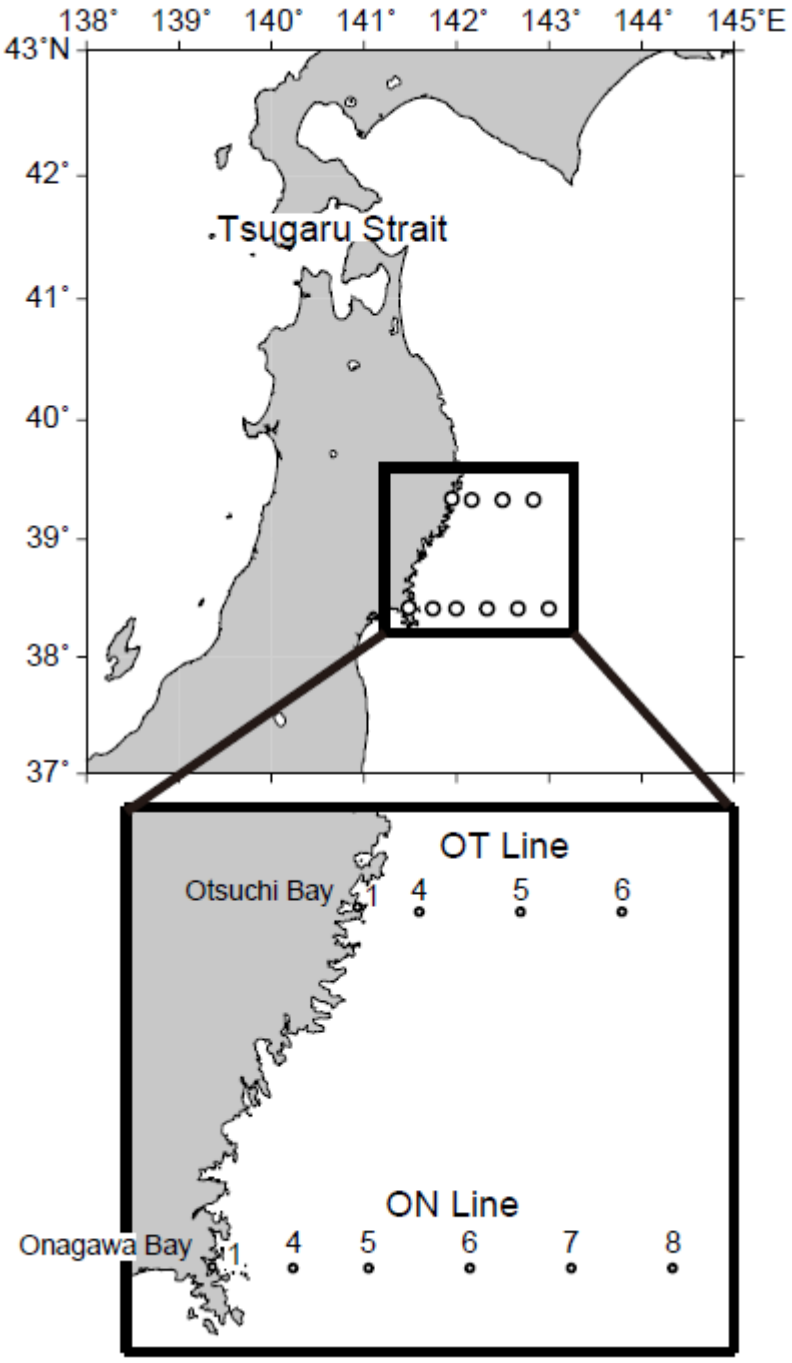
658 water column (n=73).

	Temperature	Nitrate	Ammonium	Phosphate	N/P ratio	N_2 fixation
Temperature	1					
Nitrate	-0.722**	1				
Ammonium	-0.036	0.439**	1			
Phosphate	-0.880**	0.881**	0.119	1		
N/P ratio	-0.266*	0.722**	0.751**	0.349**	1	
N_2 fixation	0.435**	-0.325**	-0.122	-0.351**	-0.219	1

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

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

661

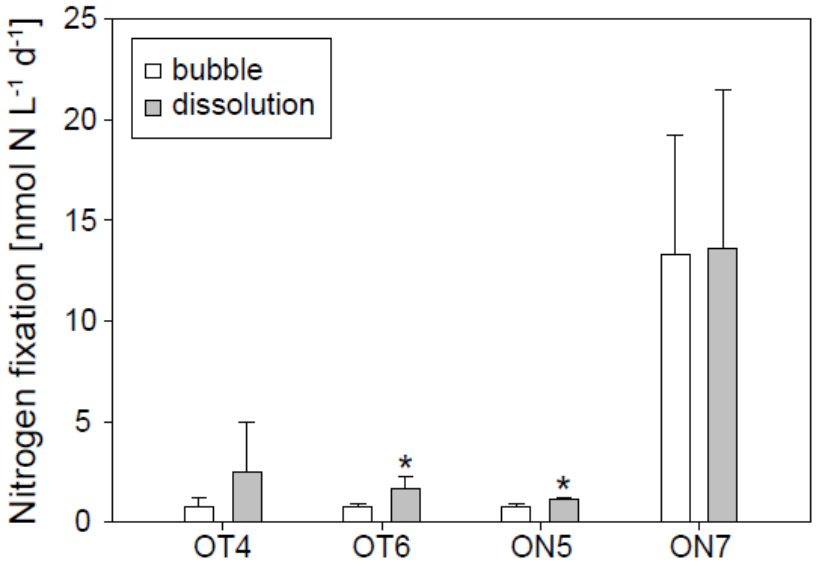


662

663

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

665



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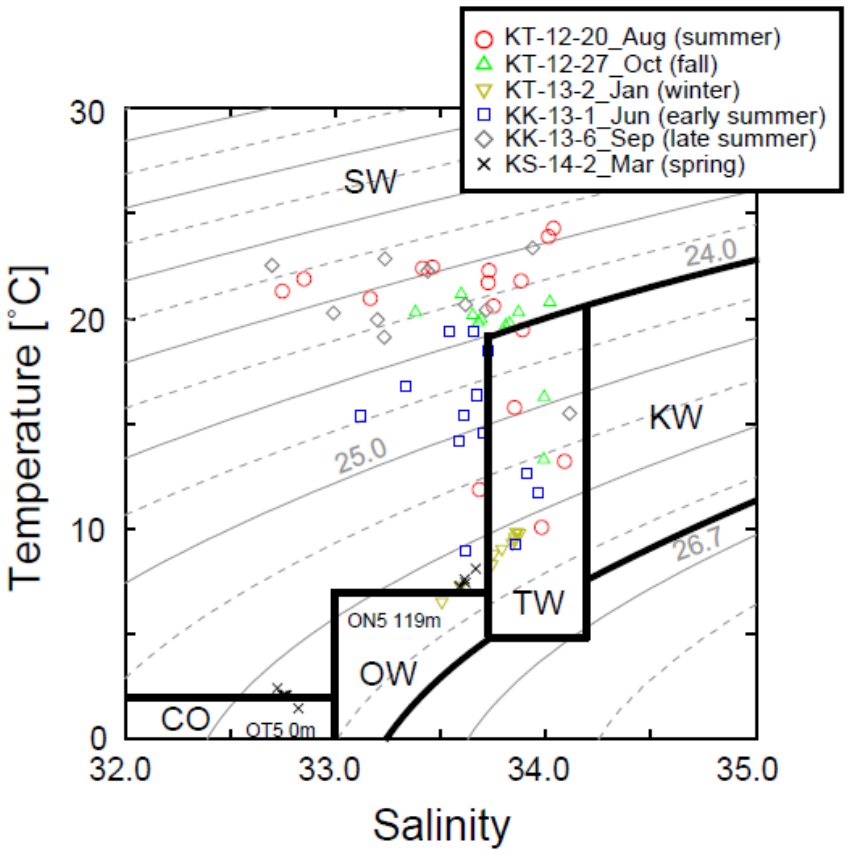
667

668 Fig.2. Nitrogen fixation rates estimated simultaneously by the $^{15}N_2$ gas bubble and

669 dissolution methods during the KK-13-6_Sep cruise. An asterisk indicates a significant

670 difference between the two methods (p <0.05).

671



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673

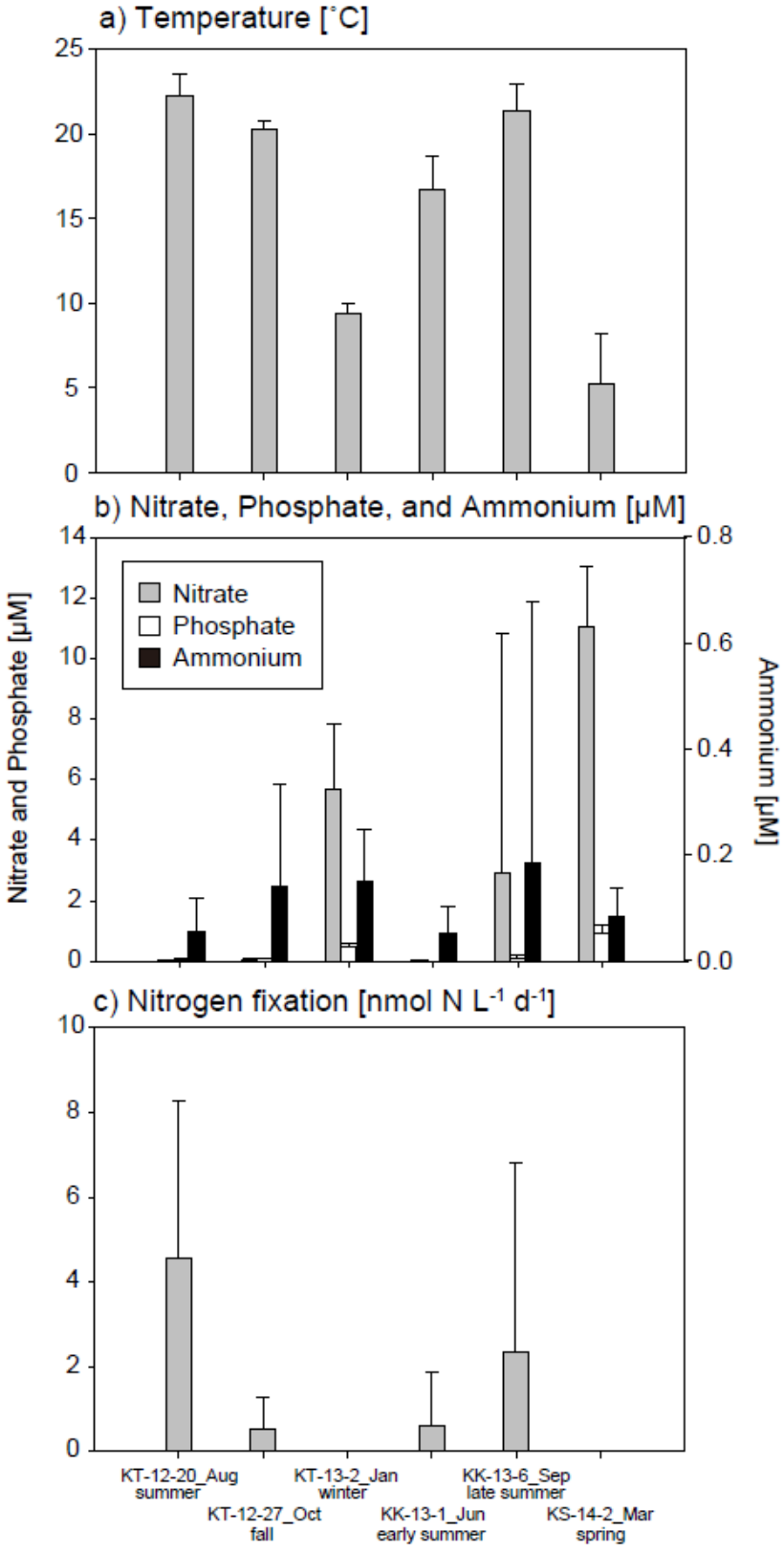
674 Fig. 3. Temperature-salinity diagram at each sampling point. The water classification was

675 defined by Hanawa and Mitsudera (1986). SW, KW, TW, OW, and CO denote the surface

676 layer water system, Kuroshio water system, Tsugaru Warm Current water system, Oyashio

677 water system, and Coastal Oyashio water system, respectively.

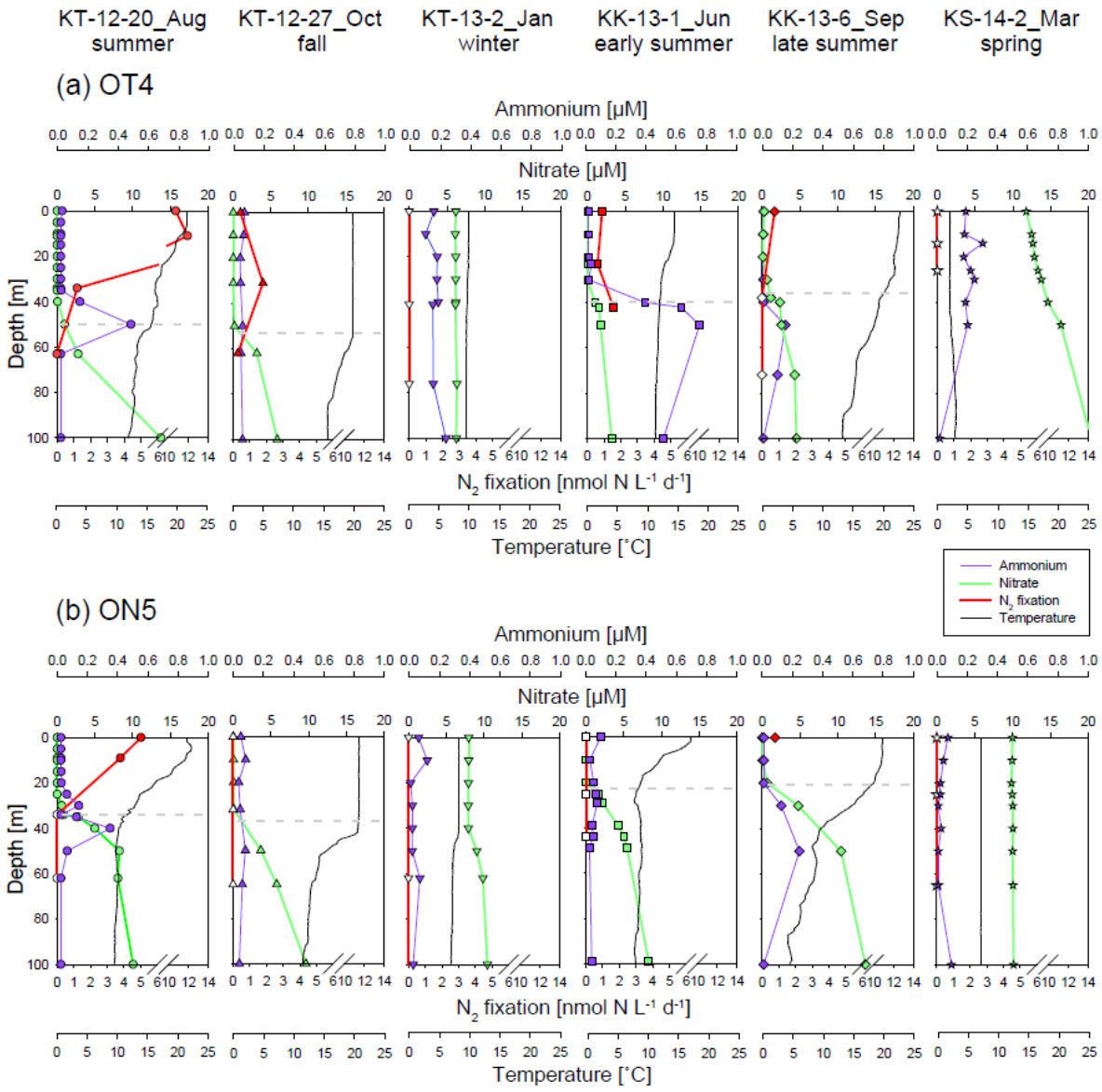
678



679

680 Fig. 4. Average (a) temperature [ºC], (b) nitrate, phosphate, and ammonium concentrations

681 [µM], and (c) nitrogen fixation [nmol N L^{-1} d^{-1}] at the surface during each cruise.



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683

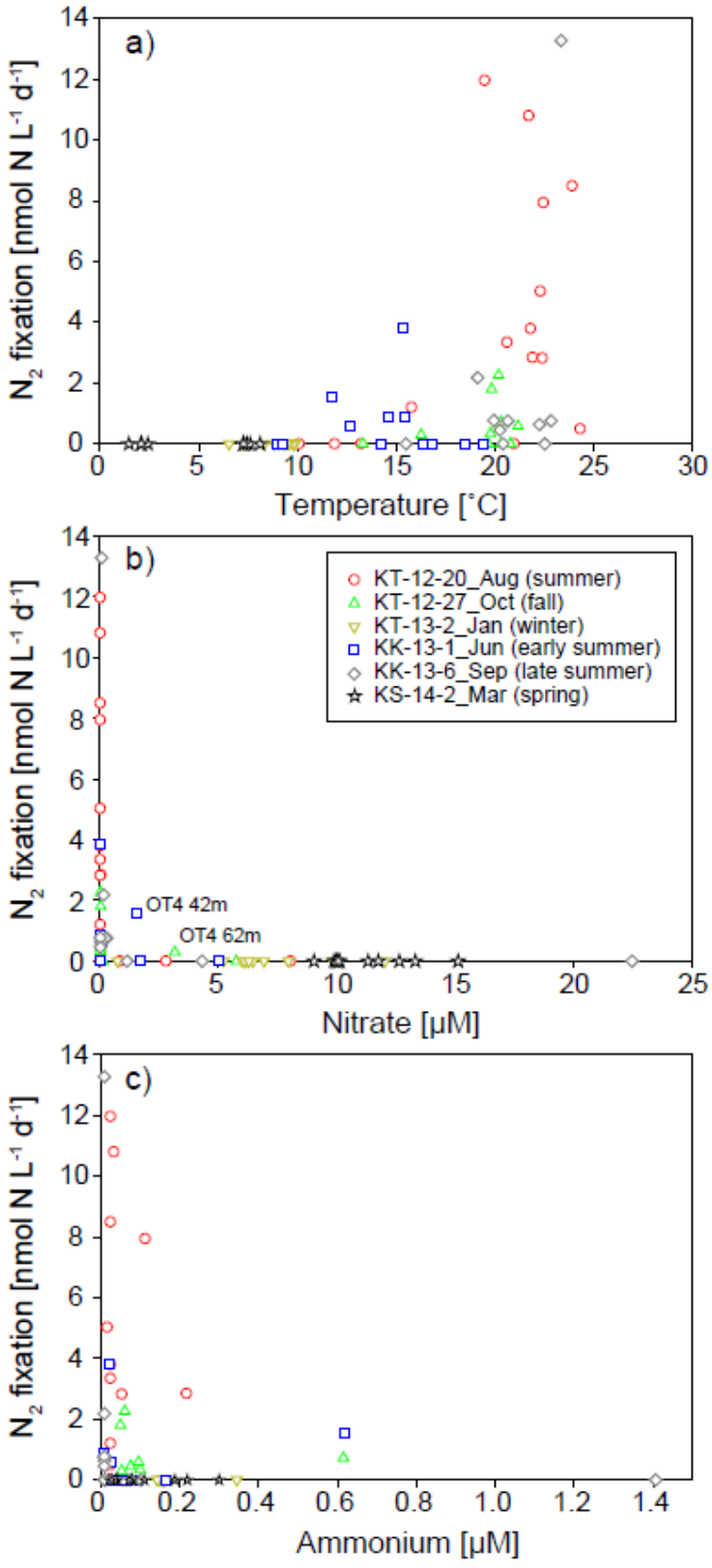
684 Fig. 5. Time-series variations in the vertical profiles of temperature [ºC] (black),

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

686 N L^{-1} d^{-1}] at Stns (a) OT4 and (b) ON5. Open symbols indicate that nitrogen fixation was

687 not detected. The horizontal dashed line indicates the nitracline depth. The strait lines of

688 temperature and nitrate were ascribable to strong mixing.

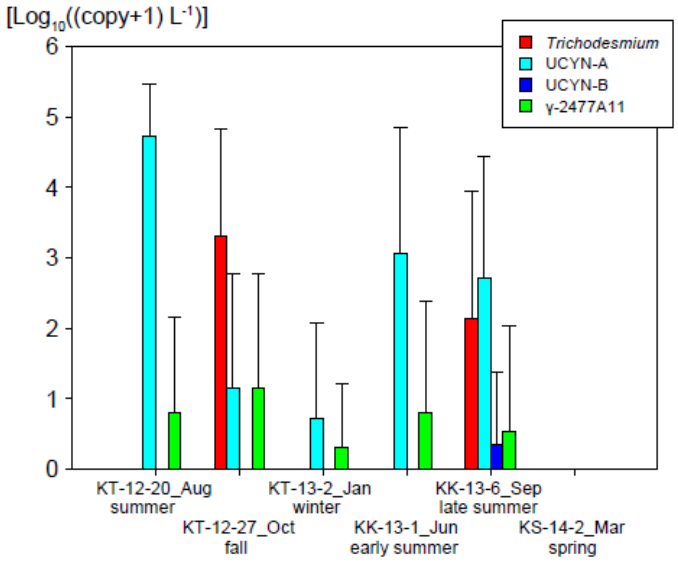


689

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

691 nitrate [µM], and (c) ammonium [µM] for all six cruises.

692



693

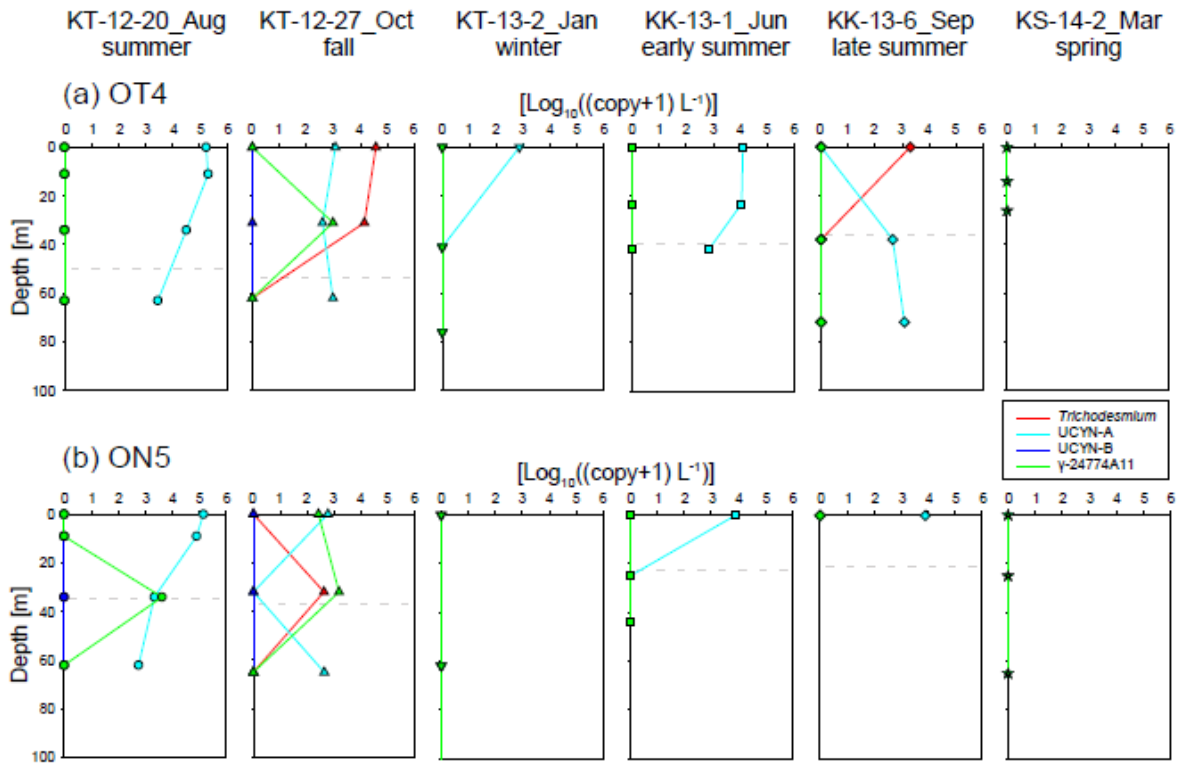
694

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

696 (blue), and γ-24774A11 (green) [Log_{10}((copy+1) L^{-1})] at the surface during each cruise.

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

698



699

700

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

702 (light blue), UCYN-B (blue), and γ-24774A11 (green) [Log_{10}((copy+1) L^{-1})] at Stns. (a) OT4

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