# 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 14.2º C (but was lower than 24.3º C) and the surface nitrate concentration was low (≤0.30 μM), although we also detected nitrogen fixation in subsurface layers (42–62 m)

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

## 1. Introduction

The amount of bioavailable nitrogen introduced into the global ocean via nitrogen fixation is considered to be roughly balanced at the large spatiotemporal scale by nitrogen loss through denitrification, as indicated by the sedimentary nitrogen isotope record during the Holocene epoch (Brandes and Devol, 2002; Deutsch et al., 2004). However, 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 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 (<~20ºC),
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 (<10ºC) 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 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

The experiments were conducted during six cruises in the temperate coastal region of the western North Pacific. These cruises covered a full seasonal cycle, including spring (KS-14-2_Mar, 14–19 March 2014), early summer (KK-13-1_Jun, 24–29 June 2013), summer (KT-12-20_Aug, 7–12 August 2012), late summer (KK-13-6_Sep, 14–21 September 2013), fall (KT-12-27_Oct, 15–22 October 2012), and winter (KT-13-2_Jan, 19–25 January 2013). Sampling stations were located along the transect lines OT (39º20’N, 141º56’–142º50’E) and ON (38º25’N, 141º29’–142º20’E). Eight stations were located offshore (OT4–6, ON4–8), while two stations were deployed in the Otsuchi (OT1) and Onagawa (ON1) bays (Fig. 1). Just before the KK-13-6_Sep cruise, Typhoon Man-yi passed from southwest to northeast in the study area (Fig. S1).

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

depths corresponding to 10% and 1% of the surface light intensities at Stns. OT4 and ON5. Light attenuation was determined using a submersible PAR sensor.

### 2.1. Nutrients

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

### 2.2. 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 $^{15}N_2$ gas 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 were 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 not detected, 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 (-80º C) until onshore analysis. Total DNA was extracted

using a ChargeSwitch Forensic DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) with slight modification of the manufacturer's protocol (Shiozaki et al., 2014a). Partial *nifH* fragments were amplified using a nested PCR strategy (Zehr and Turner, 2001) from samples collected from surface water at Stns. OT4, ON1, ON5, and ON7 during the KT-12-20_Aug and KT-12-27_Oct cruises, at Stns. OT4, ON1, and ON5 during the KT-13-2_Jan and KS-14-2_Mar cruises, at Stns. OT4, ON1, ON5, and ON8 during the KK-13-1_Jun cruise, and at Stns OT4, ON5, ON7 during the KK-13-6_Sep cruise (Table 1). PCR reagents were applied as described by Shiozaki et al. (2014a). The first and second PCRs were run using the same cycling conditions: 95º C for 30 s followed by 30 cycles of 98º C for 10 s, 52º C for 30 s, and 72º C for 30 s; followed by a final extension at 72º C for 7 min. Sterile distilled water was used as the negative control. After PCR analysis, we confirmed there was no band in agarose gel of electrophoresis from the negative control. The PCR products were cloned and sequenced according to Shiozaki et al. (2014a). The present study obtained 197 *nifH* sequences in total. The *nifH* sequences were translated into amino acid sequences and searched against the protein database of the National Center for Biotechnology Information using the BLASTp algorithm. Clones with 100% amino acid sequence similarity were defined as the same operational taxonomic unit (OTU) using the CD-HIT suite (Huang et al., 2010). The amino acid sequences were aligned using multiple sequence comparisons by the

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

2.4.2. 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 duing these seasons. In addition, UCYN-B which is considered to be a major diazotroph in the tropical and subtropical oligotrophic ocean (Moisander et al., 2010), was quantified. TaqMan primer and probe sets previously designed for these four *nifH* phylotypes were used for quantification (Shiozaki et al., 2014a,c). The 20 µL qPCR reactions contained 10 µL 2 × Premix Ex Taq (Probe qPCR; Takara), 5.6 µL of nuclease-free water, 1 µL each of the forward and reverse primers, 0.4 µL of TaqMan probe, and 2 µL of template DNA. The

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

## 3. RESULTS

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

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

### 3.2. Seasonal variations in nitrogen fixation rates

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

Sea surface temperatures (SST) (range, 1.5 to 24.3º C) (Figs. 4a and S1) and surface nitrate and phosphate concentrations determined during each cruise were averaged to emphasize the seasonal variability of these parameters (Fig. 4b). In general, surface nitrate and phosphate concentrations were low (≤0.07 µM and ≤0.20 µM, respectively) in the warmer seawaters (14.2–24.3º C) sampled in early summer (KK-13-1_Jun), summer (KT-12-20_Aug), and fall (KT-12-27_Oct), whereas they were relatively high (≥0.75 µM and ≥0.28 µM, respectively) in the colder seawaters (1.5–9.8º C) sampled 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. The seasonal variation pattern of the average ammonium concentration at the surface differed from those of nitrate and phosphate concentrations (Fig. 4b), characterized by low concentrations (≤ ~1 μM) throughout the year. The high variation in surface ammonium concentration during the KK-13-6_Sep cruises were due to relatively high ammonium concentrations at Stn. OT1 (1.41 μM) (Fig. S2).

Nitrogen fixation was detected in the surface waters of most samples collected during the four cruises conducted in early summer (KK-13-1_Jun), summer (KT-12-20_Aug), late summer (KK-13-6_Sep), and fall (KT-12-27_Oct), and varied in the range of 0.33–13.6 nmol N $L^{-1}$ $d^{-1}$ (Figs. 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 not detected in seawater samples collected during the winter and spring cruises, even after addition of mannitol (KS-14-2_Mar). Furthermore, nitrogen fixation was not detected in DIN-replete water at

235 Stn. OT1 in late summer (KK-13-6_Sep).

236 The rates of nitrogen fixation in samples collected at different depths (0–119 m) were
237 examined at Stns. OT4 and ON5 (Fig. 5). Nitrogen fixation was detectable only during the
238 four cruises conducted in early summer (KK-13-1_Jun), summer (KT-12-20_Aug), late
239 summer (KK-13-6_Sep), and fall (KT-12-27_Oct), the same seasons during which surface
240 nitrogen fixation was observed (Fig. 4). Nitrogen fixation rates tended to be higher at the
241 surface than in the deeper layers during summer (KT-12-20_Aug) and late summer
242 (KK-13-6_Sep (at Stn. OT4)), whereas this vertical trend was less evident during fall
243 (KT-12-27_Oct) and early summer (KK-13-1_Jun). At Stn. OT4, nitrogen fixation was
244 detectable even in deeper layers below the nitracline, where nitrate concentrations were
245 relatively high (KT-12-27_Oct, depth = 62 m; KK-13-1_Jun, depth = 42 m). In this layer,
246 the ammonium concentrations were 0.05 μM (KT-12-27_Oct) and 0.62 μM (KK-13-1_Jun).
247 The nitrogen fixation rate below the nitracline (1.56 nmol N $L^{-1}$ $d^{-1}$) was higher than that at
248 the surface (0.87 nmol N $L^{-1}$ $d^{-1}$) during the KK-13-1_Jun cruise. The maximum
249 depth-integrated nitrogen fixation (294 μmol N $m^{-2}$ $d^{-1}$) was observed at Stn. OT4 during
250 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 11.7º C, with
255 higher rates (>6 nmol N $L^{-1}$ $d^{-1}$) noted in waters warmer than 19.5º 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 15.4º C, while it was low (undetectable) at higher temperatures.
259 Nitrogen fixation rates were negatively correlated with nitrate and phosphate concentrations
260 ($p < 0.01$) (Table 2). There was no significant correlation between nitrogen fixation rates
261 and ammonium concentration ($p > 0.05$). We also found no significant correlation between
262 nitrogen fixation rates and the ratio of total inorganic nitrogen (nitrate + nitrite + ammonium)
263 to phosphate (Table 2). A plot of the nitrogen fixation against nitrate concentrations
264 indicated that nitrogen fixation was generally detectable only when nitrate was depleted (Fig.
265 6b), except that relatively high nitrogen fixation rates were determined in the subsurface layer
266 of Stn. OT4 (KT-12-27_Oct and KK-13-1_Jun). Active nitrogen fixation tended to occur at
267 low ammonium concentration $\leq$ ~0.1 μM. However, seasonal variation in ammonium
268 concentration was small and no statistically significant relationship with nitrogen fixation
269 was observed (Fig. 6c).
270

## 3.4. Seasonal variation in the diazotroph community

### 3.4.1. Diazotroph community

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

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

The detected cyanobacterial sequences belonged to *Trichodesmium*, UCYN-A, and *Leptolyngbya*. The *nifH* sequences of UCYN-B, UCYN-C, and *Richelia intracellularis* were not recovered. The *nifH* sequence of *Trichodesmium* was recovered only during the KT-12-27_Oct cruise (Table 1). UCYN-A was generally observed from early summer to fall, while *nifH of Leptolyngbya* was detected during winter. During the KS-14-2_Mar

289 cruise, all recovered sequences were derived from heterotrophic bacteria, and were
290 dominated by Cluster III diazotrophs at Stns. OT4 and ON5. The Cluster III diazotroph
291 *nifH* sequences were recovered on all cruises except the KK-13-1_Jun cruise. Note that 58
292 out of 187 sequences displayed >97% similarity, at the amino acid level, to terrestrial
293 diazotroph sequences derived from soil, mudflats, and lakes (Fig. S3, S4, and S5). These
294 sequences were mainly affiliated with α- and δ-proteobacterial diazotrophs, with 29 of 39
295 α-proteobacterial sequences and 22 of 24 δ-proteobacterial sequences being similar to
296 terrestrial diazotroph sequences.
297 3.4.2. Diazotrophs abundances
298 The *nifH* sequence of *Trichodesmium* was detected by qPCR assay during the KT-12-27_Oct
299 and KK-13-6_Sep cruises (Fig. 7 and 8). During these two cruises, the abundance of
300 *Trichodesmium* ranged from below the detection limit to $8.7 \times 10^4$ copies $L^{-1}$ at all depths.
301 *Trichodesmium* abundance at the surface was higher than those of UCYN-A, UCYN-B, and
302 γ-24774A11 at most stations during the KT-12-27_Oct cruise (Fig. 7 and S6). UCYN-A
303 was detected on all cruises except for the KS-14-2_Mar cruise (Fig. 7 and 8). The
304 maximum abundance of UCYN-A generally occurred at the surface except at Stn. OT4
305 during the KK-13-6_Sep cruise where the peak ($1.2 \times 10^3$ copies $L^{-1}$) was observed at 72 m
306 (Fig. 8). The abundance of UCYN-A varied from below the detection limit to $2.6 \times 10^5$

307 copies $L^{-1}$ at all depths. At the surface, UCYN-A was the most abundant among the four
308 groups at most of the stations investigated during the KT-12-20_Aug, KT-13-2_Jan,
309 KK-13-1_Jun, and KK-13-6_Sep cruises (Fig. 7 and S6). UCYN-B was detected only at
310 Stn. ON7 during the KK-13-6_Sep cruise (Fig. 7, 8, and S6). γ-24774A11 was detected
311 during all cruises except for the KS-14-2_Mar cruise (Fig. 7 and 8). The abundance of
312 γ-24774A11 ranged from below the detection limit to $1.8 \times 10^4$ copies $L^{-1}$, with a tendency of
313 a subsurface peak at both stations (Fig. 8).
314

315 ## 4. DISCUSSION

316 ### 4.1. Seasonal variations in nitrogen fixation rates in the temperate coastal
317 ocean

318 Nitrogen fixation rates were measurable mainly from early summer to fall when nitrate was
319 generally depleted in sample seawaters, although there were some exceptions. Our
320 estimates of the nitrogen fixation rates (0.33–13.6 nmol N $L^{-1}$ $d^{-1}$) were significantly ($p$
321 <0.05) higher than the corresponding values previously reported in the temperate region of
322 the eastern North Pacific (0.15–0.31 nmol N $L^{-1}$ $d^{-1}$; Needoba et al., 2007) and the
323 oligotrophic region of the western and central North Pacific (0.17–3.62 nmol N $L^{-1}$ $d^{-1}$;
324 Shiozaki et al., 2010), whereas they were comparable to those determined in the Kuroshio

(0.54–28 nmol N $L^{-1}$ $d^{-1}$; Shiozaki et al., 2010) and the western Atlantic coastal regions (1.3–49.8 nmol N $L^{-1}$ $d^{-1}$; Mulholland et al., 2012). Higher nitrogen fixation rates have been determined in other temperate oceans, including the western English Channel (18.9±0.01 and 20.0 nmol N $L^{-1}$ $d^{-1}$; Rees et al., 2009) and the Baltic Sea estuaries (47–83 nmol N $L^{-1}$ $d^{-1}$; Bentzon-Tilia et al., 2015).

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

Blais et al. (2012) proposed that nitrogen fixation can proceed even in nutrient-replete waters,

343 if large amounts of iron and organic materials are available for consumption by bacterial
344 diazotrophs. In the present study, this possibility was examined by conducting mannitol
345 addition experiments using surface seawaters collected during spring. These waters,
346 belonging to the Oyashio Current system (Nishioka et al., 2007, 2011; Shiozaki et al., 2014b),
347 were considered to be rich in iron during spring, as indicated by a previous study (iron conc.,
348 0.79–8.46 nM; Nishioka et al. 2007). Despite potentially high iron concentrations, our
349 results showed that nitrogen fixation was undetectable even after the mannitol addition,
350 suggesting that, contrary to the Blais et al. proposition, diazotrophs remained inactive under
351 our experimental settings.
352 Our data showed that nitrogen fixation rates were below the detection limit during winter,
353 spring, and late summer (KK-13-6_Sep), when nitrate concentrations were high. These
354 results were consistent with the results of previous studies in the Pacific Ocean, which
355 indicated that nitrogen fixation rates were low or undetectable in DIN-replete waters
356 (Shiozaki et al., 2010). In contrast, Mulholland et al. (2012) reported that, in temperate
357 regions of the Atlantic Ocean, nitrogen fixation rates were high even in DIN-replete (>1 μM)
358 and cold (<10ºC) surface seawaters. Their study was conducted downstream of the Gulf
359 Stream, where diazotrophs could be delivered from subtropical oceans where DIN is depleted.
360 Previous studies have suggested that cyanobacterial diazotrophs can travel over long

361 distances (>1,000 km) in currents, without losing their capacity for $N_2$ fixation (Shiozaki et
362 al., 2013), and that activity is not lost immediately even after mixing with DIN-replete
363 seawaters (Holl and Montoya, 2005; Dekaezemacker and Bonnet, 2011). In our region,
364 because the Tsugaru Warm Current flows from north to south, diazotrophs entrained by the
365 current have little chance of meeting high-DIN water at the surface. DIN-replete water
366 during summer was observed at the inside bay station OT1 (Fig. S2). Concomitantly,
367 low-salinity surface waters spread offshore along the OT transect line (Fig. S7), suggesting
368 that anomalously high DIN concentrations were likely attributable to terrestrial surface
369 discharge enhanced by Typhoon Man-yi, which passed over the region immediately before
370 the cruise. Subramaniam et al. (2008) reported that nitrogen fixation rates near the Amazon
371 River estuary, with low salinity and high nitrate levels, were fairly low. Their results are
372 consistent with ours. Ammonium inhibits nitrogen fixation, especially when ammonium
373 concentrations exceed 1 μM, as demonstrated by *Trichodesmium* (Mulholland et al. 2001).
374 In our study, no negative relationship between nitrogen fixation and ammonium
375 concentration was found. This can likely be explained by relatively low ammonium
376 concentrations (≤ ~1 μM) throughout the year and across the investigated region. Nitrogen
377 fixation was also negatively correlated with phosphate as with nitrate, and positively
378 correlated with temperature (Table 2). Since correlation between nitrate, phosphate, and

379 temperature was significant, all these factors would not necessarily influence nitrogen
380 fixation directly. Rather, one or more the factors that varied with nitrate could
381 synergistically influence nitrogen fixation.
382
383 **4.2. Seasonal variation in the diazotroph community in the temperate**
384 **coastal ocean**
385 The qPCR analysis demonstrated that the target groups were quantifiable even at stations at
386 which their sequences were not recovered by the clone library analysis, suggesting that the
387 number of clones was not sufficient to capture the diazotroph community structure on each
388 cruise. Despite this limitation, the sequences more frequently recovered in the clone library
389 generally corresponded to the most abundant group revealed by the qPCR analysis. For
390 example, UCYN-A was frequently recovered in the library during the KT-12-20_Aug,
391 KK-13-1_Jun, and KK-13-6_Sep cruises; for these samples, the qPCR results showed that
392 UCYN-A was the most abundant group among the four examined. Similarly, qPCR data
393 indicated that *Trichodesmium* was the most abundant group during fall, when this group was
394 frequently recovered in the library (during the KT-12-27_Oct cruise). Therefore, the
395 diazotrophs targeted by the qPCR analysis were likely important for nitrogen fixation in this
396 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 by qPCR 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). UCYN-A was detected by qPCR even during winter at some stations, yet, was not observed during spring. This could be because UCYN-A abundance decreased from fall to winter with decreasing temperatures, eventually disappeared in spring.

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

415 from the adjacent subtropical region where seawater temperatures were high (>24º C). In
416 the western North Pacific subtropical region, *Trichodesmium* is abundant from July to
417 September (Marumo and Nagasawa, 1976; Chen et al., 2008). *Trichodesmium* that
418 flourished in the subtropical region during summer could be transported by the Tsugaru
419 Warm Current, displaying peak abundance during fall in the investigated region. This could
420 support the above discussion that waters containing active nitrogen fixation were delivered to
421 this region by the Tsugaru Warm Current.
422 We observed γ-24774A11 by qPCR analysis during all cruises except for the KS-14-2_Mar
423 cruise. This phylotype has not been reported previously in other temperate oceans
424 (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012). The *nifH* sequence of
425 γ-24774A11 was similar to that of *Pseudomonas stutzeri* (94% similarity at the amino acid
426 level), which was observed in waters including temperate regions (Bentzon-Tilia et al., 2015).
427 Bentzon-Tilia et al. (2015) reported that *P. stutzeri*-like *nifH* genes (99% similarity at the
428 nucleotide level) were the most abundant sequences among their samples collected from the
429 temperate Baltic Sea estuary. In the present study, we recovered *P. stutzeri*-like *nifH* genes
430 (>97% similarity at the amino acid level) from Stn. OT4 during the KT-13-2_Jan cruise by
431 the clone library analysis. However, γ-24774A11 was not detected on that occasion by
432 qPCR analysis, suggesting that γ-24774A11 was not quantified as *P. stutzeri* and that *P.*

433 *stutzeri* could not be a major diazotroph in this study region. The ecology of γ-24774A11 is
434 still fairly unknown. It remains to be seen, in future studies whether this phylotype
435 contributes to the nitrogen fixation in this region.
436 UCYN-B was not detected by qPCR except at one station. This result is consistent with
437 previous knowledge. UCYN-B becomes abundant with increasing temperature, similar to
438 *Trichodesmium* (Moisander et al., 2010), and is rarely observed in the temperate region
439 (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012; Bentzon-Tilia et al., 2015).
440 Furthermore, UCYN-B abundance is low in shallow nitracline regions (Shiozaki et al.,
441 2014a,c). The nitracline depth in this region (≤60 m) was shallower than that of >100-m
442 depths of regions where UCYN-B is abundant (Shiozaki et al., 2014a). Therefore, although
443 UCYN-B might also have been delivered from subtropical region, it could not survive in the
444 shallower nitracline region.
445 In nitrate-rich water during winter and spring, Cluster III diazotrophs were detected at most
446 of the stations. Furthermore, from early summer to fall, *nifH* sequences of Cluster III
447 diazotrophs were recovered by the clone library analysis in samples from all cruises (except
448 KK-13-1_Jan). Therefore, Cluster III diazotrophs likely presented throughout a year.
449 Cluster III diazotrophs are putative anaerobes (Hamersley et al., 2011; Farnelid et al., 2013;
450 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 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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647

Table 1. Summary of recovered *nifH* sequences belonging to *Trichodesmium* (Tri), UCYN-A (UA), Leptolyngbya (Lep), α-proteobacteria (α-Pro), β-proteobacteria (β-Pro), 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** |

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

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

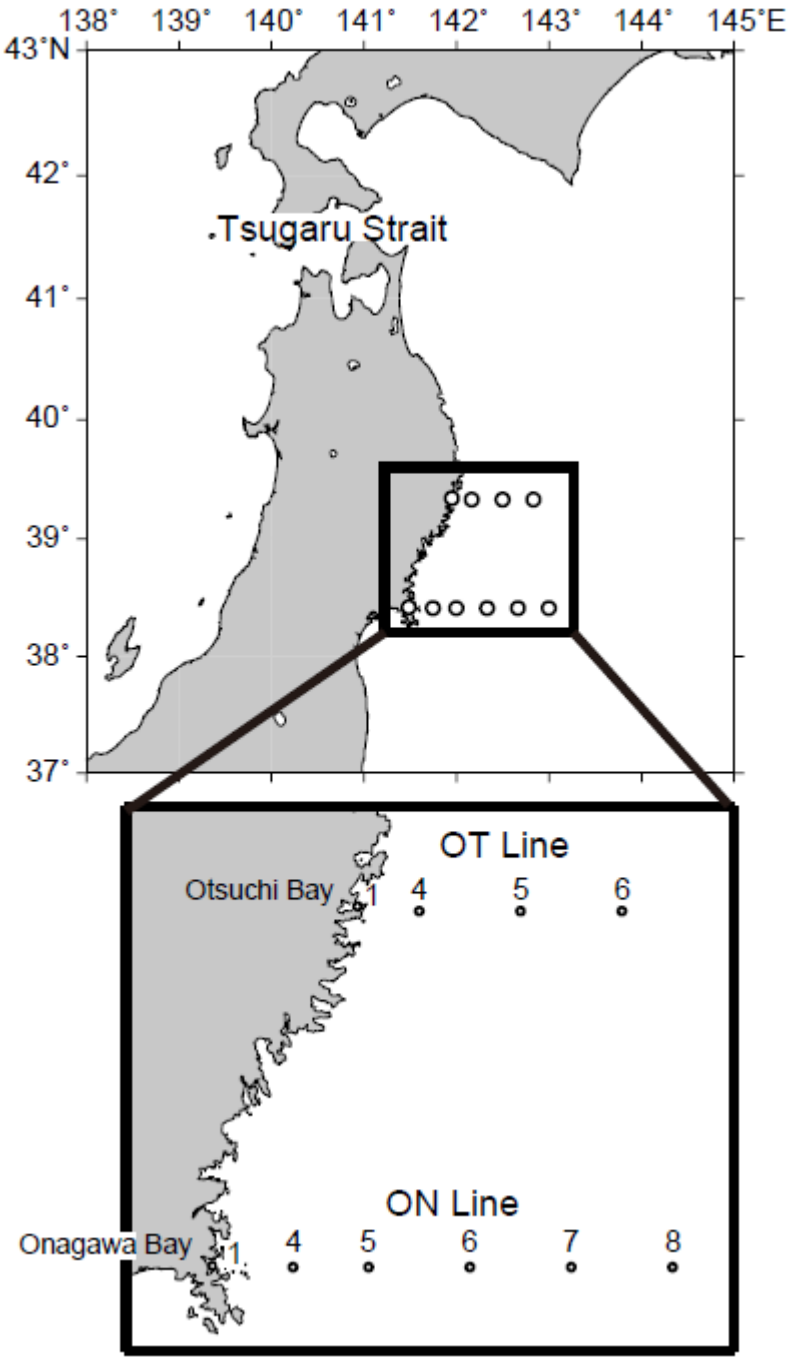
654 water column (n=73).

| | 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 |

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

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

657

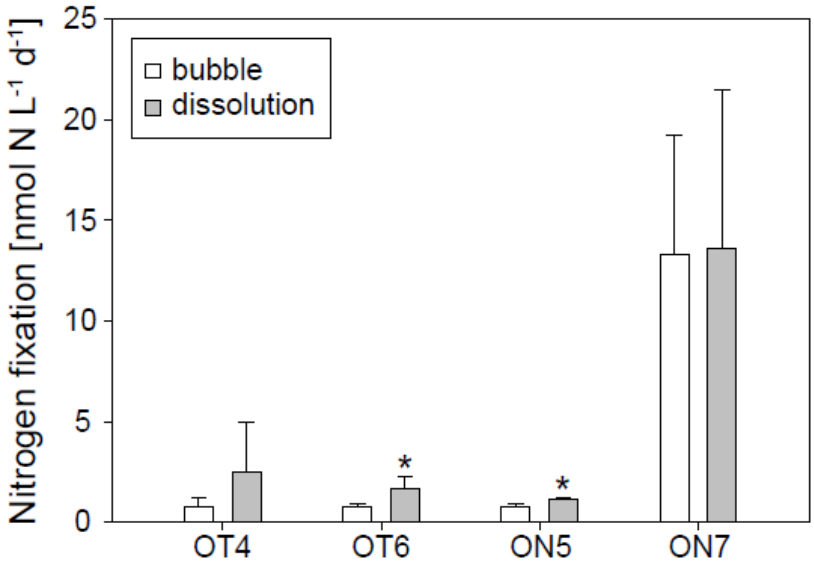



658

659

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

661

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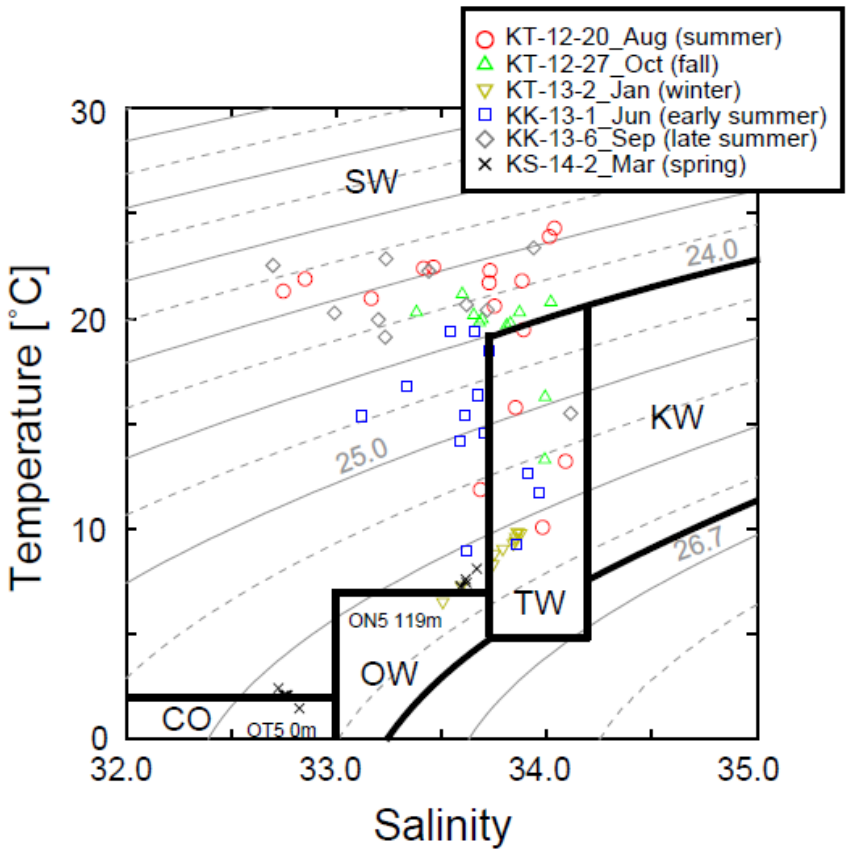
663

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

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

666 difference between the two methods ($p$ <0.05).

667

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669

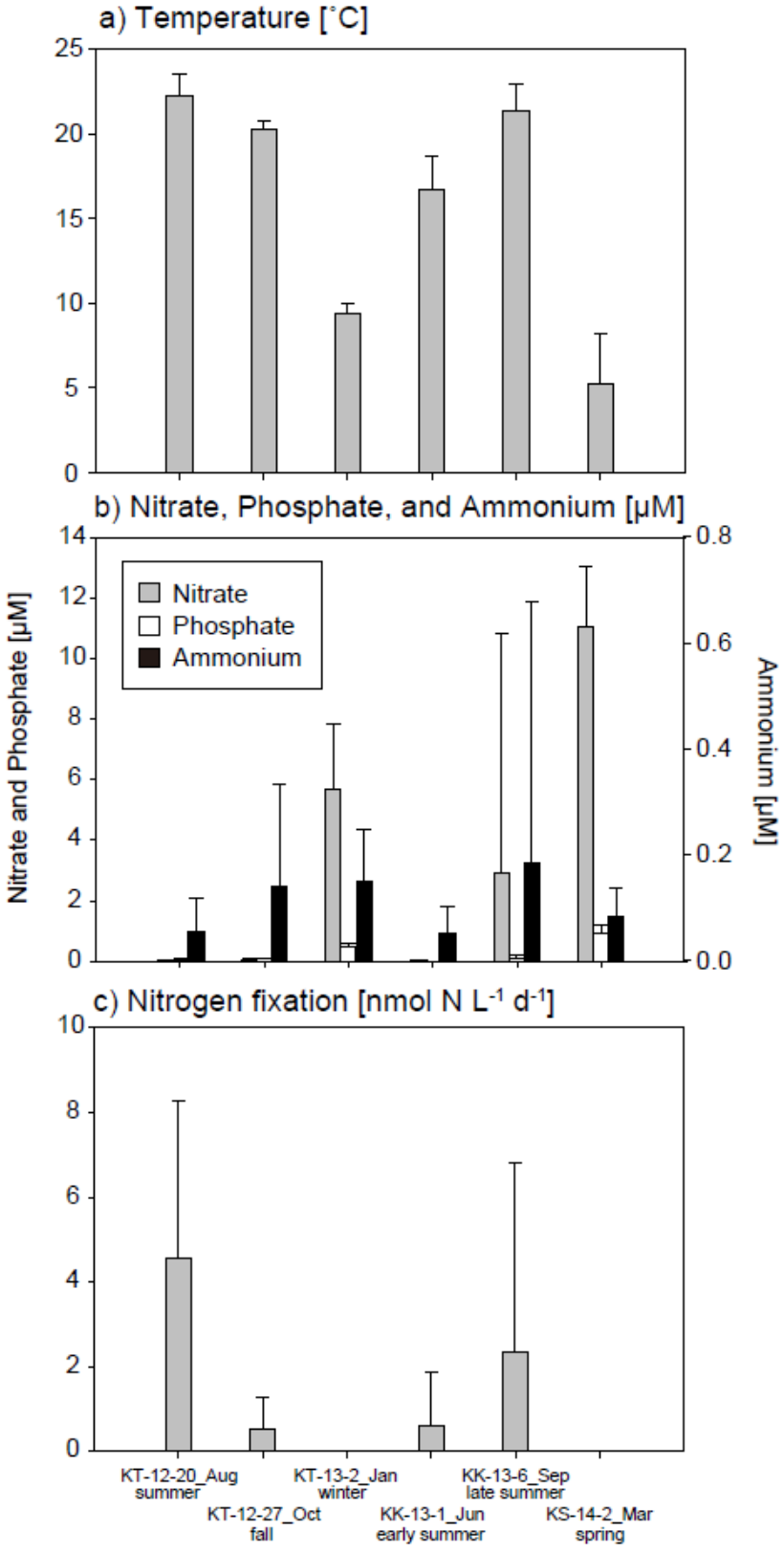
670 Fig. 3. Temperature-salinity diagram at each sampling point. The water classification was

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

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

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

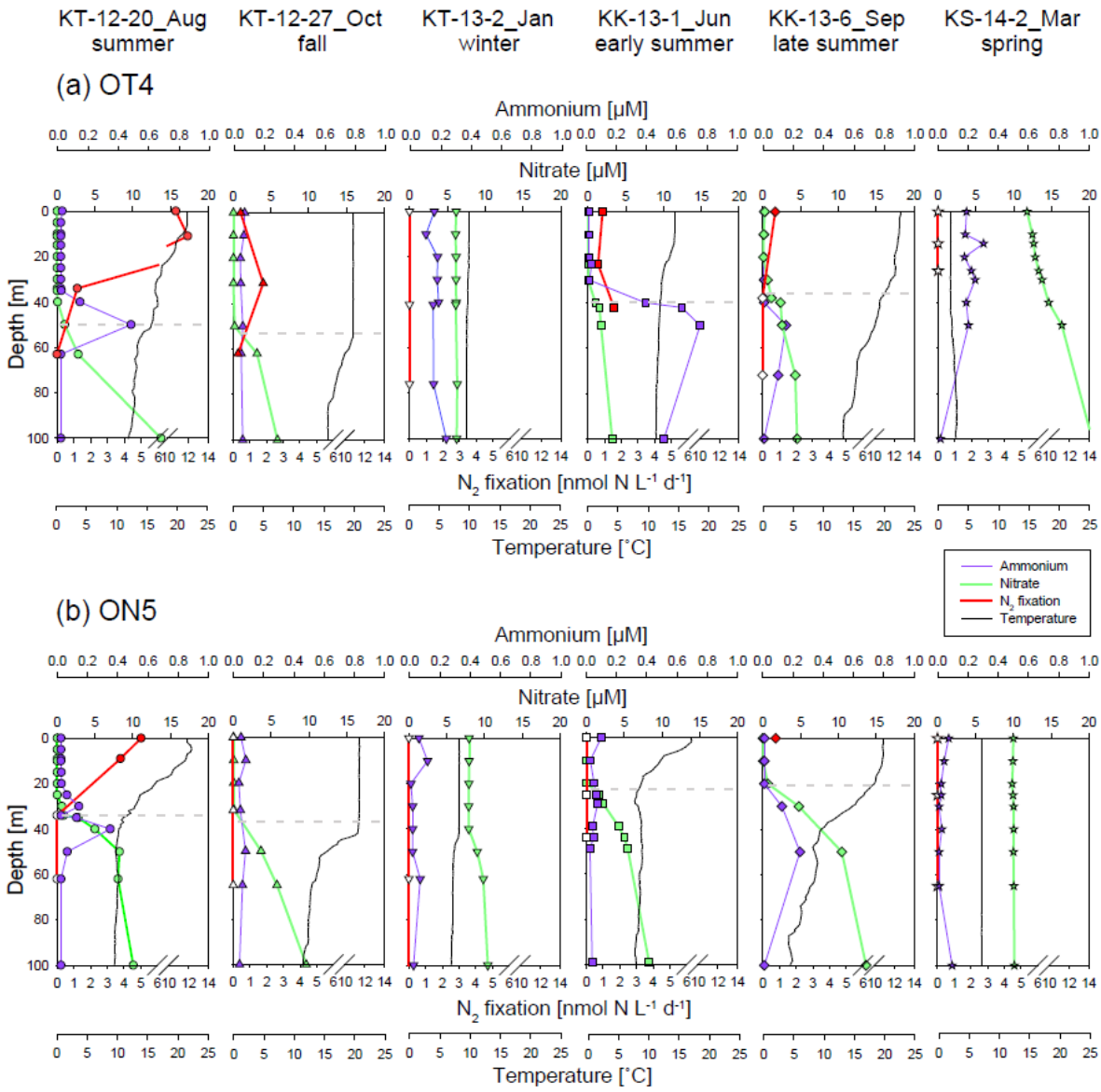
674




675

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

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

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679

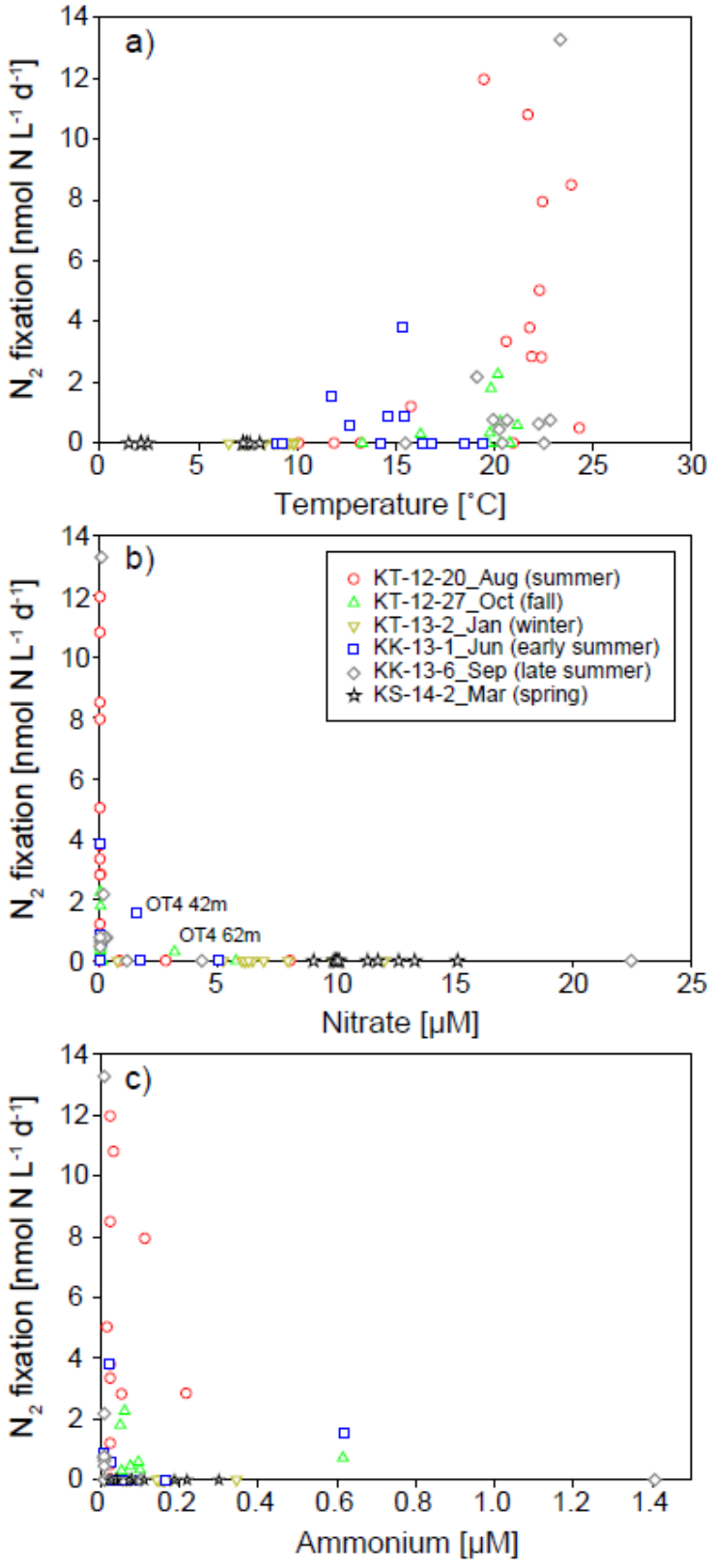
680 Fig. 5. Time-series variations in the vertical profiles of temperature [ºC] (black),

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

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

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

684 temperature and nitrate were ascribable to strong mixing.

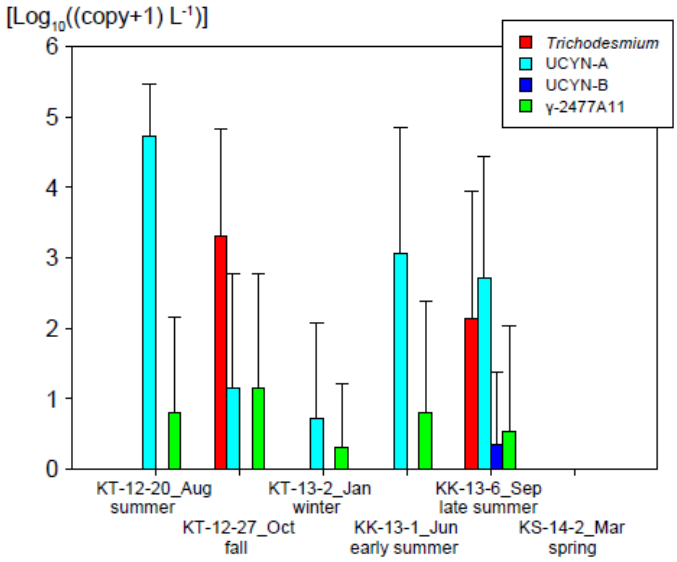



685

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

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

688

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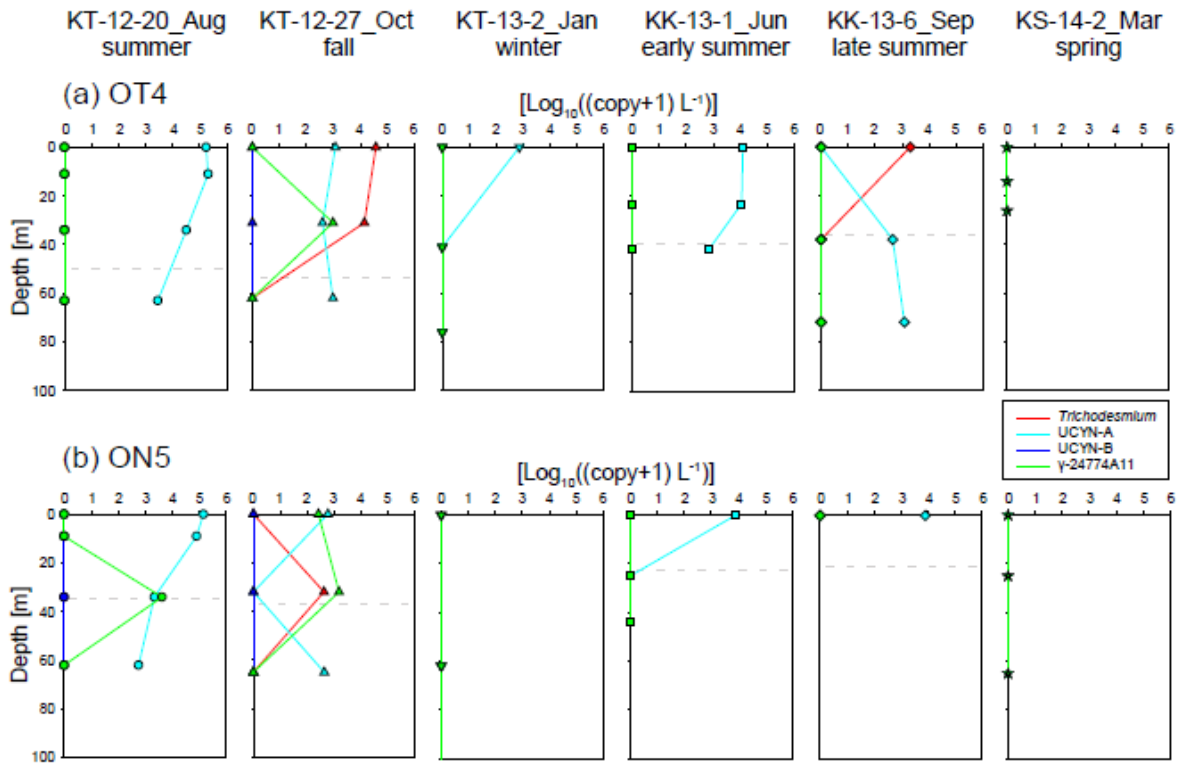
690

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

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

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

694




695

696

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

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

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