Referee #2

General comment:

The manuscript improved significantly after the previous revisions. Although insufficient sequence number of clone library is the major limitation of this study, addition of qPCR of some nifH phylotypes is helpful in compensating the limitation. As limited amount of relevant studies have been done in temperate regions of North Pacific Ocean, it is difficult for the authors to further elaborate and discuss with this set of data in a convincing way. On another hand, the data set could provide useful information for the related studies in the future. Therefore, I think this work can be accepted, after some minor revisions. There are some grammatical mistakes found in the manuscript, and the authors should do proof-reading more carefully.

We have checked the manuscript thoroughly and have revised unclear wordings. Furthermore, the English in the revised manuscript has been check by a professional editor, a native speaker of English.

Specific comments:

L.141, What does "when nitrogen fixation was not detected" mean? Did the authors mean "when nitrogen fixation was undetectable" or "not measured"? If the nitrogen fixation rate was not measured, the authors should not assume the missing data to be zero. The authors should clarify their meaning here.

We have corrected the sentence to "when nitrogen fixation was undetectable". (L140)

L.199, "underestimates" should be "underestimated".

We have corrected as suggested. (L195)

L.401, "one or more the factors" should be "one or more factors"

We have corrected as suggested. (L384)

L. 432, It is suggested to use "undetectable" to replace "disappear"

We have changed the sentence as follows. (L411)

"It appears that UCYN-A abundance decreased with decreasing temperature from fall to winter, and then became undetectable in spring."

L.455-457, The logic here is not clear enough. Since the author did not use qPCR to quantify the P. stutzeri-like nifH gene, there is no reason to say "P. stutzeri could not be a major diazotroph in this study region". Also, "γ-24774A11 was not detected on that occasion by qPCR analysis" should not be the evident suggesting "γ-24774A11 was not quantified as P. stutzeri". The authors can simply compare the sequences of γ-24774A11 and P. stutzeri nifH gene recovered in this study.

We have corrected the sentence as follows. (L436-437)
"γ-24774A11 was not detected on that occasion by qPCR analysis probably due to the difference in the sequence between γ-24774A11 and *P. stutzeri*."

L.496, It is suggested to add "coastal" after "temperate", as the study was conducted in coastal area.

We have added "coastal" as suggested. (L470)

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

T. Shiozaki[1,2], T. Nagata[1], M. Ijichi[1], K. Furuya[2]

[1]{Atmosphere and Ocean Research Institute, The University of Tokyo, Chiba, 277-8564, Japan}

[2]{Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, 113-8657, Japan}

Corresponding to: T. Shiozaki (shiozaki@aori.u-tokyo.ac.jp)

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

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

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

nitrogen fixation, presumably mediated by unicellular cyanobacteria and heterotrophic bacteria, is detectable even in the relatively cold (<10°C) and DIN-rich waters (>1 μM) of the Atlantic coast (Mulholland et al., 2012) and the Baltic Sea estuaries (Bentzon-Tilia et al., 2015). These results highlight the necessity of re-evaluating the extent, variation, and control mechanisms of nitrogen fixation in temperate oceans, with recognition of the widespread occurrence of diverse diazotrophic microbes.

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

2. Materials and Methods

The experiments were conducted during six cruises in the temperate coastal region of the western North Pacific. These cruises covered a full seasonal cycle, including spring (KS-14-2_Mar, 14–19 March 2014), early summer (KK-13-1_Jun, 24–29 June 2013), summer (KT-12-20_Aug, 7–12 August 2012), late summer (KK-13-6_Sep, 14–21 September 2013), fall (KT-12-27_Oct, 15–22 October 2012), and winter (KT-13-2_Jan, 19–25 January 2013). Sampling stations were located along the transect lines OT (39°20'N, 141°56'–142°50'E) and ON (38°25'N, 141°29'–142°20'E). Eight stations were located offshore (OT4–6, ON4–8), while two stations were deployed in 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~~ 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 $^{15}N_2$ ~~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~~ 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 ~~not detected~~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 ~~there was no band in agarose gel of electrophoresis from~~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 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~~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~~ 20-μL qPCR reactions contained 10 μL 2 × Premix Ex Taq (Probe qPCR; Takara), 5.6

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

188 ## 3. RESULTS

189 ### 3.1. Comparison of the bubble method and the dissolution method

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

method, which was used as ~~a~~the standard protocol during all ~~the~~ cruises, the possibility that some of these rates could be underestimated must be kept in mind. ~~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~~underestimated~~.~~

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 mostly belonged mostly to either the surface layer water system (SW) or the Tsugaru Warm Current water system (TW) (Fig. 3). Exceptions included the~~, with the exception of~~ waters collected from the 1% light depth (119 m) at Stn. ON5 during the KT-13-2_Jan cruise (classified as the Oyashio water system (OW)) and those collected at the surface of OT5 during the KS-14-2_Mar cruise (classified as~~, 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~~ assumed that surface waters collected during the same cruise in a particular season generally

217 belonged to the same water system that was prevalent in the investigated region at the time of
218 our sampling.
219 Sea surface temperatures (SSTs) (range, 1.5 ~~to~~ 24.3 ~~°~~°C) (Figs. 4a and S1) and surface
220 nitrate and phosphate concentrations determined during each cruise were averaged to
221 ~~emphasize~~ indicate the seasonal variability of these parameters (Fig. 4b). In general, surface
222 nitrate and phosphate concentrations were low (≤0.07 μM and ≤0.20 μM, respectively) in the
223 warmer seawaters (14.2–24.3º C) sampled in early summer (KK-13-1_Jun), summer
224 (KT-12-20_Aug), and fall (KT-12-27_Oct), whereas they were relatively high (≥0.75 μM and
225 ≥0.28 μM, respectively) in the colder seawaters (1.5–9.8º C) sampled during winter
226 (KT-13-2_Jan), and spring (KS-14-2_Mar). During the KK-13-6_Sep cruise (late summer),
227 the nitrate concentrations were relatively high and variable (mean ± SD; 2.92 ± 7.90 μM).
228 This was because the highest nitrate concentration (22.6 μM) was determined at the
229 near-shore Stn. OT1 (Fig. S2). Similar to nitrate, surface phosphate concentrations tended
230 to be high during winter (KT-13-2_Jan) and spring (KS-14-2_Mar), while they were low
231 during the warmer seasons. ~~The seasonal variation pattern of the average~~ By contrast,
232 surface ammonium concentrations were generally low ~~at the surface differed from those of~~
233 ~~nitrate and phosphate concentrations (Fig. 4b), characterized by low concentrations~~ (≤ ~1
234 μM) throughout the year (Fig. 4b), except for the high ammonium concentration determined

at Stn. OT1 (1.41 μM) during . ~~The high variation in surface ammonium concentration during~~ the KK-13-6_Sep cruise~~s 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~~ D~~d~~uring 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 ~~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~~below the detection limit in seawater samples collected during the winter and spring cruises. For those samples, nitrogen fixation was ~~unmeasurable~~undetectable~~,~~ even after the addition of mannitol (KS-14-2_Mar). ~~Furthermore, n~~Also, nitrogen fixation was ~~not detected~~ ~~unmeasurable~~undetectable in ~~DIN-replete~~ a DIN-replete water collected at Stn. OT1 in late summer (KK-13-6_Sep).

~~The rates of nitrogen fixation in~~Nitrogen fixation rates were determined for samples collected from~~at~~ different depths (0–119 m) ~~were examined~~ at Stns. OT4 and ON5 (Fig. 5). Nitrogen fixation was detected~~able~~ in surface and deeper layers~~only~~ during ~~the~~ four cruises conducted

253 in early summer (KK-13-1_Jun), summer (KT-12-20_Aug), late summer (KK-13-6_Sep), and
254 fall (KT-12-27_Oct)~~, the same seasons during which surface nitrogen fixation was observed~~
255 (Fig. 4). Nitrogen fixation rates tended to be higher at the surface than in the deeper layers
256 during summer (KT-12-20_Aug) and late summer (KK-13-6_Sep (at Stn. OT4)), whereas this
257 vertical trend was less evident during fall (KT-12-27_Oct) and early summer (KK-13-1_Jun).
258 At Stn. OT4, nitrogen fixation was detected~~able~~ even ~~even in deeper layers~~in the layers below
259 the nitracline (KT-12-27_Oct, depth = 62 m; KK-13-1_Jun, depth = 42 m). During
260 KK-13-1_Jun cruise, the nitrogen fixation rate determined at the depth of 42 m (1.56 nmol N
261 L^{-1} d^{-1}) was 1.8 fold higher than the corresponding rate at the surface (0.87 nmol N L^{-1} d^{-1}).
262 The concentrations of nitrate and ammonium in these layers varied in the range of
263 <0.02–22.5 µM and <0.01–1.41 µM, respectively. ~~, where nitrate concentrations were~~
264 ~~relatively high (KT-12-27_Oct, depth = 62 m; KK-13-1_Jun, depth = 42 m). In this layer,~~
265 ~~the ammonium concentrations were 0.05 µM (KT-12-27_Oct) and 0.62 µM (KK-13-1_Jun).~~
266 ~~The nitrogen fixation rate below the nitracline (1.56 nmol N L^{-1} d^{-1}) was higher than that at~~
267 ~~the surface (0.87 nmol N L^{-1} d^{-1}) during the KK-13-1_Jun cruise.~~ The maximum
268 depth-integrated nitrogen fixation (294 µmol N m^{-2} d^{-1}) was ~~observed~~ found at Stn. OT4
269 during summer (KT-12-20_Aug).

270 **3.3. Relationship between nitrogen fixation rates and environmental**

variables

Nitrogen fixation rates tended to increase with temperature ($p < 0.01$) (Fig. 6a and Table 2). Nitrogen fixation was detected only when seawater temperatures exceeded 11.7° C, with higher rates (>6 nmol N L^{-1} d^{-1}) noted in waters warmer than 19.5° C. However, there were exceptions to this general relationship between the nitrogen fixation rate and temperature. For example, from the data collected during the KK-13-1_Jun cruise the nitrogen fixation rate was highest at 15.4° C, while it was low (~~undetectable~~below the detection limit) at higher temperatures.

Nitrogen fixation rates were negatively correlated with nitrate and phosphate concentrations ($p < 0.01$) (Table 2), whereas they were not significantly correlated with . ~~There was no significant correlation between nitrogen fixation rates and~~ ammonium concentrations ($p > 0.05$) (Table 2). We also found no significant correlation between nitrogen fixation rates and the ratio of total inorganic nitrogen (nitrate + nitrite + ammonium) to phosphate (Table 2).

~~A plot of the nitrogen fixation against nitrate concentrations indicated that ni~~Nitrogen fixation was generally detectable only when nitrate was depleted (Fig. 6b), except that relatively high nitrogen fixation rates were determined in the subsurface layer of Stn. OT4 (KT-12-27_Oct and KK-13-1_Jun). ~~Active~~ High n~~n~~itrogen fixation rates tended to be detected when ammonium concentrations were low (~~occur at low ammonium concentration~~ ≤ ~0.1 μM),

although there was no statistically significant relationship between nitrogen fixation rates and ammonium concentrations. ~~However, seasonal variation in ammonium concentration was small and no statistically significant relationship with nitrogen fixation was observed (Fig. 6c).~~

3.4. Seasonal variation in the 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, some sequences recovered from the samples obtained during the KK-13-6_Sep and KS-14-2_Mar cruises (10 clones in total) were judged to be the contaminants in PCR reagents (~~with similarity (~~>97% similarity ~~)~~ at the amino acid level was used as a criterion). ~~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 the 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~~ recovered 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~~ recovered from early summer to fall, while *nifH of Leptolyngbya* was ~~detected~~ recovered during winter. The present study detected the sequences of γ-24774A11 during the KT-12-27_Oct and KK-13-6_Sep cruises. This heterotrophic bacterial phylotype is considered to significantly contribute to nitrogen fixation in a wide range of oceanic environments (Moisander et al., 2014). During the KS-14-2_Mar cruise, all ~~recovered~~ of the sequences that we recovered were derived from heterotrophic bacteria, and were dominated by Cluster III diazotrophs at Stns. OT4 and ON5. The Cluster III diazotroph *nifH* sequences were recovered during~~on~~ all cruises except for the KK-13-1_Jun cruise. Note that 58 out of 187 sequences displayed >97% similarity, at the amino acid level, to terrestrial diazotroph sequences derived from soil, mudflats, and lakes (Fig. S3, S4, and S5). These sequences were mainly affiliated with α- and δ-proteobacterial diazotrophs, with 29 of 39 α-proteobacterial sequences and 22 of 24 δ-proteobacterial sequences being similar to terrestrial diazotroph sequences.

325 3.4.2. Diazotrophs abundances

326 The *nifH* sequence of *Trichodesmium* was detected by qPCR assay during the KT-12-27_Oct
327 and KK-13-6_Sep cruises (Fig. 7 and 8). During these two cruises, the abundance of
328 *Trichodesmium* ranged from below the detection limit to 8.7×10^4 copies L^{-1} at all depths.
329 *Trichodesmium* abundance at the surface was higher than those of UCYN-A, UCYN-B, and
330 γ-24774A11 at most stations during the KT-12-27_Oct cruise (Fig. 7 and S6). UCYN-A
331 was detected on all cruises except for the KS-14-2_Mar cruise (Fig. 7 and 8). The
332 maximum abundance of UCYN-A generally occurred at the surface except at Stn. OT4
333 during the KK-13-6_Sep cruise where the peak (1.2×10^3 copies L^{-1}) was observed at 72 m
334 (Fig. 8). The abundance of UCYN-A varied from below the detection limit to 2.6×10^5
335 copies L^{-1} at all depths. At the surface, UCYN-A was the most abundant among the four
336 groups at most of the stations investigated during the KT-12-20_Aug, KT-13-2_Jan,
337 KK-13-1_Jun, and KK-13-6_Sep cruises (Fig. 7 and S6). UCYN-B was detected only at
338 Stn. ON7 during the KK-13-6_Sep cruise (Fig. 7, 8, and S6). γ-24774A11 was detected
339 during all cruises except for the KS-14-2_Mar cruise (Fig. 7 and 8). The abundance of
340 γ-24774A11 ranged from below the detection limit to 1.8×10^4 copies L^{-1}, with a tendency of
341 a subsurface peak at both stations (Fig. 8).

342

4. DISCUSSION

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

Nitrogen fixation rates were measurable mainly from early summer to fall when nitrate was generally depleted in sample seawaters, although there were some exceptions. Our estimates of the nitrogen fixation rates (0.33–13.6 nmol N L^{-1} d^{-1}) were significantly (p <0.05) higher than the corresponding values previously reported in the temperate region of the eastern North Pacific (0.15–0.31 nmol N L^{-1} d^{-1}; Needoba et al., 2007) and the oligotrophic region of the western and central North Pacific (0.17–3.62 nmol N L^{-1} d^{-1}; Shiozaki et al., 2010), whereas they were comparable to those determined in the Kuroshio (0.54–28 nmol N L^{-1} d^{-1}; Shiozaki et al., 2010) and the western Atlantic coastal regions (1.3–49.8 nmol N L^{-1} d^{-1}; Mulholland et al., 2012). Higher nitrogen fixation rates have been determined in other temperate oceans, including the western English Channel (18.9±0.01 and 20.0 nmol N L^{-1} d^{-1}; Rees et al., 2009) and the Baltic Sea estuaries (47–83 nmol N L^{-1} d^{-1}; Bentzon-Tilia et al., 2015).

In our study, spatiotemporal variability in 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

361 therefore enhance nitrogen fixation in our study region. This is supported by the fact that
362 nitrogen fixation rates during individual cruises tended to be higher at Stn. OT4 than at Stn.
363 ON5. These stations were located up- and down-stream of the Tsugaru Warm Current,
364 respectively. In addition, variations in nitrogen fixation rates among stations and seasons
365 might also be related to the extent of vertical mixing in the Tsugaru Warm Current. It has
366 been suggested that vertical mixing may introduce iron-rich subsurface water to the surface
367 of the Tsugaru Strait (Saitoh et al., 2008). Such input of iron may enhance nitrogen fixation
368 rates. Consistent with this notion, our results showed that the nitrogen fixation rate was
369 relatively high at Stn. OT4, where the nitracline was relatively deep.
370 Blais et al. (2012) proposed that nitrogen fixation can proceed even in nutrient-replete waters,
371 if large amounts of iron and organic materials are available for consumption by bacterial
372 diazotrophs. In the present study, this possibility was examined by conducting mannitol
373 addition experiments using surface seawaters collected during spring. These waters,
374 belonging to the Oyashio Current system (Nishioka et al., 2007, 2011; Shiozaki et al., 2014b),
375 were considered to be rich in iron during spring, as indicated by a previous study (iron conc.,
376 0.79–8.46 nM; Nishioka et al. 2007). Despite potentially high iron concentrations, our
377 results showed that nitrogen fixation was undetectable even after the mannitol addition,
378 suggesting that, contrary to the Blais et al. proposition, diazotrophs remained inactive under

379 our experimental settings.

380 Our data showed that nitrogen fixation rates were below the detection limit during winter,
381 spring, and late summer (KK-13-6_Sep), when nitrate concentrations were high. These
382 results were consistent with the results of previous studies in the Pacific Ocean, which
383 indicated that nitrogen fixation rates were low or undetectable in DIN-replete waters
384 (Shiozaki et al., 2010). In contrast, Mulholland et al. (2012) reported that, in temperate
385 regions of the Atlantic Ocean, nitrogen fixation rates were high even in DIN-replete (>1 μM)
386 and cold (<10ºC) surface seawaters. Their study was conducted downstream of the Gulf
387 Stream, where diazotrophs could be delivered from subtropical oceans where DIN is depleted.
388 Previous studies have suggested that cyanobacterial diazotrophs can travel over long
389 distances (>1,000 km) in currents, without losing their capacity for N_2 fixation (Shiozaki et
390 al., 2013), and that activity is not lost immediately even after mixing with DIN-replete
391 seawaters (Holl and Montoya, 2005; Dekaezemacker and Bonnet, 2011). In our region,
392 because the Tsugaru Warm Current flows from north to south, diazotrophs entrained by the
393 current have little chance of meeting high-DIN water at the surface. DIN-replete water
394 during summer was observed at the inside bay station OT1 (Fig. S2). Concomitantly,
395 low-salinity surface waters spread offshore along the OT transect line (Fig. S7), suggesting
396 that anomalously high DIN concentrations were likely attributable to terrestrial surface

discharge enhanced by Typhoon Man-yi, which passed over the region immediately before the cruise. Subramaniam et al. (2008) reported that nitrogen fixation rates near the Amazon River estuary, with low salinity and high nitrate levels, were fairly low. Their results are consistent with ours. Ammonium inhibits nitrogen fixation, especially when ammonium concentrations exceed 1 µM, as demonstrated for~~by~~ *Trichodesmium* (Mulholland et al. 2001). In our study, ammonium concentrations were generally low (≤ ~1 µM) throughout the investigation, and no negative relationship between nitrogen fixation and ammonium concentration was found. ~~. This can likely be explained by relatively low ammonium concentrations (≤ ~1 µM) throughout the year and across the investigated region.~~ Our data showing that nitrogen fixation rates were negatively correlated with nitrate concentrations (Table 2) are consistent with the general notion that nitrogen fixation rates are generally low in nitrate replete waters (Falkowski, 1983). Our data also showed nitrogen fixation rates tended to increase with increasing temperature and with decreasing phosphate concentrations (~~Nitrogen fixation was also negatively correlated with phosphate as with nitrate, and positively correlated with temperature (~~Table 2). ~~Since correlation between~~Because t~~-nitrate, phosphate, and t~~emperature and phosphate concentrations were correlated with nitrate concentrations, ~~was significant, all~~ these factors would not necessarily influence nitrogen fixation directly. Rather, one or more ~~the~~ factors that varied with nitrate could

synergistically influence nitrogen fixation.

4.2. Seasonal variation in the diazotroph community in the temperate coastal ocean

The qPCR analysis demonstrated that the target groups were quantifiable even at stations at which their sequences were not recovered by the clone library analysis, suggesting that the number of clones was not sufficient to capture the diazotroph community structure on each cruise. Despite this limitation, the sequences more frequently recovered in the clone library generally corresponded to the most abundant group revealed by the qPCR analysis. For example, UCYN-A was frequently recovered in the library during the KT-12-20_Aug, KK-13-1_Jun, and KK-13-6_Sep cruises; for these samples, the qPCR results showed that UCYN-A was the most abundant group among the four examined. Similarly, qPCR data indicated that *Trichodesmium* was the most abundant group during fall, when this group was frequently recovered in the library (during the KT-12-27_Oct cruise). 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 ~~by qPCR~~ in all seasons except spring (KS-14-2_Mar), suggesting that

433 this group of diazotrophs could be important agents of nitrogen fixation in this region.
434 Especially from early to late summer, the abundance of UCYN-A was generally higher than
435 that of *Trichodesmium*, UCYN-B, and γ-24774A11. UCYN-A has been widely detected in
436 temperate regions, and is considered to be one of the major diazotrophs of these locations
437 (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012; Bentzon-Tilia et al., 2015).
438 UCYN-A is known to be most abundant in relatively warm waters around ~20º C (Needoba
439 et al., 2007; Moisander et al., 2010). In our study, UCYN-A was detected ~~by qPCR even~~
440 during winter at some stations. It appears that ~~, yet, was not observed during spring. This~~
441 ~~could be because~~ UCYN-A abundance decreased with decreasing temperature from fall to
442 winter, and then became undetectable~~decreased from fall to winter~~ in spring. ~~with~~
443 ~~decreasing temperatures, eventually disappeared in spring.~~
444 *Trichodesmium* was detected from late summer to fall ~~by qPCR analysis~~, when water
445 temperatures ranged from 19.1 to 23.4º C at the surface. Given that the optimal growth
446 temperature for *Trichodesmium* has been reported to be high (24–30º C) (Breitbarth et al.,
447 2007), *Trichodesmium* detected in the investigated region likely existed under suboptimum
448 conditions. The relatively high abundance of *Trichodesmium* observed during fall, despite
449 the suboptimal temperature conditions, might indicate that *Trichodesmium* was transported
450 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 ~~observed~~ detected γ-24774A11 ~~by qPCR analysis~~ 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, ~~T~~th~~is phylotype~~e γ-24774A11 sequence has not been detected~~reported~~ previously in other temperate oceans (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012). The ~~The *nifH* sequence of~~ γ-24774A11 sequence was similar (94% similarity at the amino acid level) to the *nifH* sequence ~~at~~ of *Pseudomonas stutzeri*, which ~~was observed~~has been reported to be present in temperate estuaries~~waters including temperate regions~~ (Bentzon-Tilia et al., 2015). Bentzon-Tilia et al. (2015) reported that *P. stutzeri*-like *nifH* genes (99% similarity at the nucleotide level) were the most abundant sequences among their samples collected from the ~~temperate~~ Baltic Sea estuary. In the present study, we recovered *P. stutzeri*-like *nifH* genes (>97% similarity at the amino acid level) only at~~from~~

Stn. OT4 during the KT-13-2_Jan cruise by the clone library analysis, and –γ-24774A11 was not detected on that occasion by qPCR analysis probably due to the difference in the sequence between γ-24774A11 and *P. stutzeri*., ~~suggesting that γ-24774A11 was not quantified as *P. stutzeri* and that *P. stutzeri* could not be a major diazotroph in this study region~~ The ecology of γ-24774A11 is still fairly unknown. It remains to be seen, in future studies whether this phylotype contributes to the nitrogen fixation in this region.

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

In nitrate-rich water during winter and spring, Cluster III diazotrophs were detected at most 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 ~~likely presented~~appeared to be present throughout the investigation period.~~a year.~~ 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

505 This study demonstrated that nitrogen fixation can and does proceed at high rates, depending
506 on the season, in the temperate coastal region of the northwestern North Pacific, although we
507 failed to detect nitrogen fixation in DIN-replete cold waters. *nifH* sequences were
508 omnipresent and recovered throughout the year, displaying a marked seasonality in their
509 composition. UCYN-A was a major diazotroph during summer, whereas *Trichodesmium*
510 was abundant during fall, despite low temperatures. It has been suggested that
511 *Trichodesmium* was laterally transported from the adjacent subtropical region, which displays
512 high temperatures. Although the Cluster III diazotrophs were recovered almost throughout
513 a year, they were considered to be inactivated in oxic water columns.
514

515 **Author Contributions**

516 T.S., T.N., and K.F. designed the experiment and T.S. collected the samples at sea. T.S.
517 determined nitrogen fixation and nutrient concentrations and analyzed satellite datasets. T.S.
518 and M.I. conducted the genetic analyses. T.S. prepared the manuscript with contributions
519 from all co-authors.
520

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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,

554 doi:10.1029/2001GB001856, 2002.

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

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

557 53-61, 2007.

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

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

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

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

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

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

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

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

566 173-203, 2004.

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

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

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

570 2009.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

604 1178-1183, 2005.

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

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

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

608 blue-green alga, *Trichodesmium* in the Kuroshio water, Bull. Plankton Soc. Japan, 23(1),
609 19-25, 1976 (in Japanese with English abstract).
610 Moisander, P. H., Beinart, R. A., Hewson, I., White, A. E., Johnson, K. S., Carlson, C. A.,
611 Montoya, J. P., and Zehr, J. P.: Unicellular cyanobacterial distributions broaden the oceanic
612 N_2 fixation domain, Science, 327, 1512-1514, 2010.
613 Moisander, P.H., Zhang, R., Boyle, E.A., Hewson, I., Montoya, J.P., Zehr, J.P.: Analogous
614 nutrient limitations in unicellular diazotrophs and *Prochlorococcus* in the South Pacific
615 Ocean, ISME J., 6, 733-744, 2011.
616 Moisander, P. H., Serros, T., Paerl, R. W., Beinart, R. A., and Zehr, J.P.:
617 Gammaproteobacterial diazotrophs and *nifH* gene expression in surface waters of the South
618 Pacific Ocean, ISME J., 8, 1962-1973, 2014.
619 Mohr, W., Großkopf, T., Wallace, D. W. R., and LaRoche, J.: Methodological
620 underestimation of oceanic nitrogen fixation rates, PLoS ONE, 5(9), e12583,
621 doi:10.1371/journal.pone.0012583, 2010.
622 Montoya, J. P., Voss, M., Kähler, P., and Capone, D. G.: A simple, high-precision,
623 high-sensitivity tracer assay for N_2 fixation, Appl. Environ. Microbiol., 62(3), 986-993, 1996.
624 Mulholland, M. R., Ohki, K., and Capone, D. G.: Nutrient controls on nitrogen uptake and
625 metabolism by natural populations and cultures of *Trichodesmium* (Cyanobacteria), J.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

644 Riemann, L., Farnelid, H., and Steward, G. F.: Nitrogenase genes in non-cyanobacterial
645 plankton: prevalence, diversity and regulation in marine waters, Aquat. Microbial Ecol. 61,
646 235-247, 2010.
647 Rees, A. P., Gilbert, J. A., and Kelly-Gerreyn, B. A.: Nitrogen fixation in the western English
648 Channel (NE Atlantic Ocean), Mar. Ecol. Progr. Ser., 374, 7-12, 2009.
649 Saitoh, Y., Kuma, K., Isoda, Y., Kuroda, H., Matsuura, H., Wagawa, T., Takana, H.,
650 Kobayashi, N., Nagao, S., and Nakatsuka, T.: Processes influencing iron distribution in the
651 coastal waters of the Tsugaru Strait, Japan, J. Oceanogr., 64, 815-830, 2008.
652 Shiozaki, T., Furuya, K., Kodama, T., and Takeda, S.: Contribution of N_2 fixation to new
653 production in the western North Pacific Ocean along 155ºE, Mar. Ecol. Progr. Ser. 377,
654 19-32, 2009.
655 Shiozaki, T., Furuya, K., Kodama, T., Kitajima, S., Takeda, S., Takemura, T., and Kanda, J.:
656 New estimation of N_2 fixation in the western and central Pacific Ocean and its marginal seas,
657 Global. Biogeochem. Cycles., 24, GB1015, doi:10.1029/2009GB003620, 2010.
658 Shiozaki, T., Kodama, T., Kitajima, S., Sato, M., and Furuya, K.: Advective transport of
659 diazotrophs and importance of their nitrogen fixation on new and primary production in the
660 western Pacific warm pool, Limnol. Oceanogr., 58(1), 49-60, 2013.
661 Shiozaki. T., Ijichi, M., Kodama, T., Takeda, S., and Furuya, K.: Heterotrophic bacteria are

major nitrogen fixers in the euphotic zone of the Indian Ocean, Glob. Biogeochem. Cycles, 28, doi:10.1002/2014GB004886, 2014a.

Shiozaki, T., Ito S.-I., Takahashi, K., Saito, H., Nagata, T., and Furuya, K.: Regional variability of factors controlling the onset timing and magnitude of spring algal blooms in the northwestern North Pacific, J. Geophys. Res., 119, 1-13, doi:10.1002/2013JC009187, 2014b.

Shiozaki, T., Chen, Y.-L. L., Lin, Y.-H., Taniuchi, Y., Sheu, D.-S., Furuya, K., and Chen, H.-Y.: Seasonal variations of unicellular diazotroph groups A and B, and *Trichodesmium* in the northern South China Sea and neighboring upstream Kuroshio Current, Cont. Shelf Res., 80, 20-31, 2014c.

Subramaniam, A., Yager, P. L., Carpenter, E. J., Mahaffey, C., Björkman, K., Cooley, S., Kustka, A. B., Montoya, J. P., Sañudo-Wilhelmy, S. A., Shipe, R., and Capone, D. G.: Amazon River enhances diazotrophy and carbon sequestration in the tropical North Atlantic Ocean, Proc. Natl. Acad. Aci. U.S.A., 108, 2184-2189, 2008.

Tamura, K., Peterson, N., Peterson, G., Stecher, M., Nei, M., and Kumar, S.: MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Mol. Biol. Evol., 28, 2731-2739, 2011.

Zehr, J. P., and Turner, P. J.: Nitrogen fixation: nitrogenase genes and gene expression, Meth. Microbiol., 30, 271-285, 2001.

680 Zehr, J. P., Jenkins, B. D., Short, S. M., and Steward, G. F.: Nitrogenase gene diversity and
681 microbial community structure: a cross-system comparison, Environ. Micorbiol., 5(7),
682 539-554, 2003a.
683 Zehr, J. P., Crumbliss, L. L., Church, M. J., Omoregie, E. O., Jenkins, B. D.: Nitrogenase
684 genes in PCR and RT-PCR reagents: implications for studies of diversity of functional genes,
685 BioTechniques, 35, 996-1005, 2003b.
686 Zehr, J. P.: Nitrogen fixation by marine cyanobacteria, Trends Microbiol. 19, 162-173, 2011.
687

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

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

690 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**

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

692 acid level to terrestrial diazotroph sequences.

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

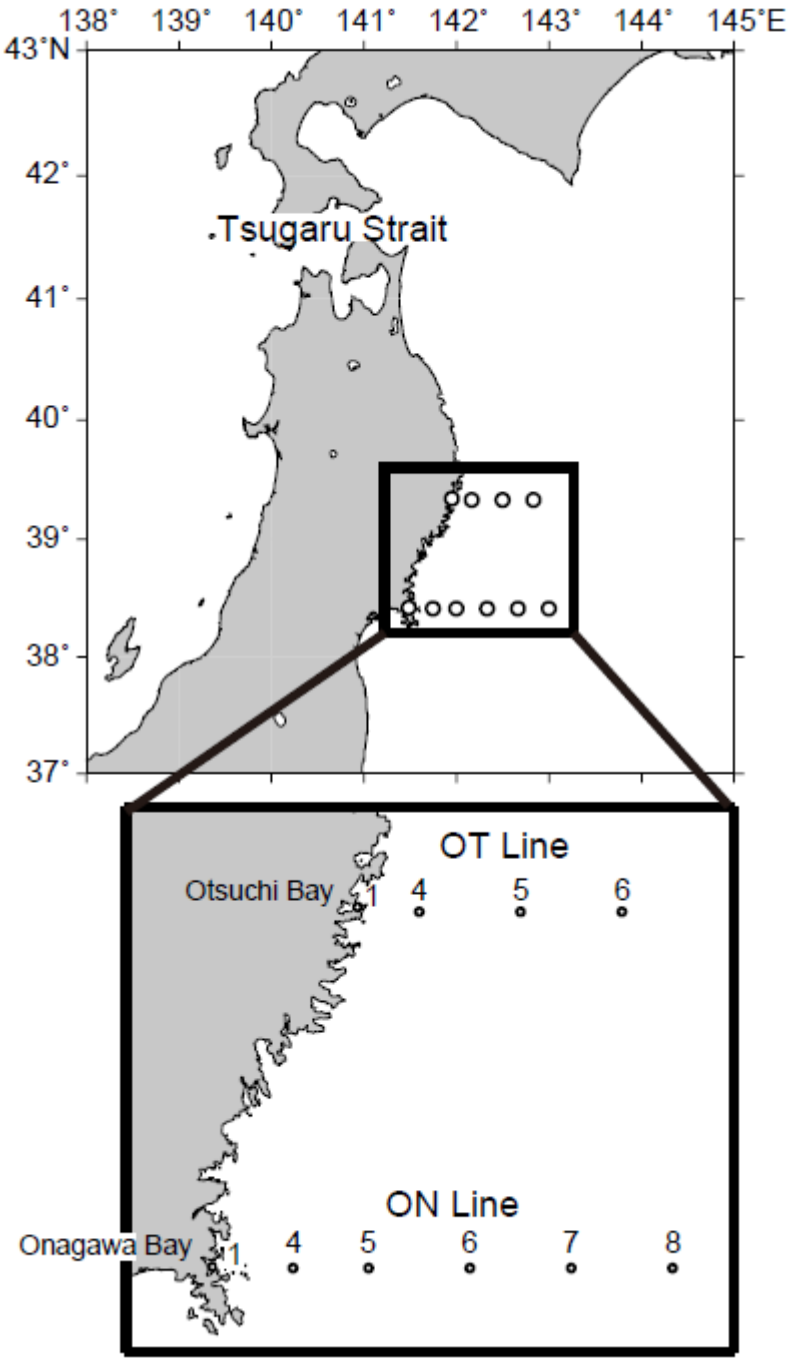
694 water column (n=73).

	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

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

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

697



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

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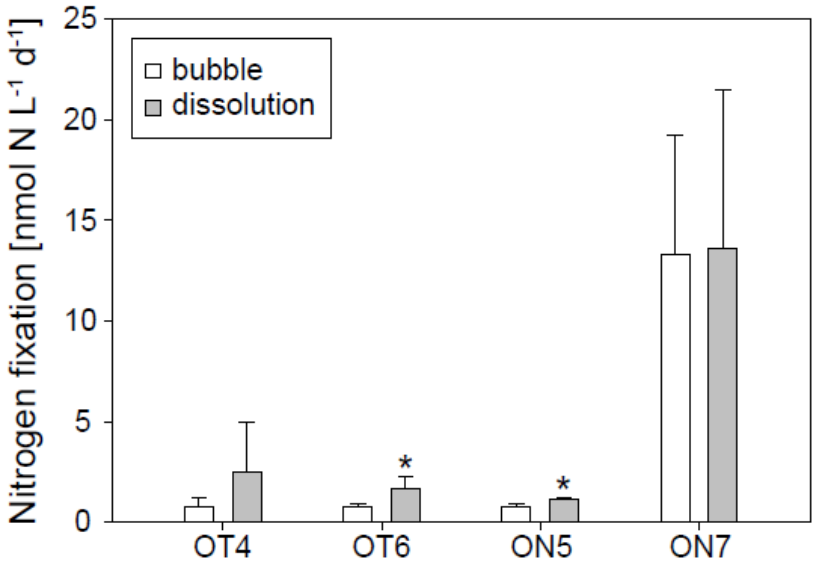
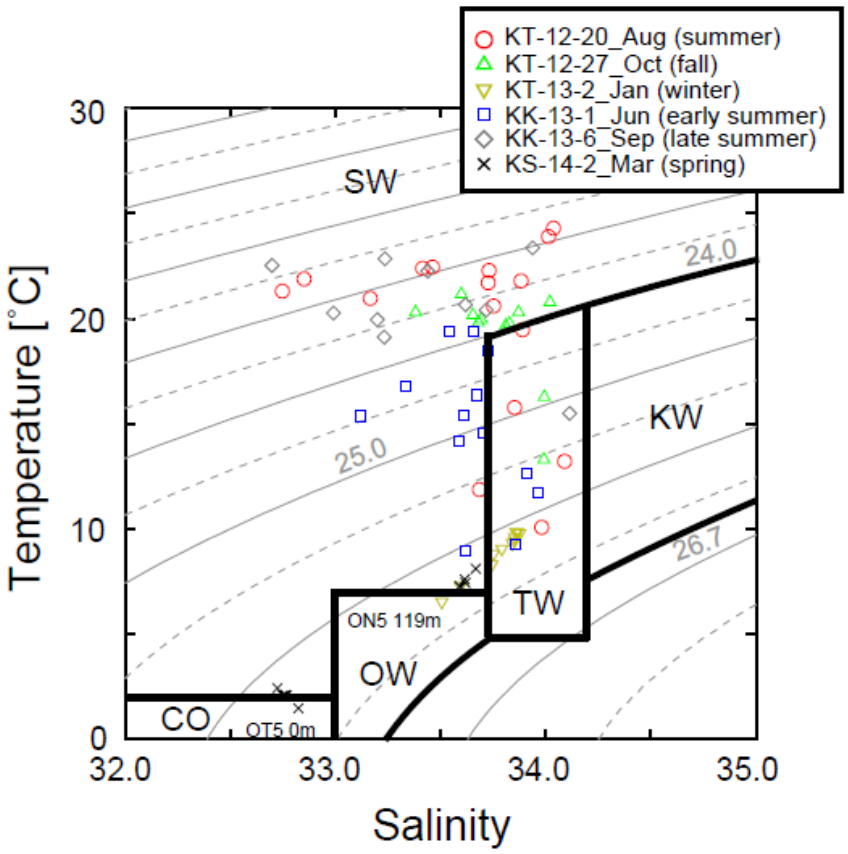


Fig.2. Nitrogen fixation rates estimated simultaneously by the $^{15}N_2$ gas bubble and dissolution methods during the KK-13-6_Sep cruise. An asterisk indicates a significant difference between the two methods (p <0.05).



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709

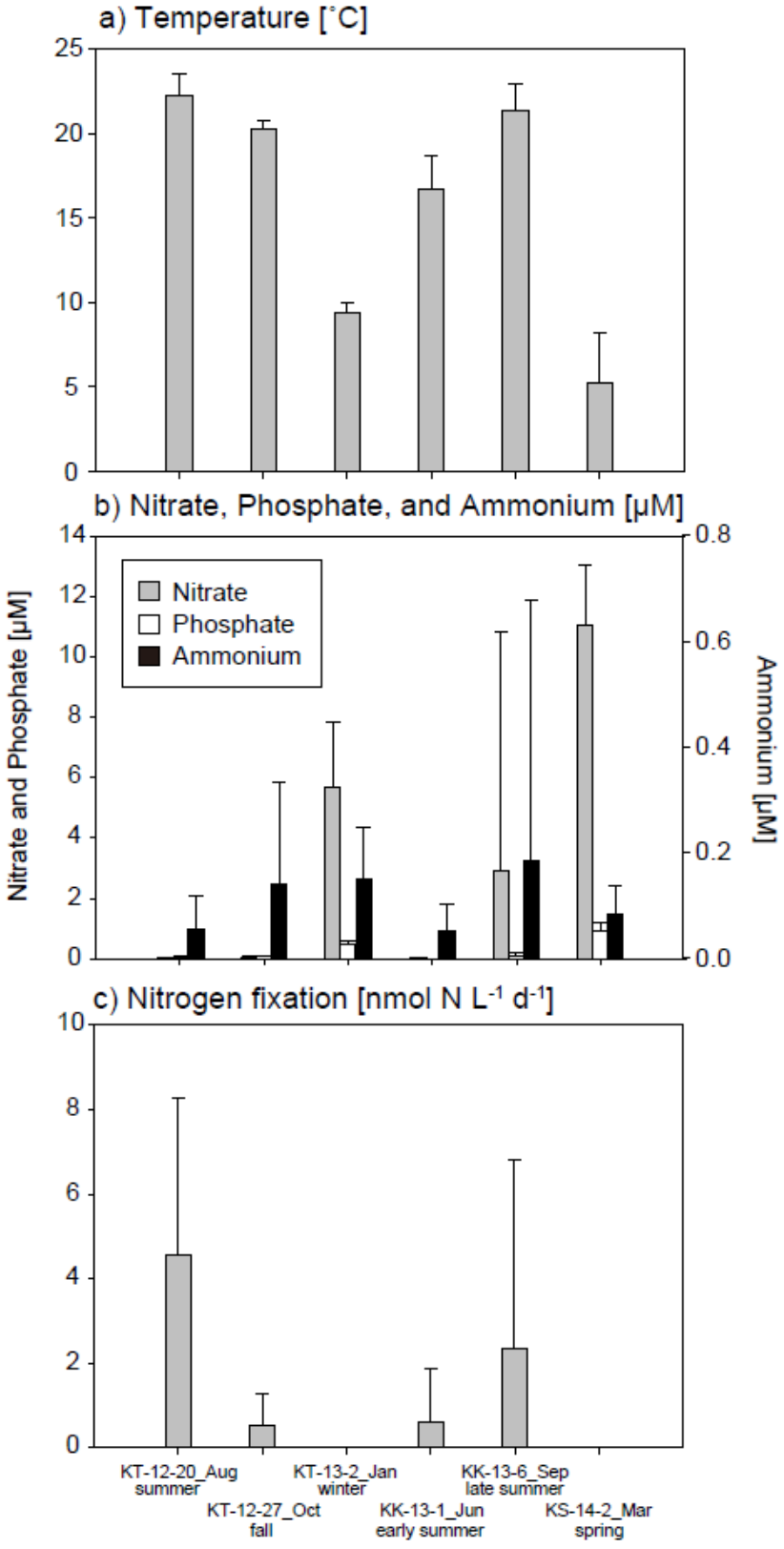
710 Fig. 3. Temperature-salinity diagram at each sampling point. The water classification was

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

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

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

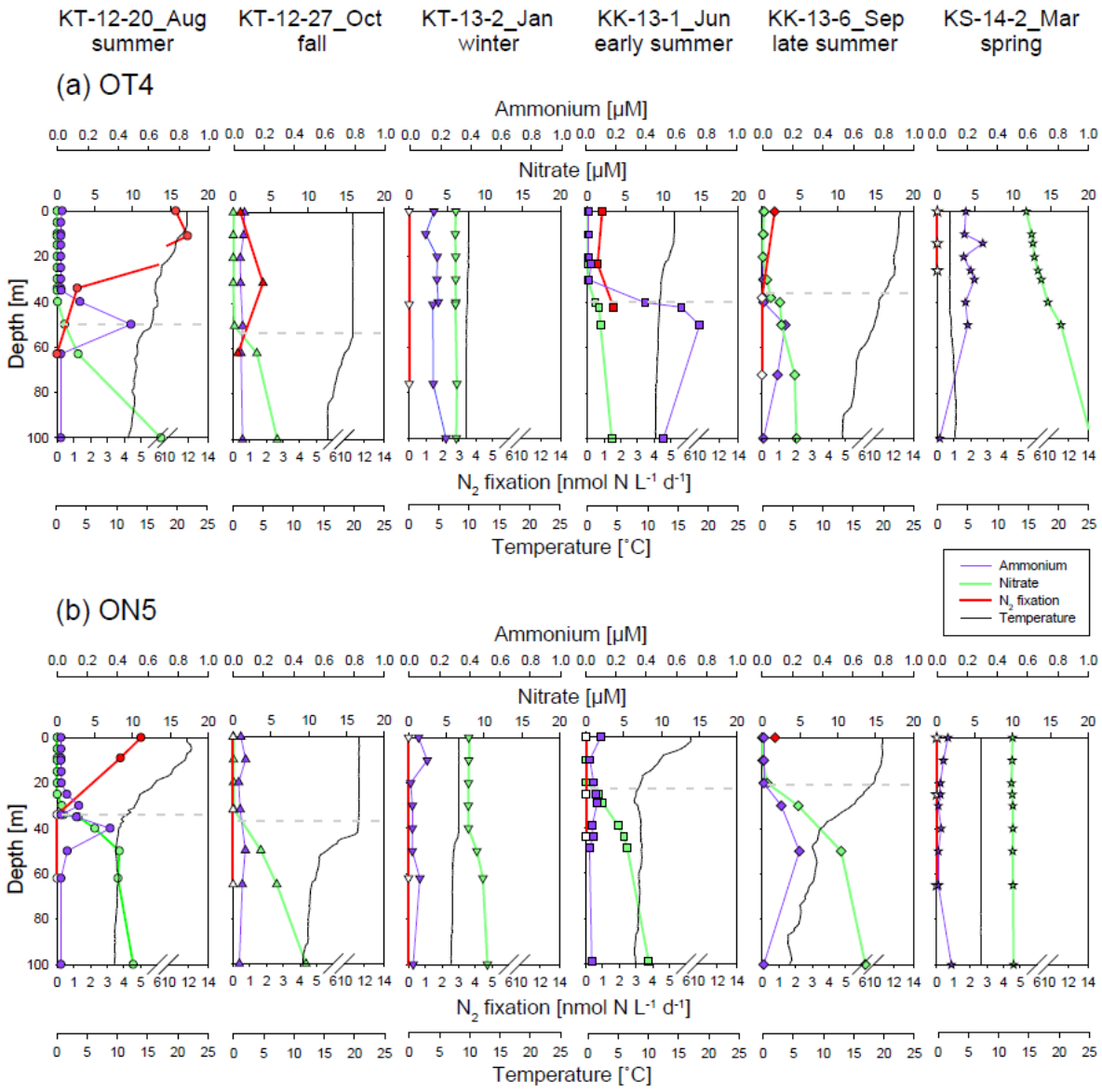
714



715

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

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



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719

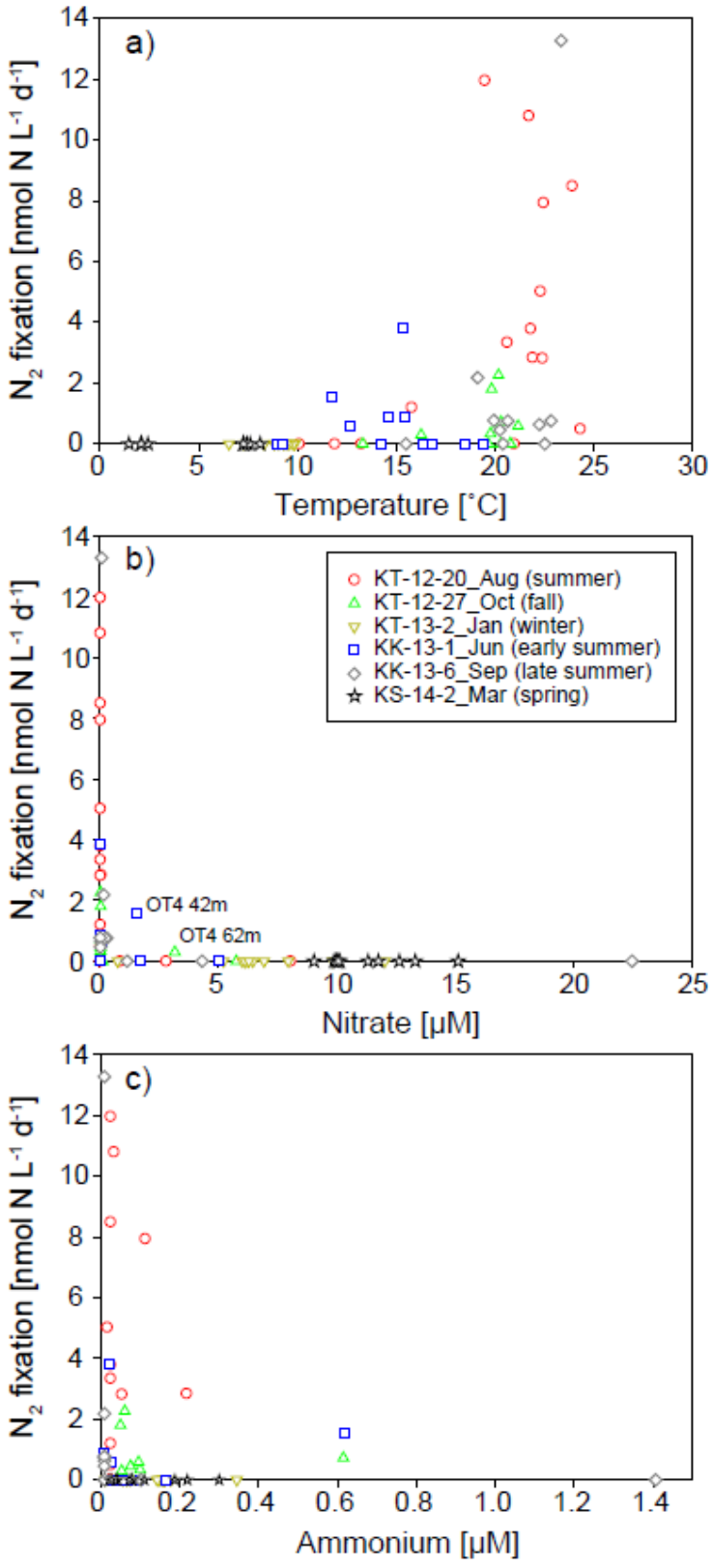
720 Fig. 5. Time-series variations in the vertical profiles of temperature [ºC] (black),

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

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

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

724 temperature and nitrate were ascribable to strong mixing.

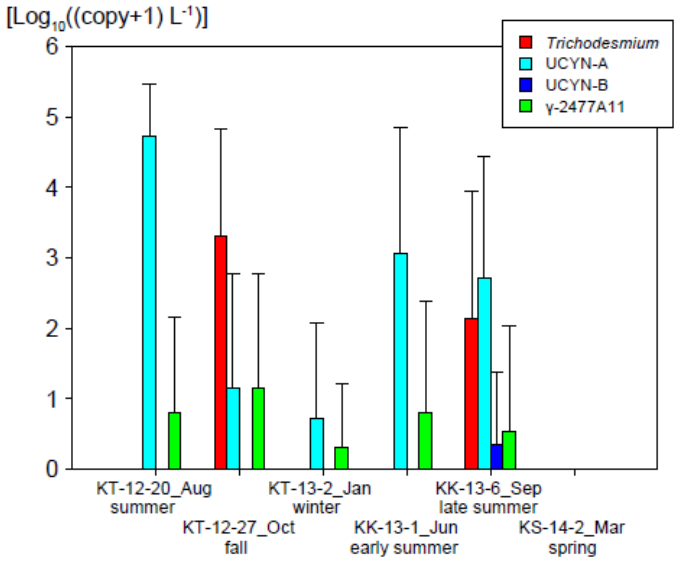


725

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

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

728



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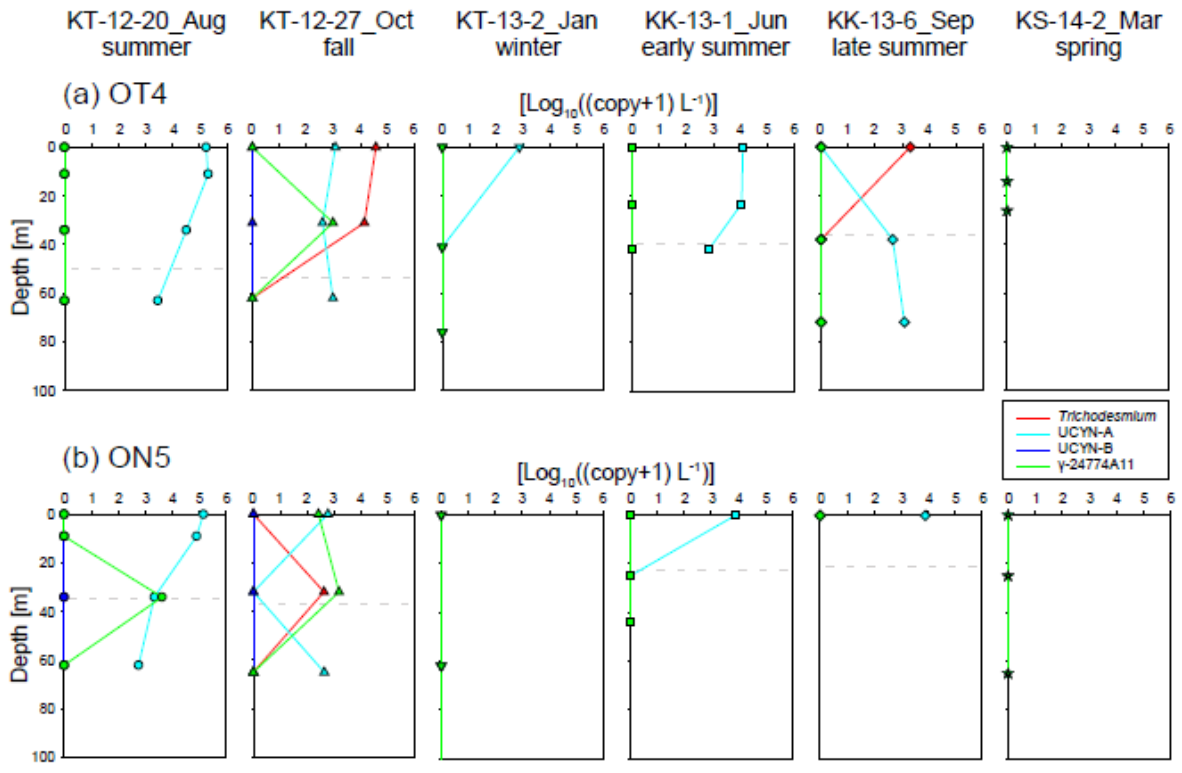
730

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

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

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

734



735

736

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

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

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