Referee #2

General comment:

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

Specific comments:

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

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

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

We have corrected to "L" throughout the manuscript.

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

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

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

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

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

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

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

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

P.24, L.415: "disappearing during in spring" seems grammatically incorrect to me.

We have corrected to "disappeared in spring".

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

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

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

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

Referee #3

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

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

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

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

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

Line 399-402: The result of clone library analysis cannot say "seasonal trend".

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

Line 452-453: The result of clone library analysis cannot say "dominant".

We have changed the sentence as follows. (L445-446)
"In nitrate-rich water during winter and spring, Cluster III diazotrophs were detected at

most of the stations."

Line 455-456: The number of the recovered sequence is not enough to say "major diazotroph".

We have not used the word "major diazotroph" in the revised manuscript. We have changed the sentence as follows. (L448)
"Therefore, Cluster III diazotrophs likely presented throughout a year."

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

We agree. We have changed the sentence as follows. (L461-462)
"Because nitrogen fixation was not detected during the KT-13-2_Jan cruise, the organism was considered not to perform nitrogen fixation."

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

We have changed it with log scale.

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 14.2º 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

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

The temperate coastal ocean is one of the regions where nitrogen fixation rates have been understudied and potentially underestimated. Conventionally, nitrogen fixation in temperate oceans has been assumed to be low because of the relatively low temperatures (<~20ºC), which generally 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

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

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

To examine the possibility of underestimation of nitrogen fixation as determined by the bubble method (Mohr et al., 2010; Großkopf et al., 2012), we compared the nitrogen fixation rates determined using the $^{15}N_2$ gas dissolution method (hereafter, the dissolution method; Mohr et al., 2010) with those determined using the bubble method (see above) during the KK-13-6_Sep and KS-14-2_Mar cruises. For the dissolution method, $^{15}N_2$-enriched seawater was prepared according to Mohr et al. (2010) and Großkopf et al. (2012). Briefly, filtered seawater was degassed using a Sterapore membrane unit (20M1500A: Mitsubishi Rayon Co., Ltd., Tokyo, Japan) at a flow rate of ~500 mL min^{-1} (recirculation period, 10 min). Degassed seawater was stored in 1-L Tedlar bags without headspaces and ~~10 mL~~ $^{15}N_2$ gas was added at a ratio of 10 ml $^{15}N_2$ per 1L seawater. After complete dissolution, the $^{15}N_2$-enriched seawater was added to seawater samples contained in 2-L PC bottles, which were incubated 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.

~~2.3.~~2.4. DNA analysis

~~2.3.1.~~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

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

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

~~2.3.2.~~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

235 samples collected during the winter and spring cruises, even after addition of mannitol
236 (KS-14-2_Mar). Furthermore, nitrogen fixation was not detected in DIN-replete water at
237 Stn. OT1 in late summer (KK-13-6_Sep).
238 The rates of nitrogen fixation in samples collected at different depths (0–119 m) were
239 examined at Stns. OT4 and ON5 (Fig. 5). Nitrogen fixation was detectable only during the
240 four cruises conducted in early summer (KK-13-1_Jun), summer (KT-12-20_Aug), late
241 summer (KK-13-6_Sep), and fall (KT-12-27_Oct), the same seasons during which surface
242 nitrogen fixation was observed (Fig. 4). Nitrogen fixation rates tended to be higher at the
243 surface than in the deeper layers during summer (KT-12-20_Aug) and late summer
244 (KK-13-6_Sep (at Stn. OT4)), whereas this vertical trend was less evident during fall
245 (KT-12-27_Oct) and early summer (KK-13-1_Jun). At Stn. OT4, nitrogen fixation was
246 detectable even in deeper layers below the nitracline, where nitrate concentrations were
247 relatively high (KT-12-27_Oct, depth = 62 m; KK-13-1_Jun, depth = 42 m). In this layer,
248 the ammonium concentrations were 0.05 µM (KT-12-27_Oct) and 0.62 µM (KK-13-1_Jun).
249 The nitrogen fixation rate below the nitracline (1.56 nmol N L^{-1} d^{-1}) was higher than that at
250 the surface (0.87 nmol N L^{-1} d^{-1}) during the KK-13-1_Jun cruise. The maximum
251 depth-integrated nitrogen fixation (294 µmol N m^{-2} d^{-1}) was observed at Stn. OT4 during
252 summer (KT-12-20_Aug).

3.3. Relationship between nitrogen fixation rates and environmental variables

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

Nitrogen fixation rates were negatively correlated with nitrate and phosphate concentrations ($p < 0.01$) (Table 2). There was no significant correlation between nitrogen fixation rates and ammonium concentration ($p > 0.05$). We also found no significant correlation between nitrogen fixation rates and the ratio of total inorganic nitrogen (nitrate + nitrite + ammonium) to phosphate (Table 2). A plot of the nitrogen fixation against nitrate concentrations indicated that nitrogen fixation was generally detectable only when nitrate was depleted (Fig. 6b), except that relatively high nitrogen fixation rates were determined in the subsurface layer of Stn. OT4 (KT-12-27_Oct and KK-13-1_Jun). Active nitrogen fixation tended to occur at low ammonium concentration $\leq$ ~0.1 μM. However, seasonal variation in ammonium concentration was small and no statistically significant relationship with nitrogen fixation

271 was observed (Fig. 6c).

272

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

274 3.4.1. Diazotroph community

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

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

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

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 cruise, all recovered sequences were derived from heterotrophic bacteria, and were dominated by Cluster III diazotrophs at Stns. OT4 and ON5. The Cluster III diazotroph *nifH* sequences were recovered on all cruises except 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, ~~and~~ 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.

3.4.2. Diazotrophs abundances

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

during the KK-13-6_Sep cruise where the peak (1.2×10^3 copies L^{-1}) was observed at 72 m (Fig. 8). The abundance of UCYN-A varied from below the detection limit to 2.6×10^5 copies L^{-1} at all depths. At the surface, UCYN-A was the most abundant among the four groups at most of the stations investigated during the KT-12-20_Aug, KT-13-2_Jan, KK-13-1_Jun, and KK-13-6_Sep cruises (Fig. 7 and ~~S5~~S6). UCYN-B was detected only at Stn. ON7 during the KK-13-6_Sep cruise (Fig. 7, 8, and ~~S5~~S6). γ-24774A11 was detected during all cruises except for the KS-14-2_Mar cruise (Fig. 7 and 8). The abundance of γ-24774A11 ranged from below the detection limit to 1.8×10^4 copies L^{-1}, with a tendency of a subsurface peak at both stations (Fig. 8).

4. DISCUSSION

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

~~The present study revealed that a significant difference between the bubble and the dissolution methods was not always present. Großkopf et al. (2012) indicated that the difference was smaller when *Trichodesmium* dominated in the diazotroph community than when unicellular cyanobacteria and γ-proteobacteria dominated presumably because *Trichodesmium* can float to the top of the bottle and directly use the added $^{15}N_2$ in the bubble~~

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

~~4.2.~~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~~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~~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~~L^{-1} d^{-1}; Shiozaki et al., 2010), whereas they were comparable to those determined in the Kuroshio (0.54–28 nmol N ~~l~~L^{-1} d^{-1}; Shiozaki et al., 2010) and the western Atlantic coastal regions (1.3–49.8 nmol N ~~l~~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~~L^{-1} d^{-1}; Rees et al., 2009) and the Baltic Sea estuaries (47–83 nmol N ~~l~~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

361 rates. Consistent with this notion, our results showed that the nitrogen fixation rate was
362 relatively high at Stn. OT4, where the nitracline was relatively deep.
363 Blais et al. (2012) proposed that nitrogen fixation can proceed even in nutrient-replete waters,
364 if large amounts of iron and organic materials are available for consumption by bacterial
365 diazotrophs. In the present study, this possibility was examined by conducting mannitol
366 addition experiments using surface seawaters collected during spring. These waters,
367 belonging to the Oyashio Current system (Nishioka et al., 2007, 2011; Shiozaki et al., 2014b),
368 were considered to be rich in iron during spring, as indicated by a previous study (iron conc.,
369 0.79–8.46 nM; Nishioka et al. 2007). Despite potentially high iron concentrations, our
370 results showed that nitrogen fixation was undetectable even after the mannitol addition,
371 suggesting that, contrary to the Blais et al. proposition, diazotrophs remained inactive under
372 our experimental settings.
373 Our data showed that nitrogen fixation rates were below the detection limit during winter,
374 spring, and late summer (KK-13-6_Sep), when nitrate concentrations were high. These
375 results were consistent with the results of previous studies in the Pacific Ocean, which
376 indicated that nitrogen fixation rates were low or undetectable in DIN-replete waters
377 (Shiozaki et al., 2010). In contrast, Mulholland et al. (2012) reported that, in temperate
378 regions of the Atlantic Ocean, nitrogen fixation rates were high even in DIN-replete (>1 μM)

379 and cold (<10ºC) surface seawaters. Their study was conducted downstream of the Gulf
380 Stream, where diazotrophs could be delivered from subtropical oceans where DIN is depleted.
381 Previous studies have suggested that cyanobacterial diazotrophs can travel over long
382 distances (>1,000 km) in currents, without losing their capacity for N_2 fixation (Shiozaki et
383 al., 2013), and that activity is not lost immediately even after mixing with DIN-replete
384 seawaters (Holl and Montoya, 2005; Dekaezemacker and Bonnet, 2011). In our region,
385 because the Tsugaru Warm Current flows from north to south, diazotrophs entrained by the
386 current have little chance of meeting high-DIN water at the surface. DIN-replete water
387 during summer was observed at the inside bay station OT1 (Fig. S2). Concomitantly,
388 low-salinity surface waters spread offshore along the OT transect line (Fig. ~~S6~~S7), suggesting
389 that anomalously high DIN concentrations were likely attributable to terrestrial surface
390 discharge enhanced by Typhoon Man-yi, which passed over the region immediately before
391 the cruise. Subramaniam et al. (2008) reported that nitrogen fixation rates near the Amazon
392 River estuary, with low salinity and high nitrate levels, were fairly low. Their results are
393 consistent with ours. Ammonium inhibits nitrogen fixation, especially when ammonium
394 concentrations exceed 1 μM, as demonstrated by *Trichodesmium* (Mulholland et al. 2001).
395 In our study, no negative relationship between nitrogen fixation and ammonium
396 concentration was found. This can likely be explained by relatively low ammonium

concentrations (≤ ~1 µM) throughout the year and across the investigated region. Nitrogen fixation was also negatively correlated with phosphate as with nitrate, and positively correlated with temperature (Table 2). Since correlation between nitrate, phosphate, and temperature 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.

~~4.3.~~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. ~~This consistency in the general results obtained by the clone library and qPCR suggests that both of these approaches captured a similar seasonal trend in community composition changes for at least the major diazotroph groups.~~ In the discussion below, we mainly discuss possible factors responsible for seasonal variation in the diazotrophs targeted by the qPCR analysis. ~~community by focusing on the major diazotroph groups.~~

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 ~20° C (Needoba et al., 2007; Moisander et al., 2010). UCYN-A was detected by qPCR even during winter at some stations, yet, was not observed during spring. This could be because UCYN-A abundance decreased from fall to winter with decreasing temperatures, eventually ~~disappearing~~ disappeared ~~during~~ in spring.

Trichodesmium was detected from late summer to fall by qPCR analysis, when water temperatures ranged from 19.1 to 23.4º C at the surface. Given that the optimal growth temperature for *Trichodesmium* has been reported to be high (24–30º C) (Breitbarth et al., 2007), *Trichodesmium* detected in the investigated region likely existed under suboptimum conditions. The relatively high abundance of *Trichodesmium* observed during fall, despite the suboptimal temperature conditions, might indicate that *Trichodesmium* was transported from the adjacent subtropical region where seawater temperatures were high (>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 γ-24774A11 by qPCR analysis during all cruises except for the KS-14-2_Mar cruise. This phylotype has not been reported previously in other temperate oceans (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012). The *nifH* sequence of γ-24774A11 was similar to that of *Pseudomonas stutzeri* (94% similarity at the amino acid level), which was observed in waters including temperate regions (Bentzon-Tilia et al., 2015).

Bentzon-Tilia et al. (2015) reported that *P. stutzeri*-like *nifH* genes (99% similarity at the nucleotide level) were the most abundant sequences among their samples collected from the temperate Baltic Sea estuary. In the present study, we recovered *P. stutzeri*-like *nifH* genes (>97% similarity at the amino acid level) from Stn. OT4 during the KT-13-2_Jan cruise by the clone library analysis. However, γ-24774A11 was not detected on that occasion by qPCR analysis, suggesting that γ-24774A11 was not quantified as *P. stutzeri* and that *P. stutzeri* ~~was~~ 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 ~~dominant~~ 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 throughout a year. ~~Because UCYN-A, *Trichodesmium*, and γ-24774A11 were scarce during winter and spring, Cluster III diazotrophs were likely to be major diazotrophs at these times.~~

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. ~~S7~~S8). Therefore, ~~it is possible that the Cluster III diazotrophs that we detected in the surface layer were derived from resuspensions of coastal marine sediments in which anoxic conditions may prevail because of organic matter decomposition.~~ ~~T~~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~~must have been inactivated after being flushed out from the coastal region~~.

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 t~~T~~he Cluster III diazotrophs were recovered almost throughout a year, they were considered to be inactivated in oxic water columns.

~~abundant in surface waters during winter, which was ascribed to their delivery from the~~

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

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.

Acknowledgements

We acknowledge K. Kogure, K. Hamasaki, A. Tsuda, Y. Tada, R. Fujimura, R. Kaneko, H. Takasu, T. Yokokawa, K. Seike, and T. Kitahashi for their assistance in the sample collection and analysis. We thank the captains, crewmembers, and participants on board the R/V Tansei-maru, No.3 Kaiyo-maru, Shinsei-maru for their cooperation at sea. We also thank H. Fukuda for providing nutrient data on the KS-14-2 cruise. We acknowledge the National Aeronautics and Space Administration (NASA) and the Archiving, Validation, and Interpretation of Satellite Data in Oceanography data center at the Centre National d'Etudes

523 Spatiales (CNES AVISO) for providing satellite data sets. This study was financially
524 supported by Tohoku Ecosystem-Associated Marine Sciences (TEAMS) sponsored by
525 Ministry of Education, Culture, Sports, Science and Technology (MEXT), by a Grant-in-Aid
526 for Scientific Research on Innovative Areas (24121001), by the Japan Science and
527 Technology Agency (JST CREST), and by Grant-in-Aid for Japan Society for the Promotion
528 of Science (JSPS) Fellows (25-7341) from MEXT
529

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,

548 doi:10.1029/2001GB001856, 2002.

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

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

551 53-61, 2007.

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

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

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

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

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

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

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

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

560 173-203, 2004.

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

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

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

564 2009.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

598 1178-1183, 2005.

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

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

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

602 blue-green alga, *Trichodesmium* in the Kuroshio water, Bull. Plankton Soc. Japan, 23(1),
603 19-25, 1976 (in Japanese with English abstract).
604 Moisander, P. H., Beinart, R. A., Hewson, I., White, A. E., Johnson, K. S., Carlson, C. A.,
605 Montoya, J. P., and Zehr, J. P.: Unicellular cyanobacterial distributions broaden the oceanic
606 N_2 fixation domain, Science, 327, 1512-1514, 2010.
607 Moisander, P.H., Zhang, R., Boyle, E.A., Hewson, I., Montoya, J.P., Zehr, J.P.: Analogous
608 nutrient limitations in unicellular diazotrophs and *Prochlorococcus* in the South Pacific
609 Ocean, ISME J., 6, 733-744, 2011.
610 Moisander, P. H., Serros, T., Paerl, R. W., Beinart, R. A., and Zehr, J.P.:
611 Gammaproteobacterial diazotrophs and *nifH* gene expression in surface waters of the South
612 Pacific Ocean, ISME J., 8, 1962-1973, 2014.
613 Mohr, W., Großkopf, T., Wallace, D. W. R., and LaRoche, J.: Methodological
614 underestimation of oceanic nitrogen fixation rates, PLoS ONE, 5(9), e12583,
615 doi:10.1371/journal.pone.0012583, 2010.
616 Montoya, J. P., Voss, M., Kähler, P., and Capone, D. G.: A simple, high-precision,
617 high-sensitivity tracer assay for N_2 fixation, Appl. Environ. Microbiol., 62(3), 986-993, 1996.
618 Mulholland, M. R., Ohki, K., and Capone, D. G.: Nutrient controls on nitrogen uptake and
619 metabolism by natural populations and cultures of *Trichodesmium* (Cyanobacteria), J.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

638 Riemann, L., Farnelid, H., and Steward, G. F.: Nitrogenase genes in non-cyanobacterial
639 plankton: prevalence, diversity and regulation in marine waters, Aquat. Microbial Ecol. 61,
640 235-247, 2010.
641 Rees, A. P., Gilbert, J. A., and Kelly-Gerreyn, B. A.: Nitrogen fixation in the western English
642 Channel (NE Atlantic Ocean), Mar. Ecol. Progr. Ser., 374, 7-12, 2009.
643 Saitoh, Y., Kuma, K., Isoda, Y., Kuroda, H., Matsuura, H., Wagawa, T., Takana, H.,
644 Kobayashi, N., Nagao, S., and Nakatsuka, T.: Processes influencing iron distribution in the
645 coastal waters of the Tsugaru Strait, Japan, J. Oceanogr., 64, 815-830, 2008.
646 Shiozaki, T., Furuya, K., Kodama, T., and Takeda, S.: Contribution of N_2 fixation to new
647 production in the western North Pacific Ocean along 155ºE, Mar. Ecol. Progr. Ser. 377,
648 19-32, 2009.
649 Shiozaki, T., Furuya, K., Kodama, T., Kitajima, S., Takeda, S., Takemura, T., and Kanda, J.:
650 New estimation of N_2 fixation in the western and central Pacific Ocean and its marginal seas,
651 Global. Biogeochem. Cycles., 24, GB1015, doi:10.1029/2009GB003620, 2010.
652 Shiozaki, T., Kodama, T., Kitajima, S., Sato, M., and Furuya, K.: Advective transport of
653 diazotrophs and importance of their nitrogen fixation on new and primary production in the
654 western Pacific warm pool, Limnol. Oceanogr., 58(1), 49-60, 2013.
655 Shiozaki. T., Ijichi, M., Kodama, T., Takeda, S., and Furuya, K.: Heterotrophic bacteria are

656 major nitrogen fixers in the euphotic zone of the Indian Ocean, Glob. Biogeochem. Cycles,
657 28, doi:10.1002/2014GB004886, 2014a.
658 Shiozaki, T., Ito S.-I., Takahashi, K., Saito, H., Nagata, T., and Furuya, K.: Regional
659 variability of factors controlling the onset timing and magnitude of spring algal blooms in the
660 northwestern North Pacific, J. Geophys. Res., 119, 1-13, doi:10.1002/2013JC009187, 2014b.
661 Shiozaki, T., Chen, Y.-L. L., Lin, Y.-H., Taniuchi, Y., Sheu, D.-S., Furuya, K., and Chen,
662 H.-Y.: Seasonal variations of unicellular diazotroph groups A and B, and *Trichodesmium* in
663 the northern South China Sea and neighboring upstream Kuroshio Current, Cont. Shelf Res.,
664 80, 20-31, 2014c.
665 Subramaniam, A., Yager, P. L., Carpenter, E. J., Mahaffey, C., Björkman, K., Cooley, S.,
666 Kustka, A. B., Montoya, J. P., Sañudo-Wilhelmy, S. A., Shipe, R., and Capone, D. G.:
667 Amazon River enhances diazotrophy and carbon sequestration in the tropical North Atlantic
668 Ocean, Proc. Natl. Acad. Aci. U.S.A., 108, 2184-2189, 2008.
669 Tamura, K., Peterson, N., Peterson, G., Stecher, M., Nei, M., and Kumar, S.: MEGA5:
670 Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance,
671 and maximum parsimony methods, Mol. Biol. Evol., 28, 2731-2739, 2011.
672 Zehr, J. P., and Turner, P. J.: Nitrogen fixation: nitrogenase genes and gene expression, Meth.
673 Microbiol., 30, 271-285, 2001.

674 Zehr, J. P., Jenkins, B. D., Short, S. M., and Steward, G. F.: Nitrogenase gene diversity and
675 microbial community structure: a cross-system comparison, Environ. Micorbiol., 5(7),
676 539-554, 2003a.
677 Zehr, J. P., Crumbliss, L. L., Church, M. J., Omoregie, E. O., Jenkins, B. D.: Nitrogenase
678 genes in PCR and RT-PCR reagents: implications for studies of diversity of functional genes,
679 BioTechniques, 35, 996-1005, 2003b.
680 Zehr, J. P.: Nitrogen fixation by marine cyanobacteria, Trends Microbiol. 19, 162-173, 2011.
681

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

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

684 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**

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

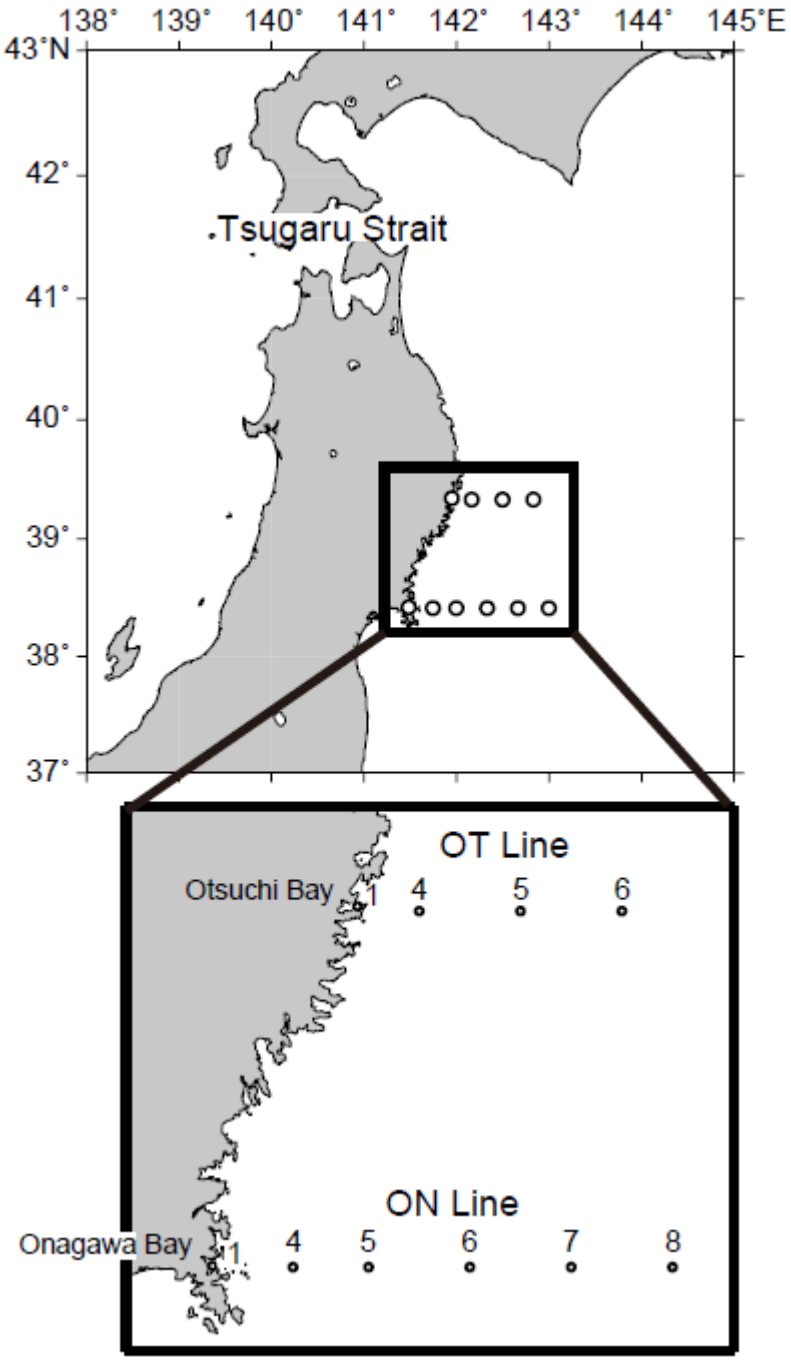
686 acid level to terrestrial diazotroph sequences.

Table 2 Pearson's correlation matrix of N_2 fixation rates and water properties in the entire water column (n=73).~~at the surface (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	-0.8~~63~~**	0.~~86~~8~~17~~**	0.~~135~~119	1		
N/P ratio	-0.~~125~~266*	0.~~216~~722**	0.~~171~~751**	0.~~009~~349**	1	
N_2 fixation	0.435**	-0.325**	-0.122	-0.~~335~~351**	-0.~~130~~219	1

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

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

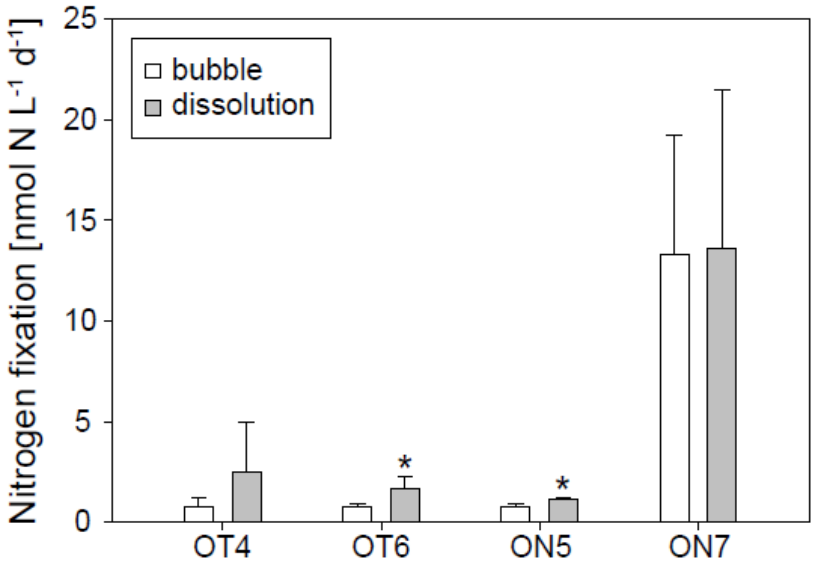


693

694

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

696



697

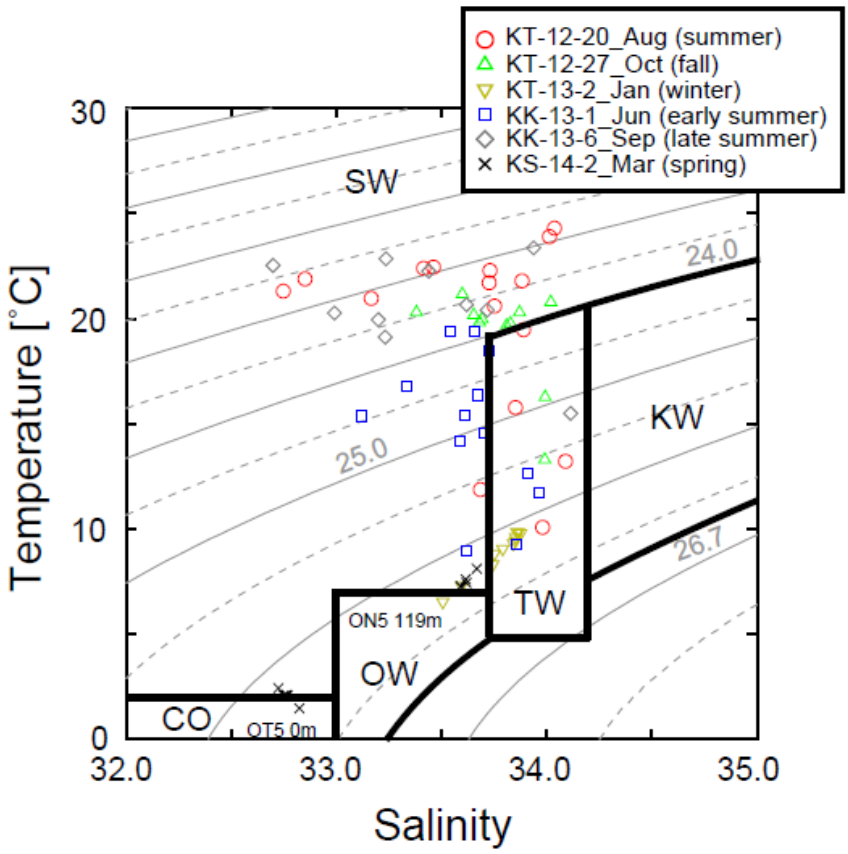
698

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

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

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

702



703

704

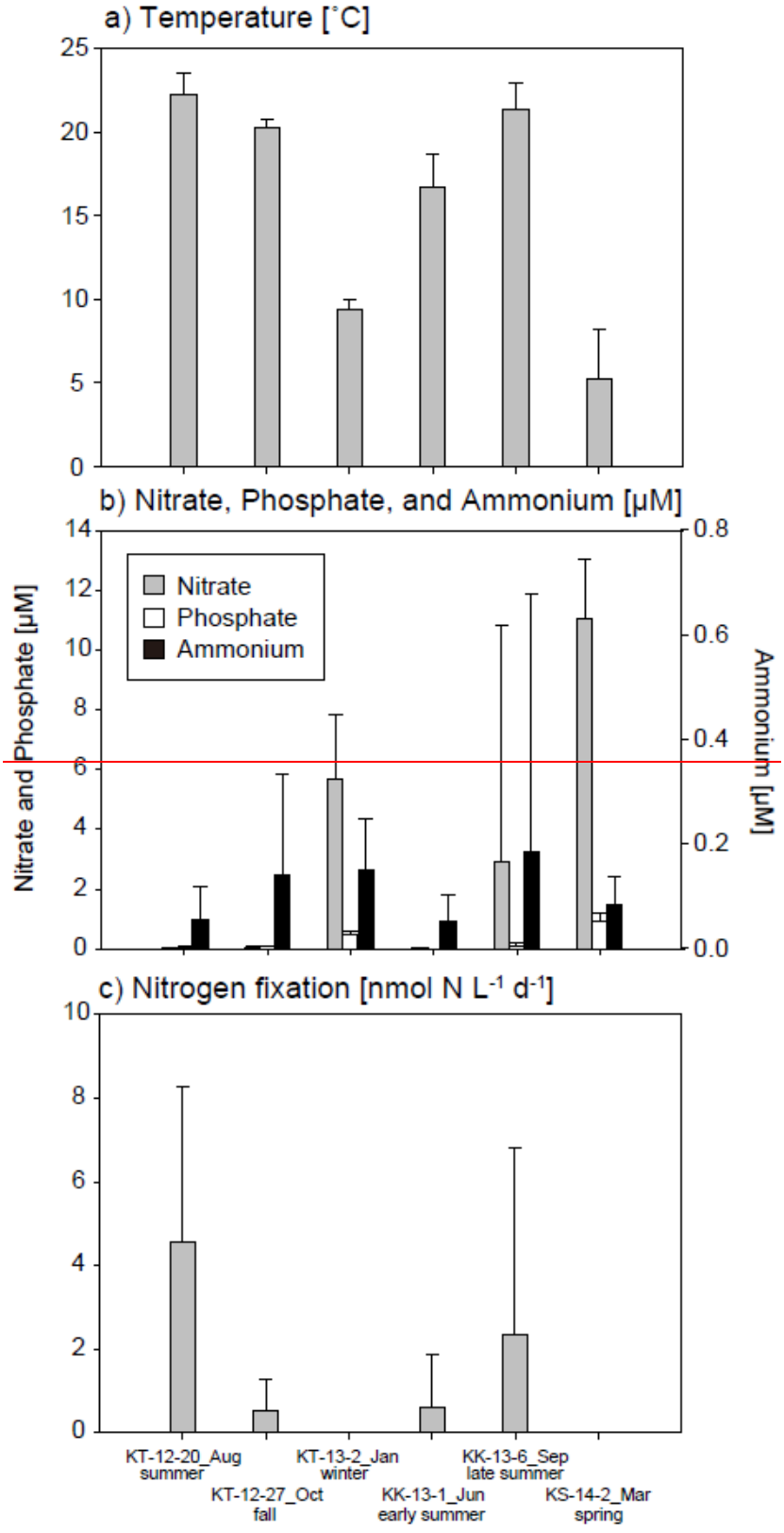
705 Fig. 3. Temperature-salinity diagram at each sampling point. The water classification was

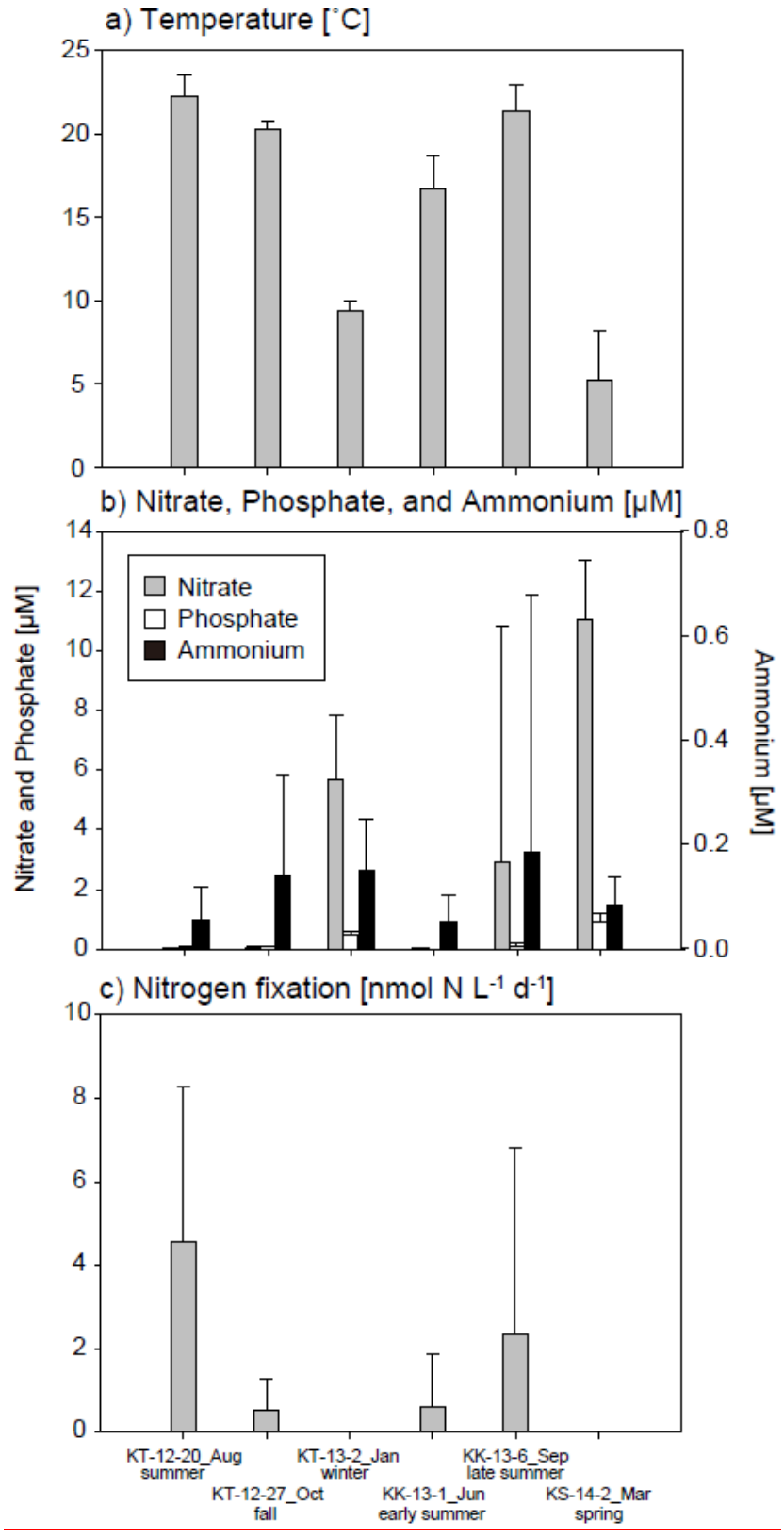
706 defined by Hanawa and Mitsudera (1986). SW, KW, TW, OW, and CO denote the surface

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

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

709

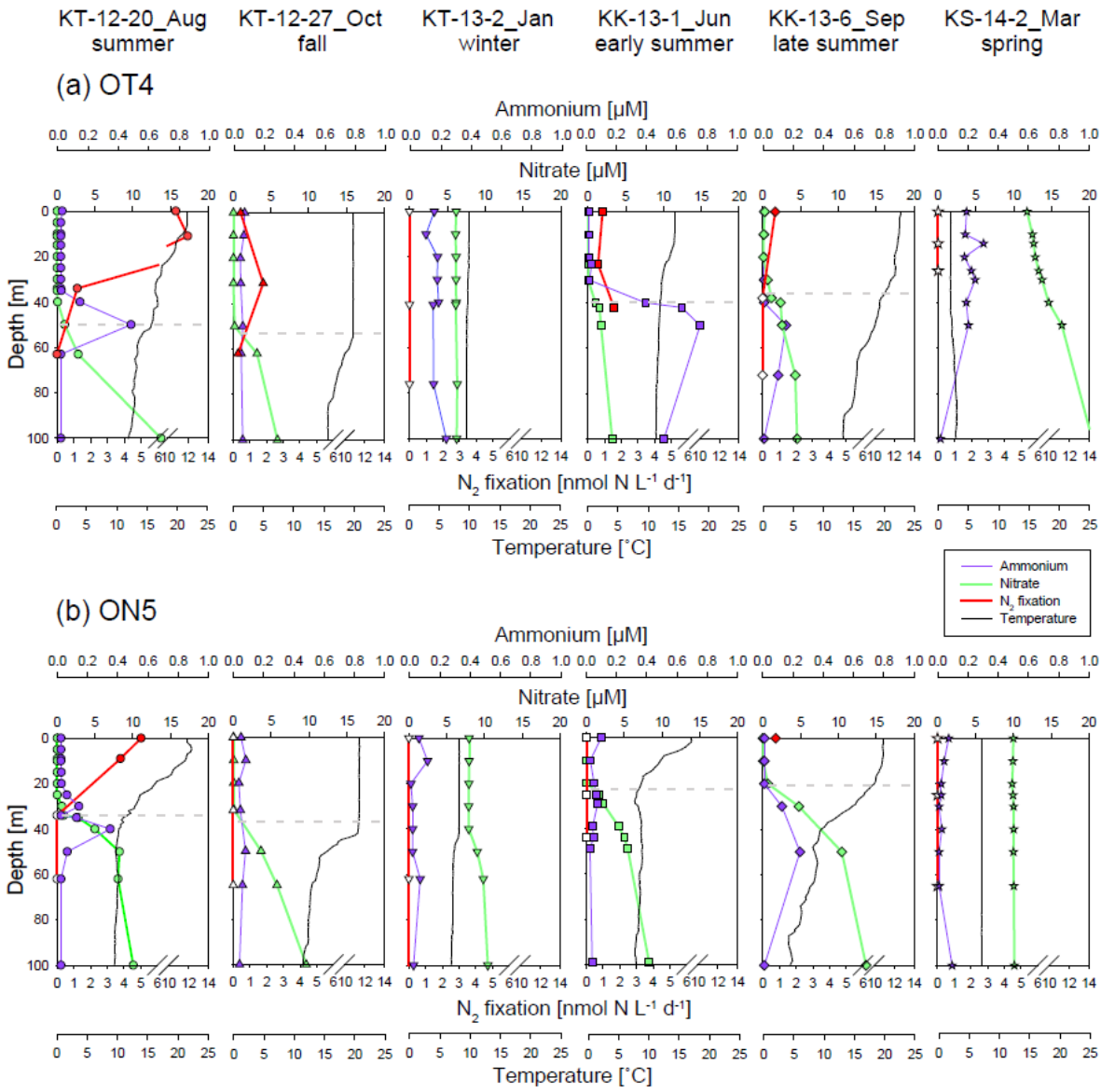

a) Temperature [˚C]
25
20
15
10
5
0
b) Nitrate, Phosphate, and Ammonium [µM]
Nitrate
Phosphate
Ammonium
Nitrate and Phosphate [µM]
Ammonium [µM]
14
12
10
8
6
4
2
0
0.8
0.6
0.4
0.2
0.0
c) Nitrogen fixation [nmol N L-1 d-1]
10
8
6
4
2
0
KT-12-20_Aug
summer
KT-12-27_Oct
fall
KT-13-2_Jan
winter
KK-13-1_Jun
early summer
KK-13-6_Sep
late summer
KS-14-2_Mar
spring



711

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

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



714

715

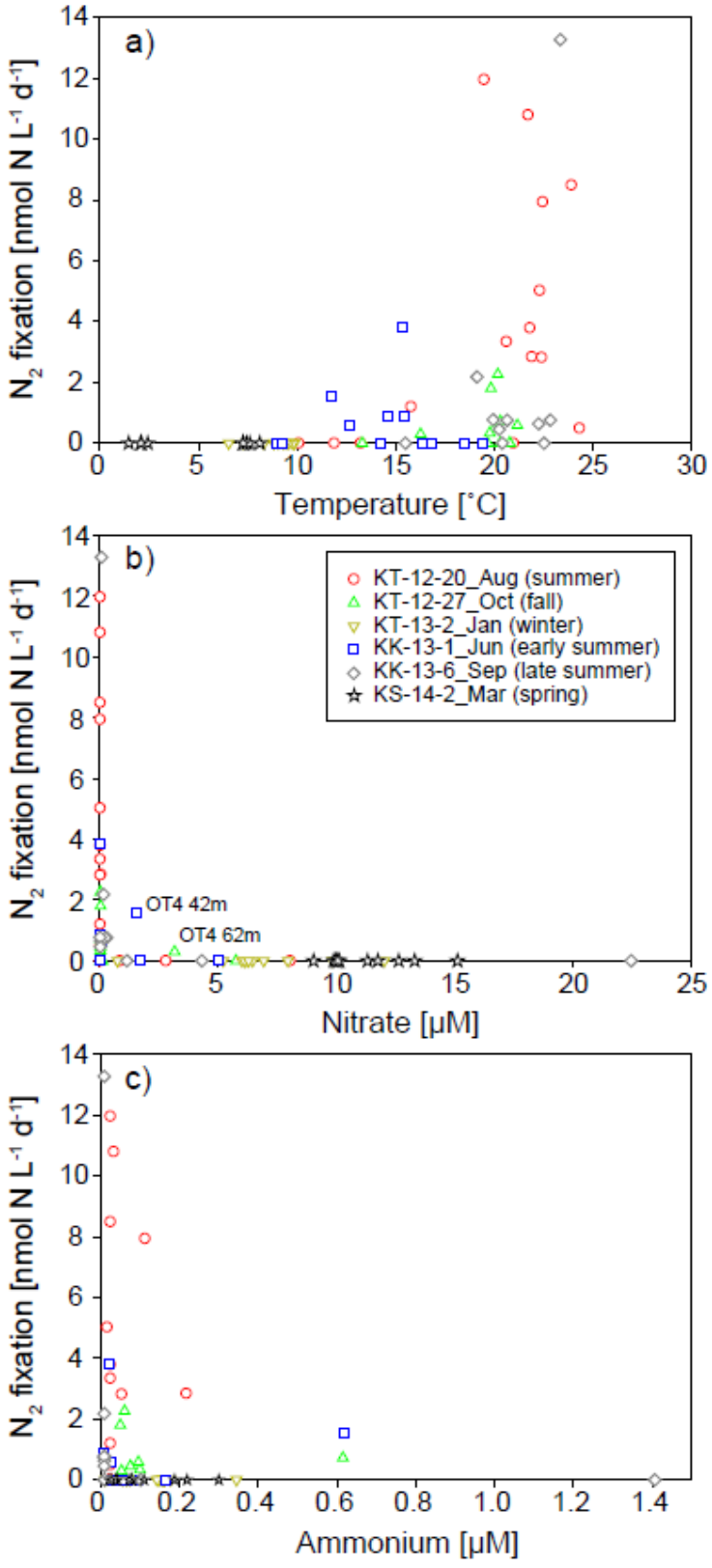
716 Fig. 5. Time-series variations in the vertical profiles of temperature [ºC] (black),

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

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

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

720 temperature and nitrate were ascribable to strong mixing.



721

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

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

724

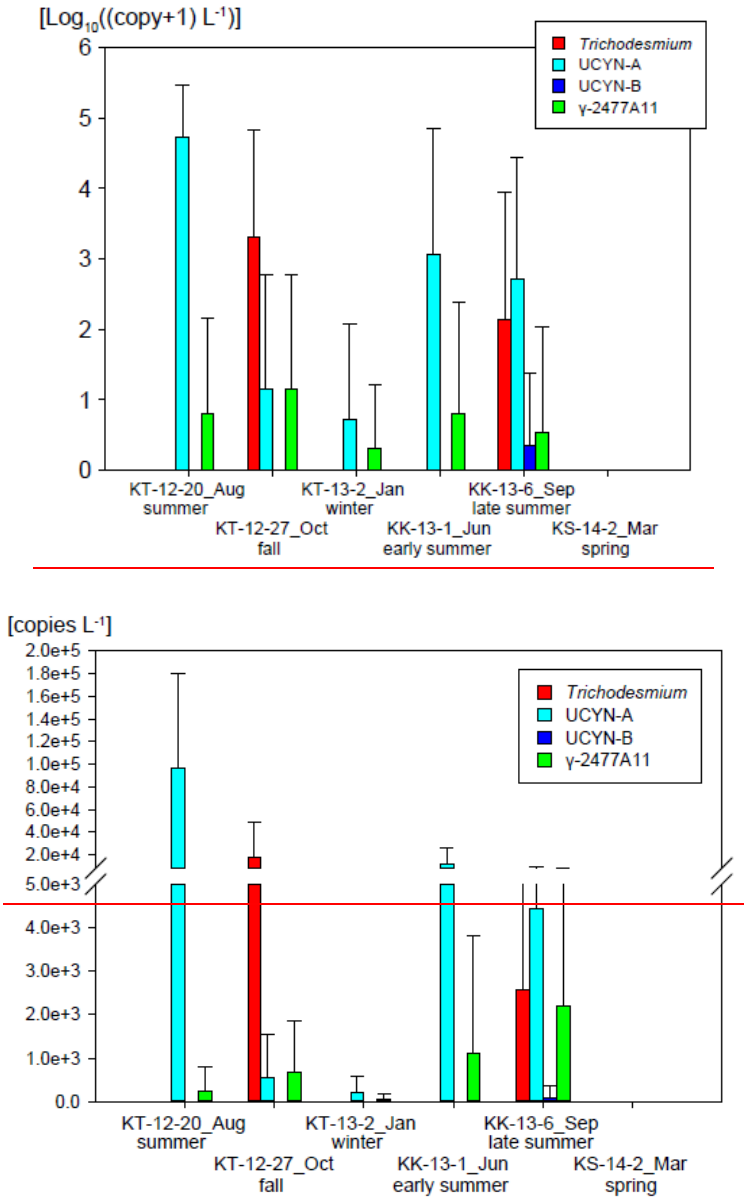
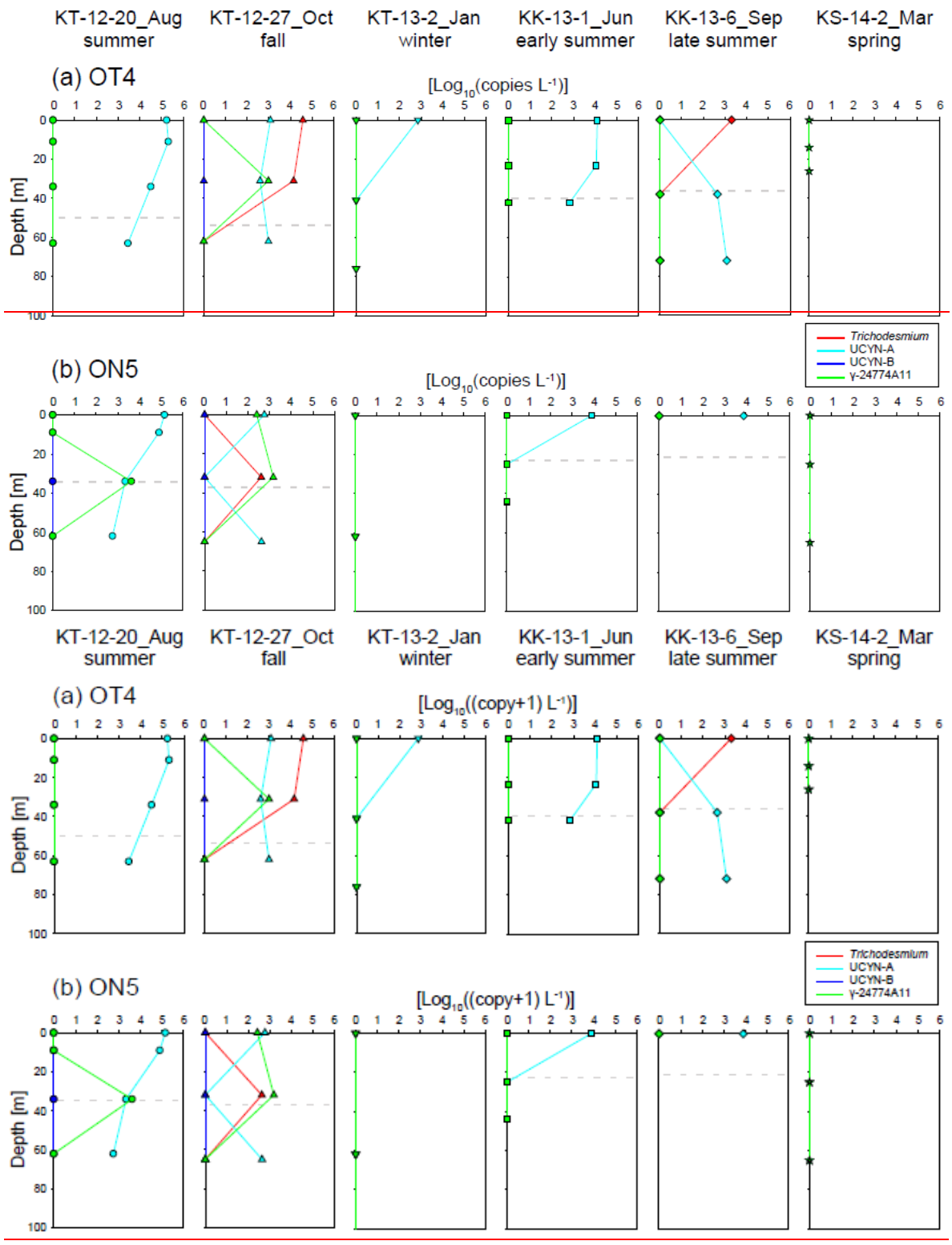


Fig. 7. Average abundances of *Trichodesmium* (red), UCYN-A (light blue), UCYN-B (blue), and γ-24774A11 (green) [Log_{10}((~~copies~~ copy+1) L^{-1})] at the surface during each cruise. When the target *nifH* gene was not detected, the copy number was assumed to be zero.



733

734

735

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

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

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