Biogeosciences, 12, 4751–4764, 2015
www.biogeosciences.net/12/4751/2015/
doi:10.5194/bg-12-4751-2015

Biogeosciences Open Access

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], **and 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

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

Received: 16 December 2014 – Published in Biogeosciences Discuss.: 15 January 2015
Revised: 14 July 2015 – Accepted: 16 July 2015 – Published: 7 August 2015

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 ($\leq$ 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 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 ($< \sim 20\,^{\circ}C$), which generally inhibit the growth of large cyanobacterial diazotrophs (Breitbarth et al., 2007), and development of high dissolved inorganic nitrogen (DIN) concentrations ($> 1\,\mu M$). High DIN concentrations are generally understood 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 ($< 10\,^{\circ}C$) and DIN-rich waters ($> 1\,\mu M$) 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 onshore–offshore transects 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.

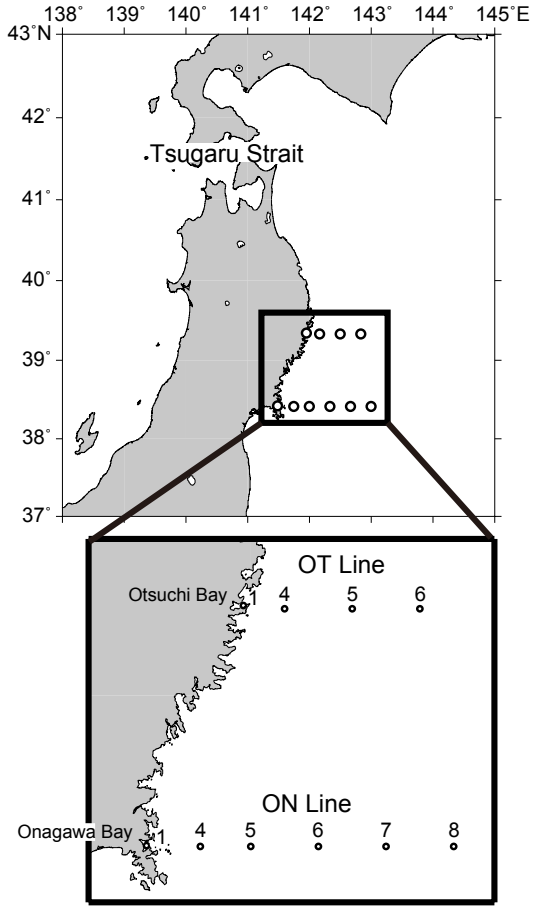

Figure 1. Sampling locations in the northwestern North Pacific Ocean.

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), mid-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 (Supplement 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 (Seabird 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 Station (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 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 were in the ranges of 0.01–0.04, 0.01–0.02, 0.01–0.03, and 0.01–0.02 µM, respectively. The nitra-

cline 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 $\sim 500\,mL\,min^{-1}$ (recirculation period, 10 min). Degassed seawater was stored in 1 L Tedlar bags without headspaces and $^{15}N_2$ gas was added at a ratio of 10 mL $^{15}N_2$ per 1 L 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 bubble method, see above) for surface seawater samples (stations ON4 and OT6 during the KS-14-2_Mar cruise) with and without addition of mannitol (final conc. 0.8 µM) ($n = 3$).

2.3 Statistical analysis

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

2.4 DNA analysis

2.4.1 DNA extraction, sequencing, and phylogenetic analysis

Samples (0.38–1 L) for DNA analysis were filtered through 0.2 µm pore-sized Nuclepore filters and stored in a deep freezer (−80 °C) until onshore analysis. Total DNA was extracted using a ChargeSwitch Forensic DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) with slight modification of the manufacturer's protocol (Shiozaki et al., 2014a). Partial *nifH* fragments were amplified using a nested PCR strategy (Zehr and Turner, 2001) from samples collected from surface water at Stns. OT4, ON1, ON5, and ON7 during the KT-12-20_Aug and KT-12-27_Oct cruises, at Stns. OT4, ON1, and ON5 during the KT-13-2_Jan and KS-14-2_Mar cruises, at Stns. OT4, ON1, ON5, and ON8 during the KK-13-1_Jun cruise, and at Stns OT4, ON5, ON7 during the KK-13-6_Sep cruise (Table 1). PCR reagents were applied as described by Shiozaki et al. (2014a). The first and second PCRs were run using the same cycling conditions: 95 °C for 30 s followed by 30 cycles of 98 °C for 10 s, 52 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 7 min. Sterile distilled water was used as the negative control. After PCR analysis, we confirmed that the negative control showed no bands in the gel. The PCR products were cloned and sequenced according to Shiozaki et al. (2014a). The present study obtained 197 *nifH* sequences in total. The *nifH* sequences were translated into amino acid sequences and searched against the protein database of the National Center for Biotechnology Information using the Basic Local Alignment Search Tool (BLASTp) algorithm. Clones with 100 % amino acid sequence similarity were defined as the same operational taxonomic unit (OTU) using the CD-HIT suite (Huang et al., 2010). The amino acid sequences were aligned using multiple sequence comparisons by the log-expectation (MUSCLE) module in the MEGA5 package (Tamura et al., 2011). A phylogenetic tree was constructed using the maximum likelihood method employing the Dayhoff matrix-based mode, and 1000 bootstrap replicates were run. The obtained sequences were assigned to bacterial groups based on known sequences included in a

cluster within the phylogenetic tree (Zehr et al., 2003a). The sequences from this study were deposited in the DNA Data Bank of Japan (DDBJ) as accession numbers LC013480 to LC013676.

2.4.2 Quantitative PCR (qPCR) analysis

The clone library analysis showed that UCYN-A, *Trichodesmium*, and γ-proteobacterial phylotype γ-24774A11 (hereafter γ-24774A11) were likely important diazotrophs from early summer to fall when nitrogen fixation occurred (see below). Therefore, the present study quantified these *nifH* phylotypes by qPCR analysis to examine their relative importance during these seasons. In addition, UCYN-B which is considered to be a major diazotroph in the tropical and subtropical oligotrophic ocean (Moisander et al., 2010), was quantified. TaqMan primer and probe sets previously designed for these four *nifH* phylotypes were used for quantification (Shiozaki et al., 2014a, c; Moisander et al., 2014). The 20 µL qPCR reactions contained 10 µL 2× Premix Ex Taq (Probe qPCR; Takara), 5.6 µL of nuclease-free water, 1 µL each of the forward and reverse primers, 0.4 µL of TaqMan probe, and 2 µL of template DNA. The qPCR assays were performed using LightCycler 480 System (Roche 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. Thus, the bubble method may have significantly underestimated the nitrogen fixation rates in some, if not all, of the samples that we analyzed. Although the nitrogen fixation rates reported in the rest of this paper are those obtained using the bubble method, which was used as the standard protocol during all cruises, the possibility that some of these rates could be underestimated must be kept in mind.

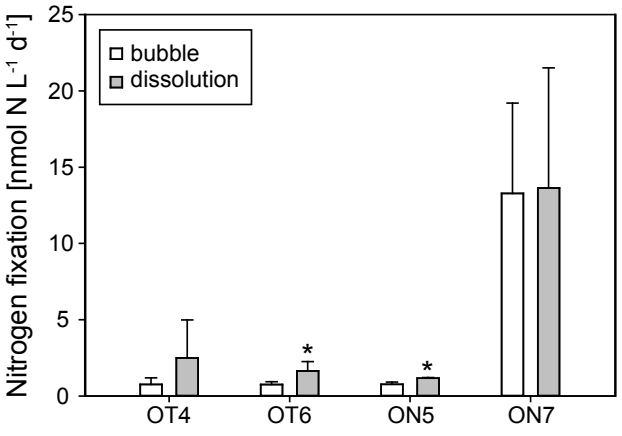

Figure 2. Nitrogen fixation rates estimated simultaneously by the $^{15}N_2$ gas bubble and dissolution methods during the KK-13-6_Sep cruise. An asterisk indicates a significant difference between the two methods ($p < 0.05$).

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 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 the Coastal Oyashio water system (CO)). 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 assumed 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 (SSTs) (range of 1.5–24.3 °C) (Figs. 4a and S1) and surface nitrate and phosphate concentrations determined during each cruise were averaged to indicate the seasonal variability of these parameters (Fig. 4b). In general, surface nitrate and phosphate concentrations were low (≤ 0.07 and ≤ 0.20 µM, respectively) in the warmer seawaters (14.2–24.3 °C) sampled in early summer (KK-13-1_Jun), mid-summer (KT-12-20_Aug), and fall (KT-12-27_Oct), whereas they were relatively high (≥ 0.75 and ≥ 0.28 µM, respectively) in the colder seawaters (1.5–9.8 °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 nearshore Stn. OT1 (Fig. S2). Similar to nitrate, surface phos-

Table 1. Summary of recovered *nifH* sequences belonging to *Trichodesmium* (Tri), UCYN-A (UA), Leptolyngbya (Lep), α-Proteobacteria (α-Pro), β-Proteobacteria (β-Pro), γ-Proteobacteria (γ-Pro), δ-Proteobacteria (δ-Pro), and Cluster III (CIII).

Cruise	Station	No. of clones	Cyanobacteria			$\alpha-$Pro	$\beta-$Pro	$\gamma-$Pro	$\delta-$Pro	CIII
			Tri	UA	Lep					
KT-12-20_Aug	OT4	12		9		3				
mid-summer	ON1	5		2						3
	ON5	8		8						
	ON7	7		1		6				
Total		32	0	20	0	9	0	0	0	3
KT-12-27_Oct	OT4	7	1							6
fall	ON1	9							4(2)	5(5)
	ON5	6						1		5
	ON7	13	6	1		5(5)		1(1)		
Total		35	7	1	0	5(5)	0	2(1)	4(2)	16(5)
KT-13-2_Jan	OT4	11			10			1		
winter	ON1	1								1
	ON5	14				5(5)			2(2)	7
Total		26	0	0	10	5(5)	0	1	2(2)	8
KK-13-1_Jun	OT4	10		2		8(8)				
early summer	ON1	15		3				2	10(10)	
	ON5	11		4		7(7)				
	ON8	1					1			
Total		37	0	9	0	15(15)	1	2	10(10)	0
KK-13-6_Sep	OT4	7							4(4)	1
late summer	ON5	11		11						
	ON7	10		2		1		7		
Total		28	0	13	0	1	0	7	4(4)	1
KS-14-2_Mar	OT4	10							1(1)	9
spring	ON1	13				3(3)	3	1(1)	3(3)	
	ON5	15				2(2)				9
Total		38	0	0	0	5(5)	3	1(1)	4(4)	18

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

phate concentrations tended to be high during winter (KT-13-2_Jan) and spring (KS-14-2_Mar), while they were low during the warmer seasons. By contrast, surface ammonium concentrations were generally low ($\leq \sim 1\,\mu M$) throughout the year (Fig. 4b), except for the high ammonium concentration determined at Stn. OT1 (1.41 µM) during the KK-13-6_Sep cruise (Fig. S2).

During the four cruises conducted in early summer (KK-13-1_Jun), mid-summer (KT-12-20_Aug), late summer (KK-13-6_Sep), and fall (KT-12-27_Oct), nitrogen fixation was measurable in most of the samples collected from surface waters: the nitrogen fixation rates varied in the range of 0.33–13.6 nmol $N\,L^{-1}\,d^{-1}$ (Figs. 4c and S2). Relatively high nitrogen fixation rates were determined for samples collected during the KT-12-20_Aug cruise, although the highest value was obtained at Stn. ON7 during the KK-13-6_Sep cruise. Nitrogen fixation was below the detection limit in seawater samples collected during the winter and spring cruises. For those samples, nitrogen fixation was undetectable even after the addition of mannitol (KS-14-2_Mar). Also, nitrogen fixation was undetectable in DIN-replete water collected at Stn. OT1 in late summer (KK-13-6_Sep).

Nitrogen fixation rates were determined for samples collected from different depths (0–119 m) at Stns. OT4 and ON5 (Fig. 5). Nitrogen fixation was detected in surface and deeper layers during four cruises conducted in early summer (KK-13-1_Jun), mid-summer (KT-12-20_Aug), late summer (KK-13-6_Sep), and fall (KT-12-27_Oct) (Fig. 4). Nitrogen fixation rates tended to be higher at the surface than in the deeper layers during mid-summer (KT-12-20_Aug)

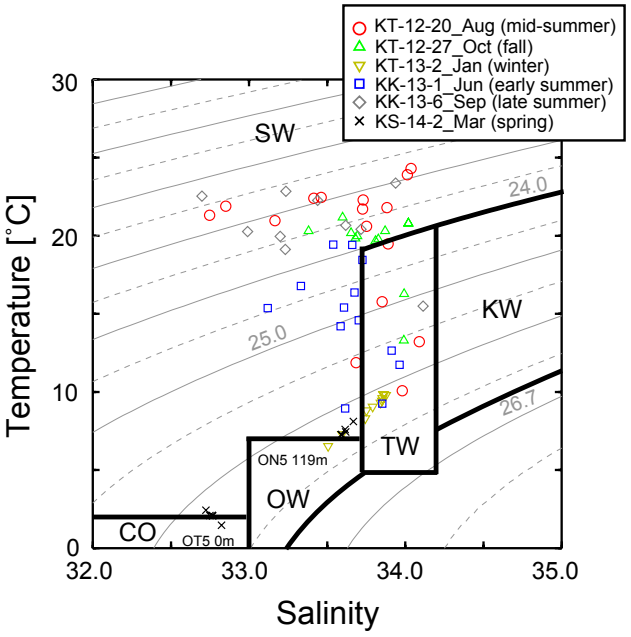

Figure 3. Temperature–salinity diagram at each sampling point. The water classification was defined by Hanawa and Mitsudera (1986). SW, KW, TW, OW, and CO denote the surface layer water system, Kuroshio water system, Tsugaru Warm Current water system, Oyashio water system, and Coastal Oyashio water system,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

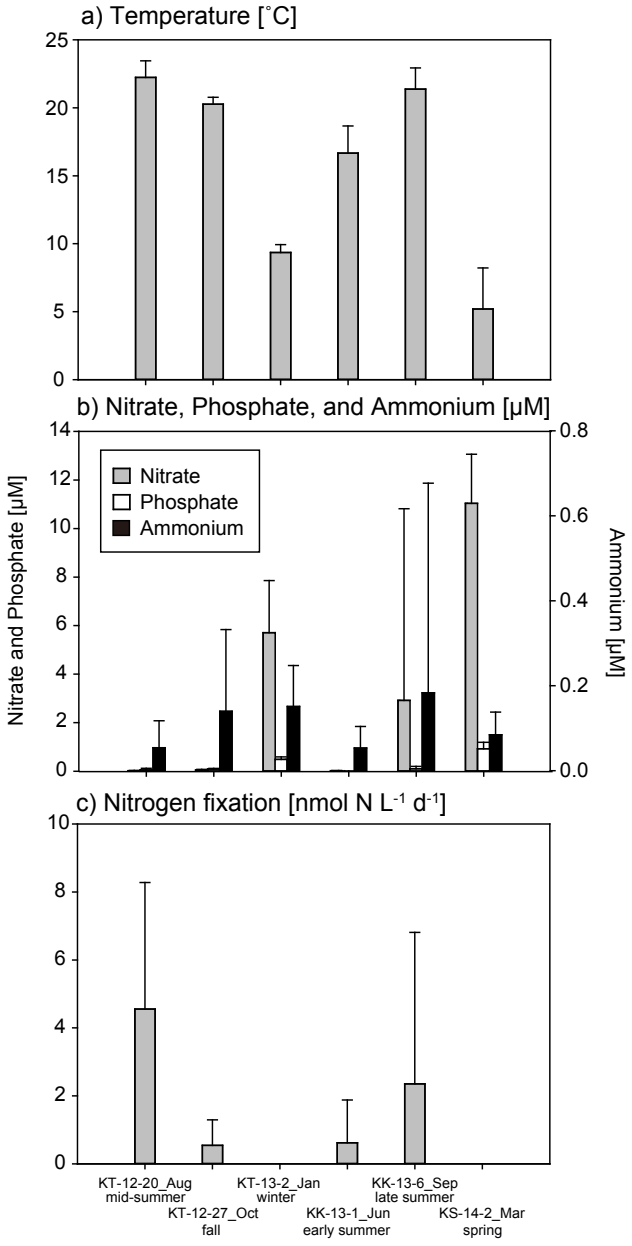

Figure 4. Average **(a)** temperature [°C], **(b)** nitrate, phosphate, and ammonium concentrations [μM], and **(c)** nitrogen fixation [nmol N L^{-1} d^{-1}] at the surface during each cruise.

and late summer (KK-13-6_Sep (at Stn. OT4)), whereas this vertical trend was less evident during fall (KT-12-27_Oct) and early summer (KK-13-1_Jun). At Stn. OT4, nitrogen fixation was detected even in the layers below the nitracline (KT-12-27_Oct, depth = 62 m; KK-13-1_Jun, depth = 42 m). During KK-13-1_Jun cruise, the nitrogen fixation rate determined at the depth of 42 m (1.56 nmol N L^{-1} d^{-1}) was 1.8 fold higher than the corresponding rate at the surface (0.87 nmol N L^{-1} d^{-1}). The concentrations of nitrate and ammonium in these layers varied in the range of < 0.02–22.5 and < 0.01–1.41 μM, respectively. The maximum depth-integrated nitrogen fixation (294 μmol N m^{-2} d^{-1}) was found at Stn. OT4 during mid-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 11.7 °C, with higher rates (> 6 nmol N L^{-1} d^{-1}) observed 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 we observed that the nitrogen fixation rate was highest at 15.4 °C, while it was low (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 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). Nitrogen fixation was generally detectable only when nitrate was depleted

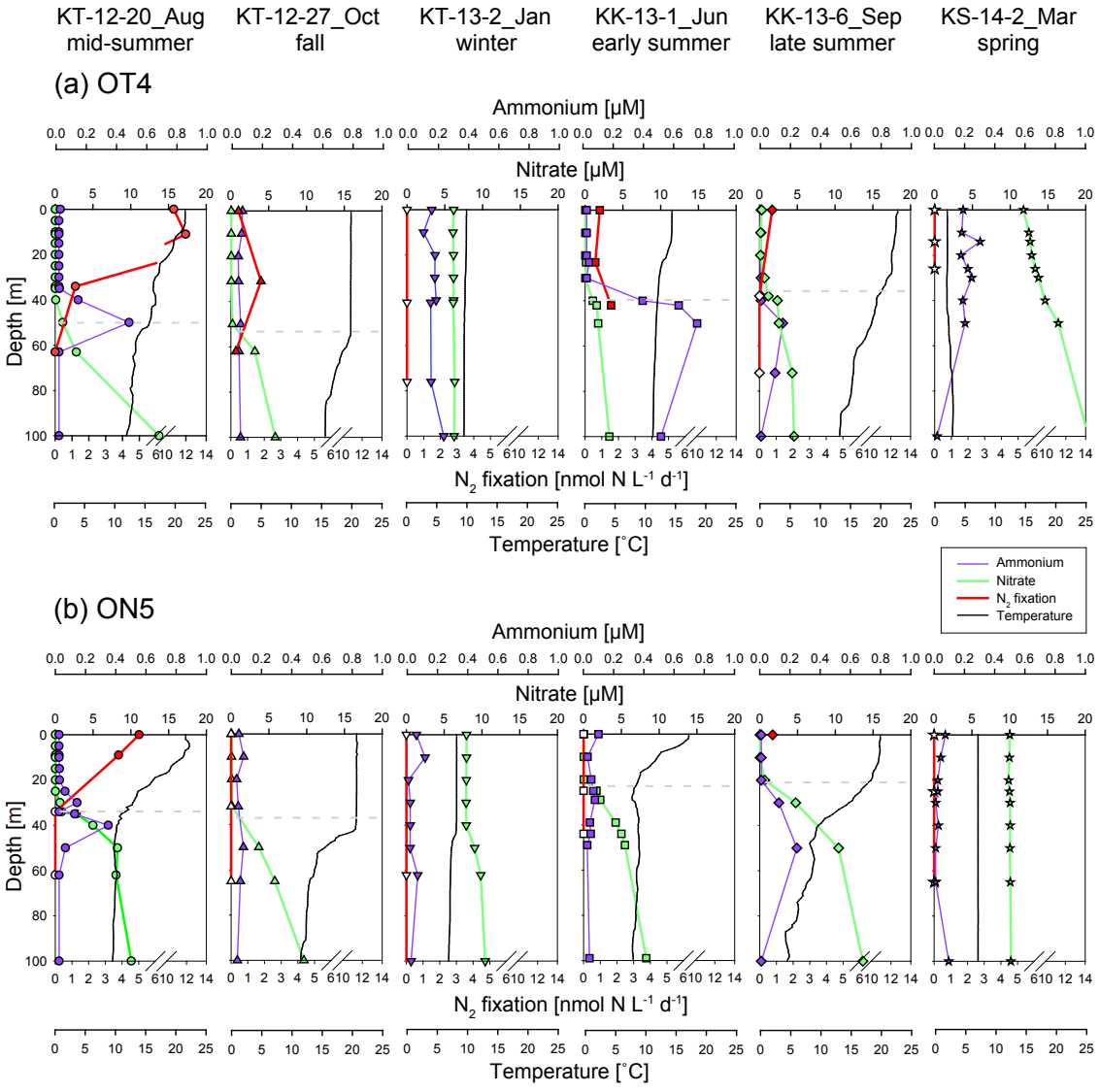

Figure 5. Time-series variations in the vertical profiles of temperature [°C] (black), ammonium (purple) and nitrate (green) concentration [μM], and nitrogen fixation (red) [nmol N L^{-1} d^{-1}] at Stns **(a)** OT4 and **(b)** ON5. Open symbols indicate that nitrogen fixation was not detected. The horizontal dashed line indicates the nitracline depth. The strait lines of temperature and nitrate were ascribable to strong mixing.

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

* $p < 0.05$, ** $p < 0.01$ N / P ratio denotes the ratio of (nitrate + nitrite + ammonium) to phosphate.

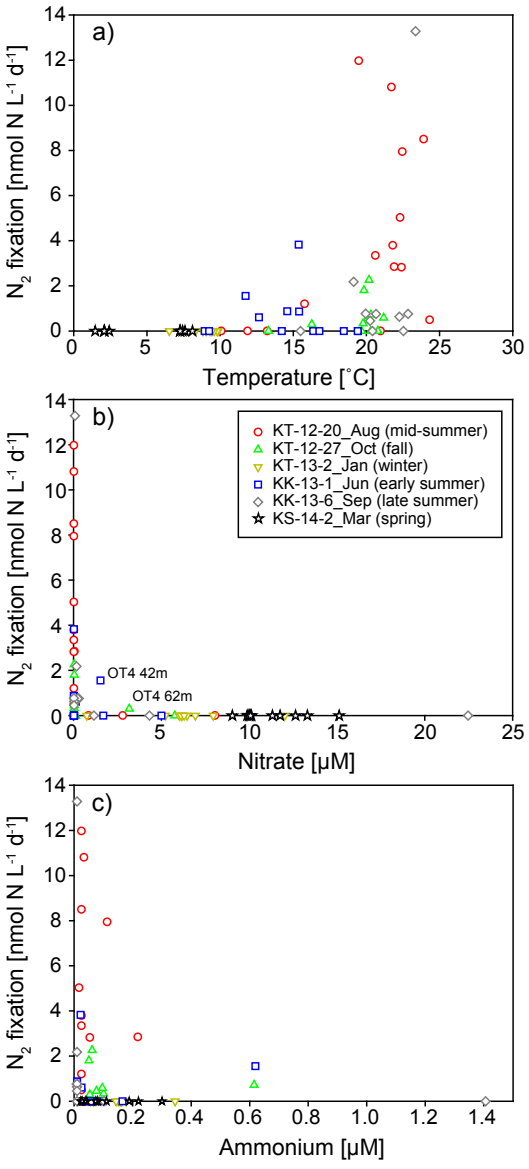

Figure 6. Relationship between nitrogen fixation [nmol N L^{-1} d^{-1}] and **(a)** temperature [°C], **(b)** nitrate [μM], and **(c)** ammonium [μM] for all six cruises.

(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). High nitrogen fixation rates tended to be detected when ammonium concentrations were low ($\leq \sim 0.1$ μM), although there was no statistically significant relationship between nitrogen fixation rates and ammonium concentrations.

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 (> 97 % similarity at the amino acid level was used as a criterion). 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 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 recovered from early summer to fall, while *nifH* of *Leptolyngbya* was 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 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 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 (Figs. 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.

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 (Figs. 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-

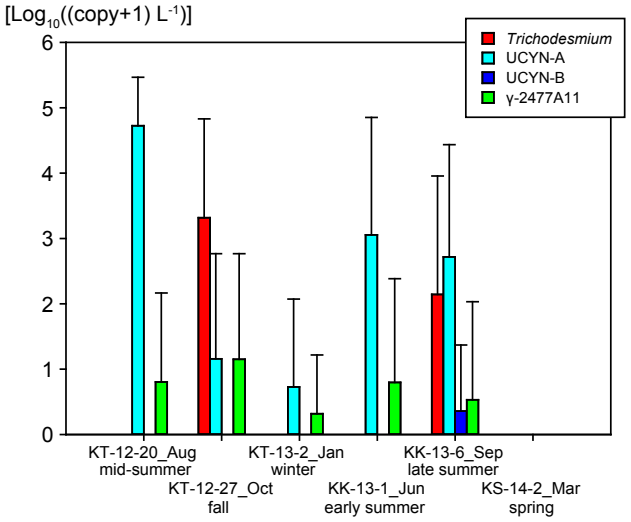

Figure 7. Average abundances of *Trichodesmium* (red), UCYN-A (light blue), UCYN-B (blue), and γ-24774A11 (green) [$\mathrm{Log_{10}((copy+1)\,L^{-1})}$] at the surface during each cruise. When the target *nifH* gene was not detected, the copy number was assumed to be zero.

12-27_Oct cruise (Figs. 7 and S6). UCYN-A was detected on all cruises except for the KS-14-2_Mar cruise (Figs. 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 $\mathrm{L^{-1}}$) was observed at 72 m (Fig. 8). The abundance of UCYN-A varied from below the detection limit to 2.6×10^5 copies $\mathrm{L^{-1}}$ 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 (Figs. 7 and S6). UCYN-B was detected only at Stn. ON7 during the KK-13-6_Sep cruise (Figs. 7, 8, and S6). γ-24774A11 was detected during all cruises except for the KS-14-2_Mar cruise (Figs. 7 and 8). The abundance of γ-24774A11 ranged from below the detection limit to 1.8×10^4 copies $\mathrm{L^{-1}}$, with a tendency of subsurface peaks at both stations (Fig. 8).

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 $\mathrm{N\,L^{-1}\,d^{-1}}$) 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 $\mathrm{N\,L^{-1}\,d^{-1}}$; Needoba et al., 2007) and the oligotrophic region of the western and central North Pacific (0.17–3.62 nmol $\mathrm{N\,L^{-1}\,d^{-1}}$; Shiozaki et al., 2010), whereas they were comparable to those determined in the Kuroshio (0.54–28 nmol $\mathrm{N\,L^{-1}\,d^{-1}}$; Shiozaki et al., 2010) and the western Atlantic coastal regions (1.3–49.8 nmol $\mathrm{N\,L^{-1}\,d^{-1}}$; 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 $\mathrm{N\,L^{-1}\,d^{-1}}$; Rees et al., 2009) and the Baltic Sea estuaries (47–83 nmol $\mathrm{N\,L^{-1}\,d^{-1}}$; Bentzon-Tilia et al., 2015).

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

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

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

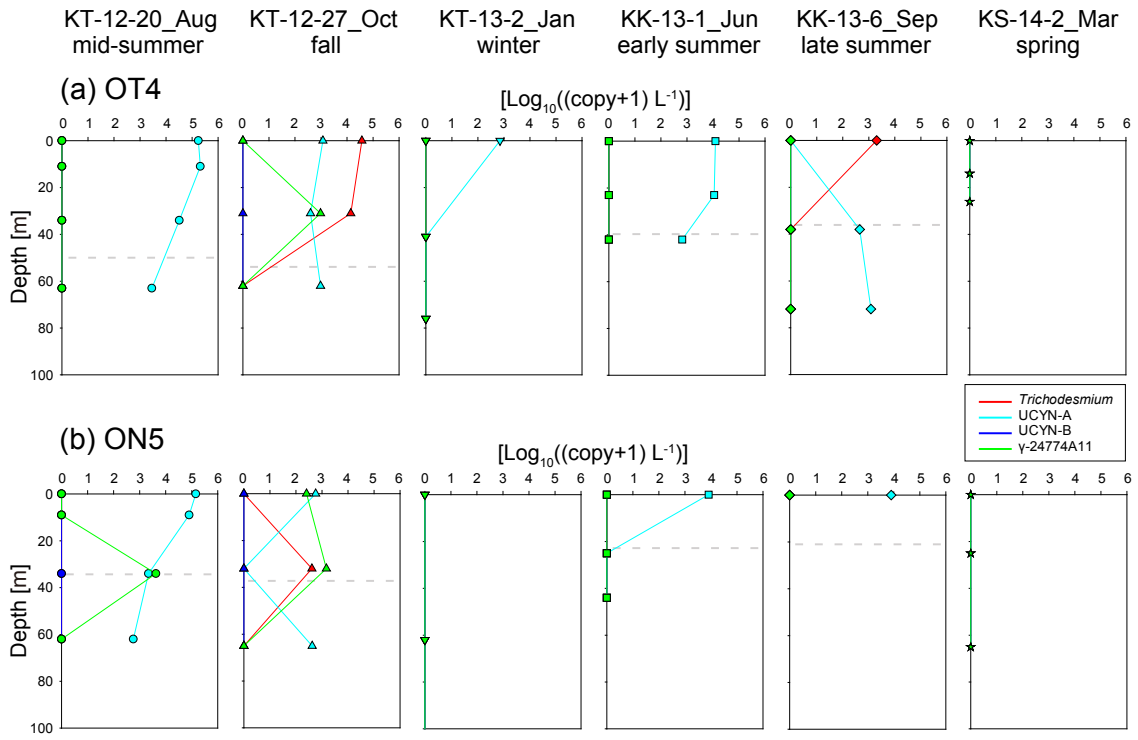

Figure 8. Time-series variations in the vertical profiles of *Trichodesmium* (red), UCYN-A (light blue), UCYN-B (blue), and γ-24774A11 (green) [$\mathrm{Log_{10}((copy{+}1)\,L^{-1})}$] at Stns. **(a)** OT4 and **(b)** ON5. The horizontal dashed line indicates the nitracline depth.

could be delivered from subtropical oceans where DIN is depleted. Previous studies have suggested that cyanobacterial diazotrophs can travel over long distances (> 1000 km) in currents, without losing their capacity for N_2 fixation (Shiozaki et al., 2013), and that activity is not lost immediately even after mixing with DIN-replete seawaters (Holl and Montoya, 2005; Dekaezemacker and Bonnet, 2011). In our region, because the Tsugaru Warm Current flows from north to south, diazotrophs entrained by the current have little chance of meeting DIN-rich water at the surface. DIN-replete water during mid-summer was observed at the inside bay station OT1 (Fig. S2). Concomitantly, low-salinity surface waters spread offshore along the OT transect line (Fig. S7), suggesting 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 *Trichodesmium* (Mulholland et al. 2001). In our study, ammonium concentrations were generally low ($\leq \sim 1$ µM) throughout the investigation, and no negative relationship between nitrogen fixation and ammonium concentration was found. 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 (Table 2). Because temperature and phosphate concentrations were correlated with nitrate concentrations, these factors would not necessarily influence nitrogen fixation directly. Rather, one or more 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 abun-

dant group during fall, when this group was frequently recovered in the library (during the KT-12-27_Oct cruise). Therefore, the diazotrophs targeted by the qPCR analysis were likely important for nitrogen fixation in this study region. In the discussion below, we mainly discuss possible factors responsible for seasonal variation in the diazotrophs targeted by the qPCR analysis.

UCYN-A was detected in all seasons except spring (KS-14-2_Mar), suggesting that this group of diazotrophs could be important agents of nitrogen fixation in this region. Especially from early to late summer, the abundance of UCYN-A was generally higher than that of *Trichodesmium*, UCYN-B, and γ-24774A11. UCYN-A has been widely detected in temperate regions, and is considered to be one of the major diazotrophs of these locations (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012; Bentzon-Tilia et al., 2015). UCYN-A is known to be most abundant in relatively warm waters around $\sim 20\,^{\circ}$C (Needoba et al., 2007; Moisander et al., 2010). In our study, UCYN-A was detected during winter at some stations. It appears that UCYN-A abundance decreased with decreasing temperature from fall to winter, and then became undetectable in spring.

Trichodesmium was detected from late summer to fall, when water temperatures ranged from 19.1 to 23.4 °C at the surface. Given that the optimal growth temperature for *Trichodesmium* has been reported to be high (24–30 °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 ($> 24\,^{\circ}$C). In the western North Pacific subtropical region, *Trichodesmium* is abundant from July to September (Marumo and Nagasawa, 1976; Chen et al., 2008). *Trichodesmium* that flourished in the subtropical region during summer could be transported by the Tsugaru Warm Current, displaying peak abundance during fall in the investigated region. This could support the above discussion that waters containing active nitrogen fixation were delivered to this region by the Tsugaru Warm Current.

We detected γ-24774A11 during all cruises except for the KS-14-2_Mar cruise. γ-24774A11 is considered to be one of the most important heterotrophic diazotrophs in the tropical and subtropical oligotrophic ocean (Moisander et al., 2014). However, the γ-24774A11 sequence has not been detected previously in other temperate oceans (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012). The γ-24774A11 sequence was similar (94 % similarity at the amino acid level) to the *nifH* sequence of *Pseudomonas stutzeri*, which has been reported to be present in temperate estuaries (Bentzon-Tilia et al., 2015). Bentzon-Tilia et al. (2015) reported that *P. stutzeri*-like *nifH* genes (99 % similarity at the nucleotide level) were the most abundant sequences among their samples collected from the Baltic Sea estuary. In the present study, we recovered *P. stutzeri*-like *nifH* genes ($> 97\%$ similarity at the amino acid level) only at 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*. The ecology of γ-24774A11 is still fairly unknown. It remains to be seen whether this phylotype contributes to the nitrogen fixation in this region, a topic for future studies.

UCYN-B was not detected 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 have survived 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 appeared to be present throughout the investigation period. Cluster III diazotrophs are putative anaerobes (Hamersley et al., 2011; Farnelid et al., 2013; Bentzon-Tilia et al., 2014), and hence, they are usually dominant in the diazotrophic community of oxygen-depleted waters (Hamersley et al., 2011; Farnelid et al., 2013) or marine sediments (Bertics et al., 2013). In this study, dissolved oxygen was not depleted (> 3.16 mL L^{-1}) in the upper winter maximum mixed layer depth in this region (~ 200 m; Shiozaki et al., 2014b) (Fig. S8). Therefore, the Cluster III activity was likely strongly suppressed in the water column because of the high oxygen concentration.

Many *nifH* sequences recovered by the clone library analysis were similar to terrestrially derived sequences. These results agree with previous data collected in coastal regions, where terrestrially derived *nifH* sequences were also found (Rees et al., 2009; Mulholland et al., 2012; Blais et al., 2012). We obtained a *Leptolyngbya*-like *nifH* gene during the KT-13-2_Jan cruise. The organism has been found on beaches and in coastal land areas (Brito et al. 2012), but not in the open ocean. Because nitrogen fixation was not detected during the KT-13-2_Jan cruise, the organism was considered not to perform nitrogen fixation.

5 Conclusion

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

The Supplement related to this article is available online at doi:10.5194/bg-12-4751-2015-supplement.

Author contributions. T. Shiozaki, T. Nagata, and K. Furuya designed the experiment and T. Shiozaki collected the samples at sea. T. Shiozaki determined nitrogen fixation and nutrient concentrations and analyzed satellite data sets. T. Shiozaki and M. Ijichi conducted the genetic analyses. T. Shiozaki 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 Spatiales (CNES AVISO) for providing satellite data sets. This study was financially supported by Tohoku Ecosystem-Associated Marine Sciences (TEAMS) sponsored by Ministry of Education, Culture, Sports, Science and Technology (MEXT), by a grant-in-aid for Scientific Research on Innovative Areas (24121001), by the Japan Science and Technology Agency (JST CREST), and by a grant-in-aid for Japan Society for the Promotion of Science (JSPS) Fellows (25-7341) from MEXT.

Edited by: K. Suzuki

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, doi:10.5194/bg-10-1243-2013, 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, Global Biogeochem. Cy., 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, 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, Global Biogeochem. Cy., 16, 1120, doi:10.1029/2001GB001856, 2002.

Breitbarth, E., Oschlies, A., and LaRoche, J.: Physiological constraints on the global distribution of *Trichodesmium* – effect of temperature on diazotrophy, Biogeosciences, 4, 53–61, doi:10.5194/bg-4-53-2007, 2007.

Brito, A., Ramos, V., Seabra, R., Santos, A., Santos, C. L., Lopo, M., Ferreira, S., Martins, A., Mota, R., Frazão, B., Martins, R., Vasconcelos, V., and Tamagnini, P.: Culture-dependent characterization of cyanobacterial diversity in the intertidal zones of the Portuguese coast: A polyphasic study, Syst. Appl. Microbiol. 35, 110–119, 2012.

Capone, D. G., Zehr, J. P., Paerl, H. W., Bergman, B., and Carpenter, E. J.: Trichodesmium, a globally significant marine cyanobacterium, Science, 276, 1221–1229, 1997.

Chen, Y.-L. L., Chen, H.-Y., Tuo, S.-H., and Ohki, K.: Seasonal dynamics of new production from *Trichodesmium* N_2 fixation and nitrate uptake in the upstream Kuroshio and South China Sea basin, Limnol. Oceanogr., 53, 1705–1721, 2008.

Codispoti, L. A.: An oceanic fixed nitrogen sink exceeding $400\,\mathrm{Tg\,N\,a^{-1}}$ vs the concept of homeostasis in the fixed-nitrogen inventory, Biogeosciences, 4, 233–253, doi:10.5194/bg-4-233-2007, 2007.

Dabundo, R., Lehmann, M. F., Treibergs, L., Tobias, C. R., Altabet, M. A., Moisander, P. H., and Granger, J.: The contamination of commercial $^{15}N_2$ gas stocks with ^{15}N-labeled nitrate and ammonium and consequences for nitrogen fixation measurements, PLoS ONE, 9, e110335, doi:10.1371/journal.pone.0110335, 2014.

Dekaezemacker, J. and Bonnet, S.: Sensitivity of N_2 fixation to combined nitrogen forms (NO_3^- and NH_4^+) in two strains of the marine diazotroph *Crocosphaera watsonii* (Cyanobacteria), Mar. Ecol.-Prog. Ser., 438, 33–46, 2011.

Deutsch, C., Sigman, D. M., Thunell, R. C., Meckler, A. N., and Haug, G. H.: Isotopic constraints on glacial/interglacial changes in the oceanic nitrogen budget, Global Biogeochem. Cy., 18, GB4012, doi:10.1029/2003GB002189, 2004.

Falkowski, P. G.: Enzymology of nitrogen assimilation, in: Nitrogen in the marine environment, edited by: Carpenter, J. and Capone, D. G., Academic Press, New York, 839–868, 1983.

Farnelid, H., Bentzon-Tilia, M., Andersson, A. F., Bertilsson, S., Jost, G., Labrenz, M., Jürgens, K., and Riemann, L.: Active nitrogen-fixing heterotrophic bacteria at and below the chemocline of the central Baltic Sea, ISME J., 7, 1413–1423, 2013.

Großkopf, T., Mohr, W., Baustian, T., Schunck, H., Gill, D., Kuypers, M. M. M., Lavik, G., Schmitz, R. A., Wallace, D. W. R., and LaRoche, J.: Doubling of marine dinitrogen-fixation rates based on direct measurements, Nature, 488, 361–364, 2012.

Halm, H., Lam, P., Ferdelman, T. G., Lavik, G., Dittmar, T., LaRoche, J., D'Hondt, S., and Kuypers, M. M. M.: Heterotrophic organisms dominate nitrogen fixation in the South Pacific Gyre, ISME J., 6, 1238–1249, 2012.

Hamersley, M. R., Turk, K. A., Leinweber, A., Gruber, N., Zehr, J. P., Gunderson, T., and Capone, D. G.: Nitrogen fixation within the water column associated with two hypoxic basins in the Southern California Bight, Aquat. Microb. Ecol., 63, 193–205, 2011.

Hanawa, K. and Mitsudera, H.: Variation of water system distribution in the Sanriku coastal area, J. Oceanogr. Soc. Jpn., 42, 435–446, 1987.

Holl, C. M. and Montoya, J. P.: Interactions between nitrate uptake and nitrogen fixation in continuous cultures of the marine diazotroph *Trichodesmium* (Cyanobacteria), J. Phycol., 41, 1178–1183, 2005.

Huang, Y., Niu, B. F., Gao, Y., Fu, L. M., and Li, W.Z.: CD-HIT Suite: a web server for clustering and comparing biological sequences, Bioinformatics, 26, 680–682, 2010.

Marumo, R. and Nagasawa S.: Seasonal variation of the standing crop of a pelagic blue-green alga, *Trichodesmium* in the Kuroshio water, Bull. Plankton Soc. Japan, 23, 19–25, 1976 (in Japanese with English abstract).

Moisander, P. H., Beinart, R. A., Hewson, I., White, A. E., Johnson, K. S., Carlson, C. A., Montoya, J. P., and Zehr, J. P.: Unicellular cyanobacterial distributions broaden the oceanic N_2 fixation domain, Science, 327, 1512–1514, 2010.

Moisander, P. H., Zhang, R., Boyle, E. A., Hewson, I., Montoya, J. P., and Zehr, J. P.: Analogous nutrient limitations in unicellular diazotrophs and *Prochlorococcus* in the South Pacific Ocean, ISME J., 6, 733–744, 2011.

Moisander, P. H., Serros, T., Paerl, R. W., Beinart, R. A., and Zehr, J. P.: Gammaproteobacterial diazotrophs and *nifH* gene expression in surface waters of the South Pacific Ocean, ISME J., 8, 1962–1973, 2014.

Mohr, W., Großkopf, T., Wallace, D. W. R., and LaRoche, J.: Methodological underestimation of oceanic nitrogen fixation rates, PLoS ONE, 5, e12583, doi:10.1371/journal.pone.0012583, 2010.

Montoya, J. P., Voss, M., Kähler, P., and Capone, D. G.: A simple, high-precision, high-sensitivity tracer assay for N_2 fixation, Appl. Environ. Microb., 62, 986–993, 1996.

Mulholland, M. R., Ohki, K., and Capone, D. G.: Nutrient controls on nitrogen uptake and metabolism by natural populations and cultures of *Trichodesmium* (Cyanobacteria), J. Phycol., 37, 1001–1009, 2001.

Mulholland, M. R., Bernhardt, P. W., Blanco-Garcia, J. L., Mannino, A., Hyde, K., Mondragon, E., Turk, K., Moisander, P. H., and Zehr, J. P.: Rates of dinitrogen fixation and the abundance of diazotrophs in North American coastal waters between Cape Hatteras and Georges Bank, Limnol. Oceanogr., 57, 1067–1083, 2012.

Needoba, J. A., Foster, R. A., Sakamoto, C., Zehr, J. P, and Johnson, K. S.: Nitrogen fixation by unicellular diazotrophic cyanobacteria in the temperate oligotrophic North Pacific Ocean, Limnol. Oceanogr., 52, 1317–1327, 2007.

Nishioka, J., Ono, T., Saito, H., Nakatsuka, T., Takeda, S., Yoshimura, T., Suzuki, K., Kuma, K., Nakabayashi, S., Tsumune, D., Mitsudera, H., Johnson, W. K., and Tsuda, A.: Iron supply to the western subarctic Pacific: Importance of iron export from the Sea of Okhotsk, J. Geophys. Res., 112, C10012, doi:10.1029/2006JC004055, 2007.

Nishioka, J., Ono, T., Saito, H., Sakaoka, K., and Yoshimura, T.: Oceanic iron supply mechanisms which support the spring diatom bloom in the Oyashio region, western subarctic Pacific, J. Geophys. Res., 116, C02021, doi:10.1029/2010JC006321, 2011.

Rahav. E., Bar-Zeev, E., Ohayon, S., Elifantz, H., Belkin, N., Herut, B., Mulholland, M. R., and Berman-Frank, I.: Dinitrogen fixation in aphotic oxygenated marine environment, Front. Microbiol., 4, 277, doi:10.3389/fmicb.2013.00227, 2013.

Rees, A. P., Gilbert, J. A., and Kelly-Gerreyn, B. A.: Nitrogen fixation in the western English Channel (NE Atlantic Ocean), Mar. Ecol.-Prog. Ser., 374, 7–12, 2009.

Riemann, L., Farnelid, H., and Steward, G. F.: Nitrogenase genes in non-cyanobacterial plankton: prevalence, diversity and regulation in marine waters, Aquat. Microb. Ecol., 61, 235–247, 2010.

Saitoh, Y., Kuma, K., Isoda, Y., Kuroda, H., Matsuura, H., Wagawa, T., Takana, H., Kobayashi, N., Nagao, S., and Nakatsuka, T.: Processes influencing iron distribution in the coastal waters of the Tsugaru Strait, Japan, J. Oceanogr., 64, 815–830, 2008.

Shiozaki, T., Furuya, K., Kodama, T., and Takeda, S.: Contribution of N_2 fixation to new production in the western North Pacific Ocean along 155° E, Mar. Ecol.-Prog. Ser., 377, 19–32, 2009.

Shiozaki, T., Furuya, K., Kodama, T., Kitajima, S., Takeda, S., Takemura, T., and Kanda, J.: New estimation of N_2 fixation in the western and central Pacific Ocean and its marginal seas, Global Biogeochem. Cy., 24, GB1015, doi:10.1029/2009GB003620, 2010.

Shiozaki, T., Kodama, T., Kitajima, S., Sato, M., and Furuya, K.: Advective transport of diazotrophs and importance of their nitrogen fixation on new and primary production in the western Pacific warm pool, Limnol. Oceanogr., 58, 49–60, 2013.

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, Global Biogeochem. Cy., 28, 1096–1110, 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, 253–265, 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, P-. Natl. Acad. Sci. USA, 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, Method. Microbiol., 30, 271–285, 2001.

Zehr, J. P., Jenkins, B. D., Short, S. M., and Steward, G. F.: Nitrogenase gene diversity and microbial community structure: a cross-system comparison, Environ. Micorbiol., 5, 539–554, 2003a.

Zehr, J. P., Crumbliss, L. L., Church, M. J., Omoregie, E. O., Jenkins, and B. D.: Nitrogenase genes in PCR and RT-PCR reagents: implications for studies of diversity of functional genes, BioTechniques, 35, 996–1005, 2003b.

Zehr, J. P.: Nitrogen fixation by marine cyanobacteria, Trends Microbiol., 19, 162–173, 2011.