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Seasonal dynamics of nitrogen fixation and the diazotroph community in the temperate coastal region of the northwestern North Pacific

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Abstract

Nitrogen fixation in temperate oceans is a potentially important, but poorly understood process that may influence the marine nitrogen budget. This study determined seasonal variations in nitrogen fixation and *nifH* gene diversity within the euphotic zone in the temperate coastal region of the northwestern North Pacific. Nitrogen fixation as high as 13.6 nmolN L^{-1} d^{-1} was measured from early summer to fall when the surface temperature exceeded 14.2 °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). During periods with high nitrogen fixation, the *nifH* sequences of UCYN-A were recovered, suggesting that these groups played a key role in nitrogen fixation. The *nifH* genes were also recovered in spring and winter when nitrogen fixation was undetectable. These genes consisted of many sequences affiliated with Cluster III diazotrophs (putative anaerobic bacteria), which hitherto have rarely been reported to be abundant in surface diazotroph communities in marine environments.

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, the data of rate measurements 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

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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 temperature ($< \sim 20\,^{\circ}C$), which generally inhibits 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 regarded to inhibit nitrogen fixation (Falkowski, 1983), especially during mixing periods. However, recent studies have indicated that nitrogen fixation, presumably mediated by unicellular cyanobacteria and heterotrophic bacteria, is detectable even in the relatively cold ($< 10\,^{\circ}C$) 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 in the temperate inside bays and open ocean located in the interfrontal zone of the northwestern North Pacific. In this 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

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best of our knowledge, the present study is the first to examine seasonal diazotrophy in the temperate ocean. The study was conducted as part of a project to monitor the dynamics of the coastal ecosystem and the recovery thereof after the 2011 Tohoku-oki tsunami, which struck the region on 11 March 2011.

2 Materials and methods

The experiments were conducted during six cruises in the temperate coastal region of the western North Pacific. These cruises covered a full seasonal cycle, including spring (KS-14-2, 14–19 March 2014), early summer (KK-13-1, 24–29 June 2013), summer (KT-12-20, 7–12 August 2012, KK-13-6, 14–21 September 2013), fall (KT-12-27, 15–22 October 2012), and winter (KT-13-2, 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), with additional stations being deployed in the Otsuchi and Onagawa bays (Fig. 1). Just before the KK-13-6 cruise, Typhoon Man-yi passed from southwest to northeast in the study area (Fig. S1 in the Supplement).

Temperature, salinity, and dissolved oxygen profiles to 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. Samples for nutrients were collected from a depth of 7–15 m in the upper 200 m at stations outside the bays and from a depth of 1–13 m in the upper bottom floor at stations inside the bays. At Stn. OT1, the CTD cast was not performed, and water samples were taken only from the surface. Samples for DNA analysis and incubation experiments were collected from the surface at 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.

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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 DNA 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 manufacturers protocol (Shiozaki et al., 2014a). Partial *nifH* fragments were amplified from samples collected from surface water at Stns. OT4, ON1, ON5, and ON7 using a nested PCR strategy (Zehr and Turner, 2001). PCR chemicals 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. The PCR products were cloned and sequenced according to Shiozaki et al. (2014a). No DNA was detected from negative controls of the PCRs. The *nifH* sequences were translated into amino acid sequences and searched against the protein database of the National Center for Biotechnology Information using the BLASTp algorithm. Clones with 100 % amino acid sequence similarity were defined as the same operational taxonomic units (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.

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The sequences from this study were deposited in the DNA Data Bank of Japan (DDBJ) with accession numbers LC013480 to LC013676.

2.3 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 acid-cleaned 2 L polycarbonate (PC) bottles and the time-zero samples immediately filtered onto precombusted GF/F filters. Two milliliters of $^{15}N_2$ gas were directly injected 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. 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).

Because of the possibility of underestimation of nitrogen fixation as determined by the bubble method (Mohr et al., 2010; Großkopf et al., 2012), we also determined nitrogen fixation rates using the $^{15}N_2$ gas dissolution method (hereafter, the dissolution method; Mohr et al., 2010) during the KK-13-6 and KS-14-2 cruises. $^{15}N_2$-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 added. 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 nitrogen fixation rate was calculated according to Mohr et al. (2010).

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 cruise) with and without addition of mannitol (final conc. 0.8 µM).

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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 and KS-14-2 cruises (Fig. 2). Both methods failed to detect nitrogen fixation in samples collected during the KS-14-2 cruise. During the KK-13-6 cruise, the nitrogen fixation rates determined by the dissolution method were significantly ($p < 0.05$) higher (1.5–2.2 fold) than those determined by the bubble method at two of four stations ($p < 0.05$). At other stations, 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 are underestimates.

3.2 Seasonal variations in nitrogen fixation rates

We found prominent seasonal variations in sea surface temperature (SST) (range, 1.5 to 24.3 °C) (Figs. 3a and S1) and surface nutrient concentrations (Figs. 3b and S2). In general, nitrate and phosphate concentrations were low (≤ 0.07 µM and ≤ 0.20 µM, respectively) in the warmer seawaters (14.2–24.3 °C) sampled in early summer (KK-13-1), summer (KT-12-20), and fall (KT-12-27), whereas they were relatively high (≥ 0.75 µM and ≥ 0.28 µM, respectively) in the colder seawaters (1.5–9.8 °C) sampled in winter (KT-13-2), and spring (KS-14-2), although the nitrate concentrations were relatively high and variable (mean ± SD; 2.92 ± 7.90 µM) in samples collected in summer during the KK-13-6 cruise. During the KK-13-6 cruise, low-salinity surface waters spread offshore along the OT transect line (Fig. S3). Concomitantly, the highest nitrate concentration (22.6 µM) was determined at the near-shore Stn. OT1 (Fig. S2). These results suggest that anomalously high nitrate concentrations were likely attributable to

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terrestrial surface discharge enhanced by Typhoon Man-yi that passed over the region immediately before the cruise.

Nitrogen fixation was detected in the surface waters of most samples collected during the four cruises conducted in early summer (KK-13-1), summer (KT-12-20 and KK-13-6), and fall (KT-12-27), and varied in the range 0.33–13.6 nmolN L^{-1} d^{-1} (Figs. 3c and S2). Relatively high nitrogen fixation rates were determined for samples collected during the KT-12-20 cruise, although the highest value was obtained at Sta. ON7 during the KK-13-6 cruise. Nitrogen fixation was not detected in seawater samples collected during the winter and spring cruises, even after addition of mannitol (KS-14-2). Furthermore, nitrogen fixation was not detected in DIN-replete water at Stn. OT1 in summer (KK-13-6).

The rates of nitrogen fixation in samples collected at different depths (0–119 m) were examined at Stns. OT4 and ON5 (Fig. 4). Nitrogen fixation was detectable only during the four cruises conducted in early summer (KK-13-1), summer (KT-12-20 and KK-13-6), and fall (KT-12-27). Nitrogen fixation rates tended to be higher at the surface than the deeper layers in summer (KT-12-20 and KK-13-6 (at Stn. OT4)), whereas this vertical trend was less evident in fall (KT-12-27) and early summer (KK-13-1). At Stn. OT4, nitrogen fixation was detectable even in deeper layers below the nitracline, where nitrogen concentrations were relatively high (KT-12-27, depth = 62 m; KK-13-1, depth = 42 m). The maximum depth-integrated nitrogen fixation (294 µmolN m^{-2} d^{-1}) was observed at Stn. OT4 in summer (KT-12-20).

3.3 Relationship between nitrogen fixation, temperature, and nitrate concentration

Nitrogen fixation was detected only when seawater temperatures exceeded 11.7 °C, with higher rates (> 6 nmolN L^{-1} d^{-1}) being noted in water warmer than 19.5 °C (Fig. 5a). However, temperature was a weak predictor of the nitrogen fixation rate in the dataset obtained from each cruise. For example, in the data collected during the KK-13-1 cruise, the nitrogen fixation rates were high at 15.4 °C, but low (undetectable)

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at higher temperatures. A plot of the nitrogen fixation against nitrate concentrations indicated that nitrogen fixation was generally only detectable when nitrate was depleted (Fig. 5b), although there were exceptions (two samples collected in the subsurface layer of OT4; see above).

3.4 Seasonal variation in the phylogenetic compositions of the *nifH* gene

The *nifH* gene was recovered from all samples that we collected during this study across different stations and seasons. Sixty-six operational taxonomic units (OTUs) were grouped from 197 *nifH* clones, based on 100 % amino acid sequence similarity. The OTUs were assigned to cyanobacteria, α-, β-, γ-, δ-proteobacteria, and Cluster III diazotrophs (Zehr et al., 2003) (Figs. S4 and S5). The detected cyanobacterial sequences belonged to *Trichodesmium*, UCYN-A, and *Leptolyngbya*. The *nifH* sequences of UCYN-B, UCYN-C, and *Richelia intracellularis* were not recovered. The *nifH* sequence of *Trichodesmium* was recovered only during the KT-12-27 cruise (Fig. 6). The UCYN-A was generally observed from early summer to fall, while *Leptolyngbya nifH* was detected in winter. On the KS-14-2 spring cruise, all recovered sequences were derived from heterotrophic bacteria, and were dominated by Cluster III diazotrophs. The Cluster III diazotroph *nifH* sequences were recovered on all cruises except the KK-13-1 cruise. Note that 69 out of 197 sequences displayed >97 % similarity, at the amino acid level, to terrestrial diazotroph sequences derived from soil, mudflats, and lakes (Figs. S4 and S5). These sequences were mainly affiliated with α- and δ-proteobacterial diazotrophs, with 39 of 49 α-proteobacterial sequences and 21 of 24 δ-proteobacterial sequences being similar to terrestrial diazotroph sequences.

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4 Discussion

4.1 Seasonal variations in nitrogen fixation rates in the temperate coastal region

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 nmolN 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 nmolN L^{-1} d^{-1}; Needoba et al., 2007) and the oligotrophic region of the western and central North Pacific (0.17–3.62 nmolN L^{-1} d^{-1}; Shiozaki et al., 2010), whereas they were comparable to those determined in the Kuroshio (0.54–28 nmolN L^{-1} d^{-1}; Shiozaki et al., 2010) and the western Atlantic coastal regions (1.3–49.8 nmolN L^{-1} d^{-1}; Mulholland et al., 2012). The relatively high nitrogen fixation rates could be due to an influence of the Tsugaru Warm Current. This current, which flows from the north (after passage thorough 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 Stn. ON5. These stations were located up- and down-stream of the Tsugaru Warm Current, respectively. In addition, variations in nitrogen fixation rates among stations and seasons might also be related to the extent of vertical mixing in the Tsugaru Warm Current. It has been suggested that vertical mixing may introduce iron-rich subsurface water to the surface of the Tsugaru Strait (Saitoh et al., 2008). Such input of iron may enhance nitrogen fixation rates. Consistent with this notion, our results showed that the nitrogen fixation rate was relatively high at Stn. OT4, where the nitracline was relatively deep.

Blais et al. (2012) proposed that nitrogen fixation can proceed even in nutrient-replete waters, if large amounts of iron and organic materials are available for consumption by bacterial diazotrophs. In this study, we examined this possibility by conducting experiments where mannitol was added to surface seawaters collected in spring when

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the iron supply was considered to be high due to intrusion of the Oyashio Current (Nishioka et al., 2007, 2011; Shiozaki et al., 2014b). However, nitrogen fixation was not detected, even in water samples to which mannitol was added. Thus, our results do not support the proposition of Blais et al. (2012).

Our data showed that nitrogen fixation rates were below the detection limit during winter, spring, and summer (KK-13-6), when nitrate concentrations were high. These results are 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 ($> 1\,\mu M$) and cold ($< 10\,^{\circ}C$) surface seawaters. Their study was conducted downstream of the Gulf Stream, where diazotrophs 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 high-DIN water at the surface. DIN-replete water in summer was observed at the inside bay station OT1, with a low salinity, attributable to the washout of terrestrial water after the passage of a typhoon. Subramaniam et al. (2008) reported that nitrogen fixation rates near the Amazon River estuary, with low salinity and high nitrate levels, were fairly low, consistent with our results.

4.2 Seasonal variation in diazotrophic community structure in the temperate coastal region

The *nifH* sequences were recovered in all seasons, even when nitrogen fixation was not detected. Many recovered *nifH* sequences were similar to terrestrially derived sequences. These results agree with previous data collected in coastal regions, where

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terrestrially derived *nifH* sequences were also found (Rees et al., 2009; Mulholland et al., 2012; Blais et al., 2012). In addition to the terrestrially derived *nifH* sequences, the cyanobacterium UCYN-A was regularly observed from early summer to fall, suggesting that this group of diazotrophs could be important agents of nitrogen fixation during these periods (although we do not have direct evidence of nitrogen fixation by UCYN-A). UCYN-A has been widely detected in oligotrophic 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). The absence of UCYN-A in spring and winter was likely to be due to the low temperature (the SST in winter and spring ranged from 2.0 to 9.4 °C), because UCYN-A is known to be most abundant in relatively warm waters around ~ 20 °C (Needoba et al., 2007; Church et al., 2009; Moisander et al., 2010).

During the KT-12-27 (fall) and KK-13-6 (summer) cruises, we detected the γ-proteobacterial diazotroph closely related to phylotype γ-24774A11 (Fig. S4). This phylotype has not been reported previously in other temperate regions (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012), although this *nifH* gene is known to occur widely in the oligotrophic subtropical and tropical oceans, where it is considered to be one of the key agents of nitrogen fixation globally (Moisander et al., 2014). It remains to be seen, in future studies, if this phylotype contributes to the nitrogen fixation that we saw in summer and fall.

In nitrate-rich water in winter and spring, Cluster III diazotrophs were dominant, excluding terrestrially derived sequences. Furthermore, from early summer to fall, Cluster III diazotrophs were observed in samples from all cruises (except KK-13-1) and were the major diazotrophs in samples collected during the KT-12-27 cruise. It is rare for Cluster III diazotrophs to be dominant in the surface diazotrophic community. Because anaerobic diazotrophs are Cluster III diazotrophs (Hamersley et al., 2011; Farnelid et al., 2013; Bentzon-Tilia et al., 2014), they are usually recovered from oxygen-depleted water (Hamersley et al., 2011; Farnelid et al., 2013) and 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)

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(Fig. S6), and hence, the Cluster III diazotrophs at the surface might be derived from coastal marine sediments. Interestingly, the ammonium concentration near the bottom at stations in the bay of the ON transect line increased during all cruises (Fig. S7), suggesting that active decomposition by microbes was in play. Because UCYN-A, *Trichodesmium*, and γ-24774A11 were not present in spring and winter, Cluster III diazotrophs were likely to be the major diazotrophs at these times. The fact that nitrogen fixation during winter and spring was undetectable was probably due to inactivation of anaerobic bacteria in oxic surface water. The 2011 Tohoku-oki tsunami substantially altered the benthic environment of the region (Seike et al., 2013; Urabe et al., 2013). Such environmental change might be related to detection of Cluster III diazotrophs at the surface. We obtained a *Leptolyngbya*-like *nifH* gene during the KT-13-2 cruise. The organism is found on beaches or coastal land areas (Brito et al. 2012), and is but rarely detected in the open ocean. Because nitrogen fixation was not observed during the KT-13-2 cruise, the bacteria must have been inactivated after being flushed out from the coastal region.

5 Conclusion and remarks on the impact of the tsunami

This study demonstrated that nitrogen fixation did indeed occur in the temperate region of the northwestern North Pacific, and that the rate could be high depending on the season. However, and unlike results obtained previously in the temperate region of the western Atlantic (Mulholland et al., 2012), nitrogen fixation was unmeasurable in DIN-replete cold waters. We found that UCYN-A and the γ-proteobacterial phylotype γ-24774A11 were generally recovered during the period when nitrogen fixation rates were high, raising the possibility that these phylotypes play key roles in the diazotrophy of our study region. This study was conducted in coastal regions heavily damaged by the 2011 Tohoku earthquake-induced tsunami. The tsunami substantially changed the coastal geography and benthic environment, and impacted on benthic ecosystems (Seike et al., 2013; Urabe et al., 2013). Given that nitrogen fixation in a temperate estu-

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ary could be an important source of new nitrogen for adjacent coastal waters, and that such fixation is influenced by the condition of the benthic environment (Bentson-Tilia et al., 2014b), the tsunami-induced alteration in and the following recovery of benthic ecosystems could affect nitrogen fixation and the distribution of the diazotroph community in the coastal waters. In future studies, continuous monitoring of nitrogen fixation and recovery of the benthic environment would produce a unique dataset that could be used to explore possible links between benthic environmental conditions and pelagic diazotrophy in temperate coastal regions.

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Author contributions. T. Shiozaki, T. Nagata, and K. Furuya designed the experiment and T. Shiozaki collected samples at sea. T. Shiozaki determined nitrogen fixation and nutrient concentration and analyzed satellite datasets. T. Shiozaki and M. Ijichi conducted the genetic analyses. T. Shiozaki prepared the manuscript with contributions from all co-authors.

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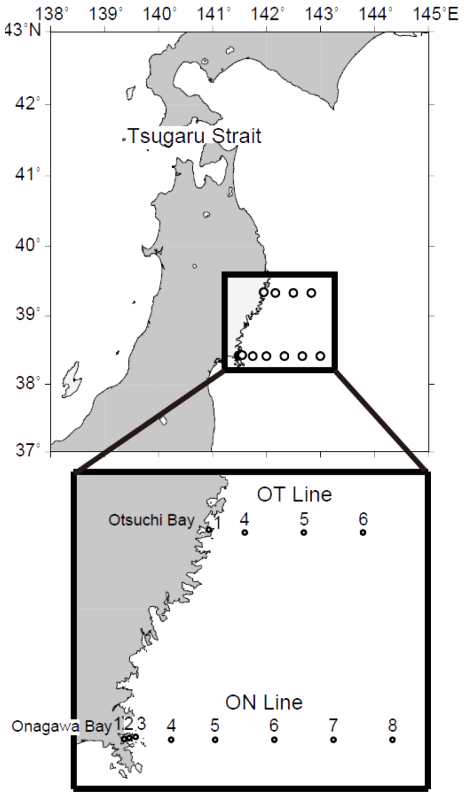

Figure 1. Sampling locations in the northwestern North Pacific Ocean.

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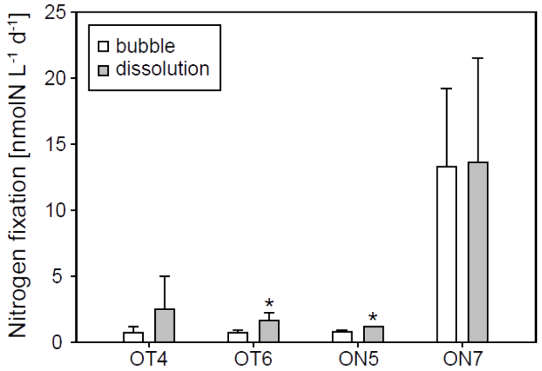

Figure 2. Nitrogen fixation rate estimated simultaneously by the $^{15}N_2$ gas bubble and dissolution methods during the KK-13-6 cruise. An asterisk indicates a significant difference between the two methods ($p < 0.05$).

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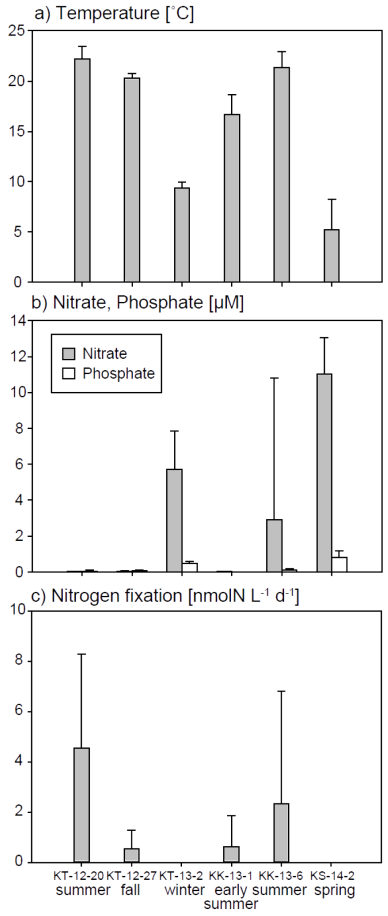

Figure 3. Average **(a)** temperature [°C], **(b)** nitrate and phosphate levels [µM], and **(c)** nitrogen fixation [nmolN L^{-1} d^{-1}] at the surface for each cruise.

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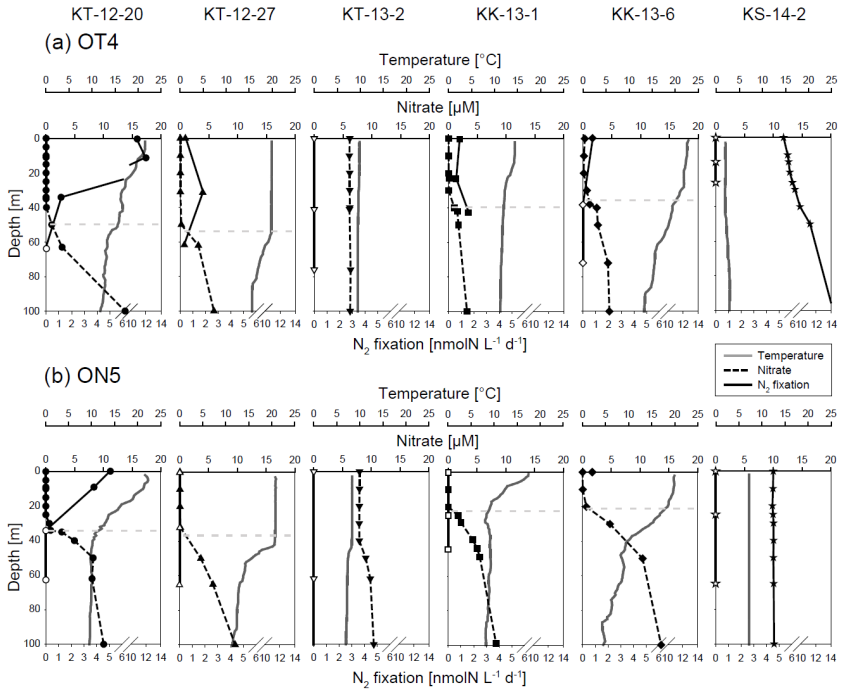

Figure 4. Time-series variations in the vertical profiles of temperature [°C], nitrate concentration [μM], and nitrogen fixation [nmolN L^{-1} d^{-1}] at Stns **(a)** OT4 and **(b)** ON5. Open symbols indicate that nitrogen fixation was not detected. The horizontal dashed line indicates the nitracline depth.

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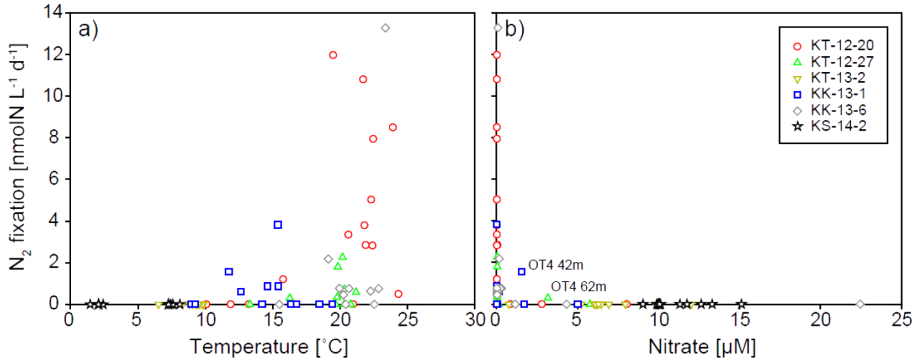

Figure 5. Relationship between nitrogen fixation [nmolN L^{-1} d^{-1}] and **(a)** temperature [°C] and **(b)** nitrate concentration [μM] for all six cruises.

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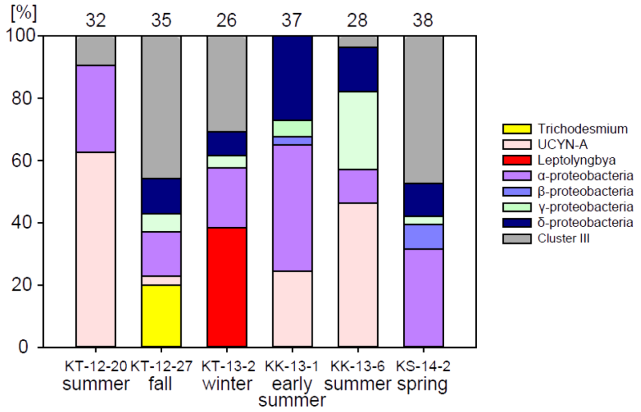

Figure 6. Relative abundance of each *nifH* group for each cruise. The number on each bar corresponds to the number of recovered *nifH* sequences.

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