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Significance of N_2 fixation in dissolved fractions of organic nitrogen

U. Konno[1], U. Tsunogai[1], D. D. Komatsu[1], S. Daita[1], F. Nakagawa[1], A. Tsuda[2], T. Matsui[3], Y.-J. Eum[3], and K. Suzuki[3]

[1]Earth and Planetary System Science, Faculty of Science, Hokkaido University, N10 W8, Sapporo 060-0810, Japan
[2]Ocean Research Institute, University of Tokyo, Tokyo 164 8639, Japan
[3]Faculty of Environmental Earth Science, Hokkaido University, N10 W5, Sapporo 060-0810, Japan

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Correspondence to: U. Konno (utakonno@mail.sci.hokudai.ac.jp)

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Abstract

Using the $^{15}N_2$ tracer method and high-sensitivity $\delta^{15}N$ analytical systems, we determined N_2 fixation rates by dividing fractions into particulate organic nitrogen (PON: >0.7 µm) and dissolved organic nitrogen (DON: <0.7 µm). While N_2 fixation in the DON fraction had been ignored in previous studies, we found significant N_2 fixation signal in the DON fraction in our study. The areal N_2 fixation rates estimated from the PON fractions varied from <1–160 µmol N m^{-2} d^{-1}, and those estimated from the DON fractions ranged from <0.5–54 µmol N m^{-2} d^{-1}. Thus, N_2 fixation in the DON fractions accounted for 50% (ranging from <10% to 84%) of the total N_2 fixation rates on an average. The new total N_2 fixation flux, which includes fixation in DON fractions, has possibility to double the original estimates; therefore, the revised influx may reduce the imbalance in the global oceanic fixed nitrogen budget.

1 Introduction

Over the last three decades, the global budget of oceanic fixed nitrogen (NO_3^-, NO_2^-, NH_4^+, particulate organic nitrogen (PON), and dissolved organic nitrogen (DON)) has been studied extensively to investigate the primary production in the ocean (e.g., Wada et al., 1975; Codispoti and Christensen, 1985; Gruber and Sarmiento, 1997; Brandes and Devol, 2002). The total fixed nitrogen is predominantly controlled by the total influx of fixed nitrogen through N_2 fixation and by the total outflux of fixed nitrogen through denitrification (Codispoti et al., 2001; Brandes and Devol, 2002; Deutsch et al., 2004). However, the estimated values of both fluxes obtained in different studies are highly variable (e.g., Wada et al., 1975; Liu, 1979; Codispoti and Christensen, 1985; Gruber and Sarmiento, 1997; Codispoti et al., 2001; Brandes and Devol, 2002; Capone and Knapp 2007; Naqvi et al., 2008). The fluxes due to both N_2 fixation and denitrification estimated in earlier studies (30 and 10 Tg yr^{-1}, respectively) (Liu, 1979) indicate that the values almost balance with the total fixed nitrogen budget. However, subsequent

improved estimates have revealed the presence of large fluxes of 95±20 Tg N yr^{-1} due to sediment denitrification (Gruber and Sarmiento, 1997) and 110±40 Tg N yr^{-1} due to oceanic N_2 fixation (Codispoti et al., 2001). Furthermore, recent studies on denitrification in sediments and water columns have revealed a considerably large outflux of 275–450 Tg N yr^{-1} (Codispoti et al., 2001; Brandes and Devol, 2002) that substantially exceeds the influx. Therefore, a large influx by N_2 fixation can be expected to balance the oceanic fixed nitrogen budget (e.g., Altabet, 2007; Brandes et al., 2007; Codispoti et al., 2007).

The two most commonly used incubation methods to estimate the N_2 fixation rate are the $^{15}N_2$ tracer method and the C_2H_2 reduction method. Because the former method is used to measure the ^{15}N uptake rate from $^{15}N_2$ to the PON fraction (Montoya et al., 1996), the estimated N_2 fixation rate may be underestimated if a considerable amount of N is released into the DON fraction during cultivation (Bronk and Glibert, 1991; Glibert and Bronk, 1994). Glibert and Bronk (1994) performed culture experiments using *Trichodesmium* spp. and found that the rates of release of fixed N release into the DON fractions accounted for 50% of the total N_2 fixation rates on an average. Therefore, in addition to PON, one should determine the initial and final values of the concentration and nitrogen isotopic composition ($\delta^{15}N=(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{AirN_2}-1$) of DON in each incubation bottle in order to estimate the total N_2 fixation rates using the $^{15}N_2$ tracer method. However, it is difficult to determine the $\delta^{15}N$ value of DON in natural samples using the conventional elemental analyzer isotope ratio mass spectrometry (EA-IRMS) techniques (Mulholland et al., 2004; Meador et al., 2007).

On the other hand, the C_2H_2 reduction method is used to measure the C_2H_4 production rate through the reduction of C_2H_2 by nitrogenase (Capone, 1993). However, this method requires the use of a conversion factor to convert the observed C_2H_2 reduction rates to N_2 fixation rates. While the theoretical reduction ratio of C_2H_2:N_2 is 3:1 (mol:mol) (Montoya et al., 1996; Postgate, 1998), little evidence has been found to support the accuracy of this ratio under natural conditions

(Mulholland et al., 2006; Tsunogai et al., 2008). Nitrogenase-dependent H_2 evolution, which is inhibited by C_2H_2, results in deviations from this theoretical stoichiometry (Robson and Postgate, 1980; Mulholland et al., 2006, 2007). Therefore, the conversion factor is generally determined using the same field measurements by comparing the N_2 fixation rate calculated using both the C_2H_2 reduction method and $^{15}N_2$-labeled PON method. Although Capone and Montoya (2001) recommended a conversion factor of 4, Mulholland et al. (2006) showed that the factor was variable with values ranging from 3.7 to 15.7 even in experiments conducted on a daily basis. Furthermore, it is impossible to estimate total N_2 fixation rates using the conversion factors estimated by the ^{15}N-labeled PON method. Therefore, it is impossible to estimate the quantitative values of the total N_2 fixation rates by the C_2H_2 reduction method.

The only way to solve the above mentioned problem is to determine the $\delta^{15}N$ values of DON and PON during $^{15}N_2$ tracer incubation. Recent developments in high-sensitivity $\delta^{15}N$ analysis of organic nitrogen have now enabled us to determine the $\delta^{15}N$ values of DON (Tsunogai et al., 2008). This is the first report on the estimation of the total N_2 fixation rates in the ocean, including the DON fractions.

2 Sampling and methods

Both the collection and incubation of water samples were performed onboard the R/V Hakuho Maru cruise in the western North Pacific region during the KH06-2 (SNIFFS 2006) expedition in June 2006, KH07-2 expedition in September 2007, and KH08-2 (SPEEDS/SOLAS 2008) expedition from August–September 2008. The date and locations at which the samples were collected are listed in Table 1. Water samples were collected from depths between 5 and 150 m using a CTD-Carousel multi-sampling system (911plus; Sea-Bird Electronics Inc.). Seawater samples were sub-sampled into 250-mL Pyrex bottles (KH06-2, KH07-2, and KH08-2) or 500-mL polycarbonate bottles (KH08-2) with septum caps without headspace. Then, 1.0 mL of $^{15}N_2$ (99 at%; Shoko

Co. Ltd., Tokyo, Japan) was injected into each bottle using a gas-tight syringe. The bottles were gently shaken and then incubated in thermostatic baths on a deck covered with screens to simulate the in situ temperature and light intensity for periods ranging from 12 to 72 h. Although the duration of incubation was variable, the incubation was mainly performed during diurnal periods (24, 48, or 72 h) to avoid the bias caused by the day-night cycle on the N_2 fixation rate. However, two samples were incubated for three different periods of 12, 36, and 60 h during the KH06-2 expedition and may have been biased by the day-night cycle. Therefore, we corrected the bias by adopting the relative variations between 36 and 12 h (corresponding to 24 h incubation), or those between 60 and 12 h (corresponding to 48 h incubation).

Immediately after incubation, the suspended particles (PON fraction) in each incubated water sample were collected on a pre-combusted (450 °C for 4 h) Whatman GF/F filter (pore size=0.7 µm) by gentle vacuum filtration. The pressure difference was strictly controlled to be <100 mm Hg to avoid the leakage of small particles from the filters. The <0.7-µm filtrate (herein considered to be the DON fraction) was collected in a light-resistant polyethylene bottle (100 mL) and frozen until analysis. The suspended particles collected on the filter were further washed with filtered clean seawater, placed in a plastic case, frozen instantaneously, and stored in a deep freezer (−80 °C) until analysis. For quantitative polymerase chain reaction (qPCR) assays targeting partial *nifH* fragments, seawater (1 L) was filtered onto 25-mm Supor filters (pore size: 0.2 µm, Pall Corporation) under gentle vacuum (<100 mm Hg). The obtained filters were frozen in a deep freezer (−80 °C) until analysis.

The concentrations and $\delta^{15}N$ values of both PON and DON, including those incubated under $^{15}N_2$ addition, were analyzed using the method developed by Tsunogai et al. (2008). This method involves the oxidation/reduction methods such as the oxidation of PON or DON to nitrate using persulfate (Knapp et al., 2005; Tsunogai et al., 2008), reduction of nitrate to nitrite using spongy cadmium, and further reduction of nitrite to nitrous oxide using sodium azide. The total recovery rate of N was around 90% for the samples. The blank level was <10 nmol N for PON (corresponding to

0.02 µmol N L^{-1} when the filtrate volume was 500 mL) and <1.0 µmol N L^{-1} for DON. All the data presented herein had already been corrected for the blank contributions. The standard deviation of the sample measurements was less than 0.3‰ for samples containing more than 50 nmol N, and less than 0.5‰ for those containing more than 20 nmol N. For the DON samples, we also needed to correct by subtracting the contributions of nitrate, nitrite, and ammonium. However, we neglected these contributions because their concentrations, which were quantified by using an AutoAnalyzer (AACS II; Bran+Luebbe), were low (mostly below detection levels) at the studied sites (Table 1). The concentrations of PON and DON ranged from 0.11 to 0.60 µmol N L^{-1} and from 4.0 to 7.0 µmol N L^{-1}, respectively (Table 2). Details will be discussed elsewhere.

The total N_2 fixation rate was calculated for each incubation bottle using the results for both the concentration and $\delta^{15}N$ values of PON/DON. ^{15}N enrichment was clearly observed over time in most of the samples of PON and DON incubated under $^{15}N_2$ addition (Fig. 1); this result indicates that part of the recently fixed-nitrogen was transferred into DON pools during the incubation experiment. The vertical distributions of the N_2 fixation rates estimated from PON fractions during the KH06-2 expedition are shown in Fig. 2. The profiles indicate that the N_2 fixation rates at the water surface were high and that these rates linearly decreased to nearly zero at depths of ca. 100 m. Therefore, we calculated the areal N_2 fixation rates by integrating the N_2 fixation rates on a volume from surface to 100 m depths, assuming linear attenuation toward zero with depths up to 100 m including the stations where the estimation was limited to the surface. If the increased $\delta^{15}N$ values estimated from the incubation experiments were less than 2‰, we classified the N_2 fixation rates as less than the detection limit and presented the maximum value.

DNA extraction was performed according to the method of Short and Zehr (2005) with slight modifications. In brief, 600 µL of XS buffer (1% potassium ethyl xanthogenate; 100 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0; 1% sodium dodecylsulfate; 800 mM ammonium acetate) and ca. 0.2 g of 0.1-mm glass beads were added to the vials containing the filters. The vials were placed in a bead beater (BioSpec Products)

and agitated three times at 4800 rpm for 50 s. The samples were incubated at 70 °C for 60 min. After incubation, the XS buffer was transferred to a 1.5-mL microtube, vortexed for 10 s, and placed on ice for 30 min. Cell debris was removed by centrifugation at 15 000 g at 5 °C for 15 min; the supernatants were then decanted into another 1.5-mL microtube with the same amount of isopropanol. The samples were incubated at room temperature for 10 min, and the precipitated DNA was pelleted by centrifugation at 15 000 g for 15 min at 5 °C. Isopropanol was decanted, and the DNA pellets were washed with 70% ethanol, vacuum dried, and resuspended in 100 µL of 10 mM Tris-HCl (pH 8.5). The obtained samples were stored at −20 °C until further analysis.

Quantitative PCR (qPCR) assays targeting partial *nifH* fragments were carried out with a Thermal Cycler Dice Real Time System (TP800; TaKaRa) using primers and TaqMan probes designed by Church et al. (2005); they determined five *nifH* phylotypes including the cyanobacteria *Crocosphaera* spp. (termed Group B), an uncultivated phylotype termed Group A that was presumed to be a unicellular cyanobacterium, *Trichodesmium* spp., heterocystous cyanobacteria, and g-proteobacteria in the North Pacific Ocean. For each set of primers and probes set, standard curves were derived using duplicate or triplicate serial dilutions of linearized pUC18 plasmids (TaKaRa) containing the positive control insert. The number of molecules of a plasmid was estimated from the amount of DNA according to the equation derived by Short and Zehr (2005). The PCR amplification mixture solution (25 µL) contained 12.5 µL of Premix Ex Taq (Perfect Real Time, TaKaRa), 0.05 µL of each primer (final conc.: 0.2 µM), 0.1 µL of the probe (final conc.: 0.4 µM), 11.3 µL of sterile Milli-Q water, and 1 µL of DNA template. In each qPCR run, environmental DNA and no template controls (i.e. sterile Milli-Q water) were also prepared in duplicate or triplicate. The thermal cycling reactions were carried out as follows: 95 °C for 10 s, and 50 cycles of 95 °C for 5 s followed by 60 °C for 30 s.

3 Results and discussion

3.1 N_2 fixation rates in the PON fractions

The areal N_2 fixation rates estimated from the PON fractions varied from <1–160 μmol N m^{-2} d^{-1} during the three expeditions undertaken in the western North Pacific (Table 2). Using the traditional $^{15}N_2$ tracer method for the PON fractions, Shiozaki et al. (2009) estimated the areal N_2 fixation rates in the western North Pacific region to be 29–152 μmol N m^{-2} d^{-1} in early spring. The areal N_2 fixation rates estimated from the PON fractions in the present study during the early summer expedition (KH06-2 cruise; 22–160 μmol N m^{-2} d^{-1}) agreed well with those reported by Shiozaki et al. (2009). On the other hand, the areal N_2 fixation rates estimated during the late summer expeditions (KH07-2 and KH08-2 cruises; <20 μmol N m^{-2} d^{-1}) were lower than those estimated during the early summer expeditions. The concentration of the sea surface chlorophyll-*a* was lower on the late summer expeditions (0.01 to 0.05 μg L^{-1}) than during the early summer expeditions (0.07 to 0.21 μg L^{-1}), indicating that the late summer expeditions coincided with the post-blooming season, when nutrients are limited. Thus, the observed difference in the areal N_2 fixation rates could be attributed to the seasonal variations in the N_2 fixation rates (Sañudo-Wilhelmy et al., 2001; Moutin et al., 2005). The lowest observed N_2 fixation rates at the two stations during the late summer expeditions (Stns. 19 and 20, KH08-2) (Table 2) could also be attributed to the lack of nutrients in post-blooming season, because the observed column-integrated quantities (from the surface to the depth of 100 m) of PO_4^{3-} (<2 μmol PO_4^{3-} m^{-2} at Stn. 19 and <1 μmol PO_4^{3-} m^{-2} at Stn. 20) were the smallest for those stations during the late summer expeditions (Table 1).

However, the column-integrated quantities of PO_4^{3-} were also small at Stns. 5, 6, and 7 (KH06-2) during the early summer expedition, where active N_2 fixation (>22 μmol N m^{-2} d^{-1}) was observed in the PON fractions. Moutin et al. (2005) also found few direct links between the P availability and accumulation of *Trichodesmium*

spp. during early summer. Therefore, explanations other than the limited availability of nutrients may be required to explain active N_2 fixation during the early summer. Owing to the ability to store P, *Trichodesmium* spp. is active for a few months after PO_4^{3-} deficiency (Thompson et al., 1994; Moutin et al., 2005). Therefore, during early summer, the N_2 fixers were using the stored P supplied during winter or spring, while they may exhaust the stored P during late summer when we found the direct links between P-availability and the N_2 fixation rate. In conclusion, the estimated values of N_2 fixation rates for the PON fractions were representative of those observed in the western North Pacific area during each season.

The $\delta^{15}N$ values of initial PON also supported our conclusions (Table 2). The lower $\delta^{15}N$ values of PON (+0‰ and +0.9‰; Stns. 1 and 5 during the KH06-2 expedition, +0.8‰ and +1.5‰; Stns. 16 and 22 during KH08-2 expedition) were found at the station where the higher N_2 fixation rate in the PON fractions was observed. Because the PON derived from N_2 fixation had nearly 0‰ of $\delta^{15}N$ derived from atmospheric N_2 (Minagawa and Wada, 1986; Carpenter et al., 1997; Montoya et al., 2002), the geographical variations in $\delta^{15}N$ of PON also support the significant N_2 fixation in the PON fractions.

3.2 N_2 fixation rates in the DON fractions

The areal N_2 fixation rates estimated in this study for the DON fractions (ranging from <0.5 to 54 $\mu mol\,N\,m^{-2}\,d^{-1}$) accounted for 50% (ranging from <10 to 84%) of the total N_2 fixation rates on average (Table 2). Glibert and Bronk (1994) also estimated that the rates of N release into the DON fractions could account for 50% on average of the total N_2 fixation rates in cultured *Trichodesmium* spp. Furthermore, Mulholland et al. (2006) estimated the rates of release of fixed nitrogen into DON fractions in the ocean on the basis of the discrepancies in the N_2 fixation rates estimated by the $^{15}N_2$ tracer method and the C_2H_2 reduction method for the same samples, and they found the DON fraction to comprise 52% (ranging from 9.1% to 81%) of the total N_2 fixation

by using the theoretical conversion factor of 3 for the C_2H_2 reduction method. Both the average N_2 fixation rate for the DON fractions within the total N_2 fixation rate and the range of the variation estimated in this study corresponded well with the estimated rate and variation in past studies. The estimated N_2 fixation rates in the DON fractions estimated in this study may be highly reliable for estimating those in the ocean.

The discrepancies in the estimates for the N_2 fixation rates between the $^{15}N_2$ tracer method and C_2H_2 reduction method have been noted in the western North Pacific region as well. Using the C_2H_2 reduction method, Kitajima et al. (2009) found high N_2 fixation rates of 0.5–12 nmol N L^{-1} d^{-1} in the western North Pacific region; this range is more than twice as high as that estimated for the PON fractions in this study (0.4–4.7 nmol N L^{-1} d^{-1} during the KH06-2 expedition) based on the $^{15}N_2$ tracer method. It is difficult to attribute the differences in the estimations to the seasonal variations since both experiments were performed during the same early summer season (May to June). Because the C_2H_2 reduction method resulted in higher N_2 fixation rates in comparison with the $^{15}N_2$ tracer method for the PON fractions when the N_2 fixation in the DON fractions was significant, the systemic difference between the estimates obtained by the two different methods for the same region implies that the N_2 fixation rates for the DON fractions were almost as significant as those for the PON fractions.

The $\delta^{15}N$ values of initial DON also support our conclusions. Within the whole $\delta^{15}N$ values of initial DON in surface water (ranging from +5.5 to +8.8‰, Table 2), which agree with those reported in a previous study in the Central Pacific region (Meador et al., 2007), the lowest $\delta^{15}N$ value of DON (+5.5‰; Stn. 16 during the KH08-2 expedition) was found at the station where the highest N_2 fixation rate in the DON fractions was observed. Because the fixation of atmospheric N_2 ($\delta^{15}N$=0‰) produces organic nitrogen with $\delta^{15}N$ values similar to atmospheric N_2 (Bourbonnais et al., 2009), the geographical variations in $\delta^{15}N$ of DON also support the significant N_2 fixation in the DON fractions. In conclusion, the estimated significant N_2 fixation rates in the DON fractions represented those in the western North Pacific region.

3.3 Mechanisms of N_2 fixation in DON fractions

The significant N_2 fixation observed in the DON fractions can be explained by the following two mechanisms: (1) active N_2 fixation in the DON fraction by small plankton such as bacterioplankton and/or picoplankton and (2) active secondary release of N into the DON fractions from the PON fractions, which includes recently fixed nitrogen such as viral cell lysis (Hewson et al., 2004), grazing (O'Neil et al., 1996), cell death (Berman-Frank et al., 2004), or direct release of N-compounds (Glibert and Bronk, 1994).

In particular, we focused on the mechanisms of the large N_2 fixation rate in the DON fractions at high latitudes (Fig. 3). In several previous studies, it has been observed that γ-proteobacteria in waters are characterized by being both cooler in temperature and richer in nutrients than waters where the usual cyanobacterial N_2 fixers are dominant (Bird et al., 2005; Langlois et al., 2005; Church et al., 2008). In particular, Church et al. (2008) found that the *nifH* gene was actively expressed in γ-proteobacterial phylotypes at stations far north up to 44° N in the north Eastern Pacific region. Because bacterioplankton and/or picoplankton having sizes ranging from ~0.2 to ~2 µm could pass through the GF/F filter (pore size: 0.7 µm) and mix with the DON fractions, they could cause active N_2 fixation in the DON fractions.

The abundance of *nifH* gene copies determined by the quantified PCR method, however, indicated that the dominant N_2 fixer at stations at high latitudes and showing active N_2 fixation in DON fractions was *Trichodesmium* spp. (Fig. 4). Although we could not directly compare the number of *nifH* gene copies with the N_2 fixation rates (Zehr et al., 2007), the large N_2 fixation rates observed in the DON fractions at high latitudes could be attributed to active secondary release of N into the DON fractions from recently fixed nitrogen by *Trichodesmium* spp. Glibert and Bronk (1994) also found that the rates of release of N from cultured *Trichodesmium* spp. into the DON fractions could account for 50% of the total N_2 fixation rates on an average. The release rate of N into the DON

fractions corresponded well with our results for the natural samples. However, further studies are essential to confirm the precise mechanisms of active N_2 fixation in DON fractions.

3.4 Implication for total N_2 fixation flux in ocean

In previous studies, the oceanic N_2 fixation rates were estimated to be in the range from 80–140 Tg N yr^{-1} on the basis of the data estimated by the $^{15}N_2$ tracer method (Brandes et al., 2007); however, these estimates only accounted for the N_2 fixation rates in the PON fractions. Using the present data, we could estimate the total N_2 fixation rates more accurately by correcting the past estimates. Using a roughly average N_2 fixation of 50% in the DON fractions that were underestimated in previous studies over oceans worldwide, the revised N_2 fixation inputs should be increased to 160–280 Tg N yr^{-1}. Codispoti et al. (2001) estimated the total influx and outflux of fixed nitrogen to be 287 and 482 Tg N yr^{-1}, respectively, for the current global fixed nitrogen budget in oceans; in this budget, the outflux exceeds the influx by ~200 Tg N yr^{-1}. The revised influx reduces the imbalance in the global fixed nitrogen budget. However, as observed during this study, the N_2 fixation rates in the DON fractions could be highly variable on different temporal and spatial scales. Further studies should be conducted to estimate the N_2 fixation rate in the DON fractions more accurately.

4 Conclusions

We found significant N_2 fixation signal in the < 0.7 µm fraction, typically characterized as dissolved organic nitrogen (DON) in the western North Pacific region; N_2 fixation in these fractions had been ignored in previous studies. As a result, N_2 fixation in the DON fractions accounted for 50% (ranging from < 10% to 84%) of the total N_2 fixation rates on an average. The abundance of *nifH* gene copies determined by quantified PCR method indicated that the large N_2 fixation rates observed in the DON fractions at high

latitudes could be attributed to active secondary N release processes to DON fractions from recently fixed-nitrogen by *Trichodesmium*. The new total N_2 fixation flux including N_2 fixation in DON fractions has possibility to double the original estimates; therefore, the revised influx may reduce the imbalance in the global oceanic fixed nitrogen budget.

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Table 1. Locations of sampling stations as well temperature (SST), salinity, and concentrations of chlorophyl-*a* and nutrients at surface (5 m depth).

sample I.D.	date	location	SST (°C)	salinity (psu)	chl-*a* ($\mu g\,L^{-1}$)	$NO_3^- + NO_2^-$ ($\mu mol\,L^{-1}$)	NH_4^+ ($\mu mol\,L^{-1}$)	PO_4^{3-} ($\mu mol\,L^{-1}$)	column PO_4^{3-} [#] ($\mu mol\,m^{-2}$)
KH06-2 Stn. 1	3 Jun 06	30° N, 137° E	21.7	34.28	0.21	<0.06	<0.15	<0.01	3
KH06-2 Stn. 5	7 Jun 06	20° N, 137° E	29.8	34.63	0.08	<0.06	<0.15	<0.01	<1
KH06-2 Stn. 6	8 Jun 06	15° N, 137° E	30.1	34.17	0.07	0.1	0.16	<0.01	<2
KH06-2 Stn. 7	17 Jun 06	15° N, 128° E	30.1	34.41	0.08	<0.09	<0.15	<0.01	<1
KH07-2 Stn. 49	3 Sep 07	18° N, 140° E	29.9	34.24	0.01	<0.04	0.39	0.05	4
KH07-2 Stn. 51	3 Sep 07	17° N, 140° E	30.2	34.39	0.01	<0.04	0.40	0.02	5
KH07-2 Stn. 55	4 Sep 07	15° N, 140° E	30.1	34.05	0.01	<0.04	0.46	0.04	13
KH07-2 Stn. 57	4 Sep 07	14° N, 140° E	30.0	33.98	0.01	<0.04	0.48	0.01	12
KH08-2 Stn. 15	28 Aug 08	37.15° N, 155° E	25.8	34.13	0.05	<0.04	<0.1	<0.01	43
KH08-2 Stn. 16	30 Aug 08	35° N, 155° E	28.6	34.00	0.03	<0.04	<0.1	<0.01	14
KH08-2 Stn. 17	1 Sep 08	32.30° N, 155° E	26.7	33.89	0.03	<0.04	<0.1	<0.01	27
KH08-2 Stn. 18	2 Sep 08	29.45° N, 155° E	27.6	34.47	0.03	<0.04	<0.1	<0.01	5
KH08-2 Stn. 19	3 Sep 08	25° N, 155° E	28.7	34.55	0.02	<0.04	<0.1	<0.01	<2
KH08-2 Stn. 20	5 Sep 08	20° N, 155° E	29.4	34.42	0.01	<0.04	<0.1	<0.01	<1
KH08-2 Stn. 21	8 Sep 08	14.55° N, 155° E	29.8	35.00	0.01	<0.04	<0.1	0.07	7
KH08-2 Stn. 22	9 Sep 08	11.5° N, 155° E	29.3	34.59	0.01	<0.04	<0.1	0.10	9

[#] integrated $[PO_4^{3-}]$ quantities from surface to 100-m depth.

Table 2. Areal N_2 fixation rates for each fraction and initial $\delta^{15}N$ values.

sample I.D.	date	PON (μmol N L^{-1})	$\delta^{15}N^{\#}$ (PON) (‰)	DON (μmol N L^{-1})	$\delta^{15}N^{\#}$ (DON) (‰)	incubation periods (h)	N_2 fixation (PON) (μmol N m^{-2} d^{-1})	N_2 fixation (DON) (μmol N m^{-2} d^{-1})	N release rate (% of N_2 fixation)
KH06-2 Stn. 1	3 Jun 06	0.30	+0	ND	ND	24	52	ND	
KH06-2 Stn. 5	7 Jun 06	0.23	+0.9	6.6	+7.5	12, 36, 60	160	41	20
KH06-2 Stn. 6	8 Jun 06	0.13	+3.5	ND	ND	24	22	ND	
KH06-2 Stn. 7	17 Jun 06	0.11	+1.6	ND	ND	12, 36, 60	42	ND	
KH07-2 Stn. 49	3 Sep 07	0.28	+2.7	ND	ND	24	7	ND	
KH07-2 Stn. 51	3 Sep 07	0.22	+2.4	ND	ND	24	13	ND	
KH07-2 Stn. 55	4 Sep 07	0.17	+2.9	ND	ND	24	20	ND	
KH07-2 Stn. 57	4 Sep 07	0.12	+3.1	ND	ND	24	8	ND	
KH08-2 Stn. 15	28 Aug 08	0.60	+8.1	4.0	+8.1	24	4	18 (15–21)	84
KH08-2 Stn. 16	30 Aug 08	0.52	+0.8	4.1	+5.5	24	17	54 (51–56)	76
KH08-2 Stn. 17	1 Sep 08	0.52	+5.1	6.0	+8.0	48	4	7 (5–9)	66
KH08-2 Stn. 18	2 Sep 08	0.35	+8.9	6.0	+6.9	48	1	4 (2–5)	82
KH08-2 Stn. 19	3 Sep 08	0.47	+9.9	7.0	+7.5	72	<0.01	<0.2	
KH08-2 Stn. 20	5 Sep 08	0.29	+5.4	6.3	+6.8	48	<0.01	<0.3	
KH08-2 Stn. 21	8 Sep 08	0.30	+9.9	6.8	+8.8	24	5	<0.5	<10
KH08-2 Stn. 22	9 Sep 08	0.26	+1.5	5.5	+7.3	24	14	2 (0–4)	14

$^{\#}\delta^{15}N=(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{Air N_2}-1$
ND, not determined

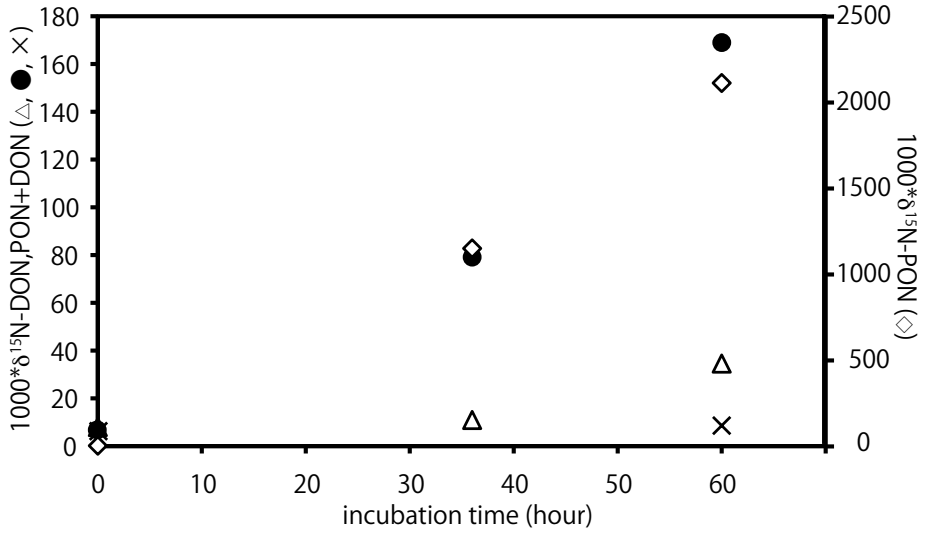

Fig. 1. Variations in δ^{15}N values for each fraction in an incubation bottle (5-m depth/KH06-2 Stn. 5) plotted as a function of the incubation period. The diamonds, triangles, circles, and crosses indicate the δ^{15}N values of PON with $^{15}N_2$, DON with $^{15}N_2$, PON+DON with $^{15}N_2$, and DON without $^{15}N_2$, respectively.

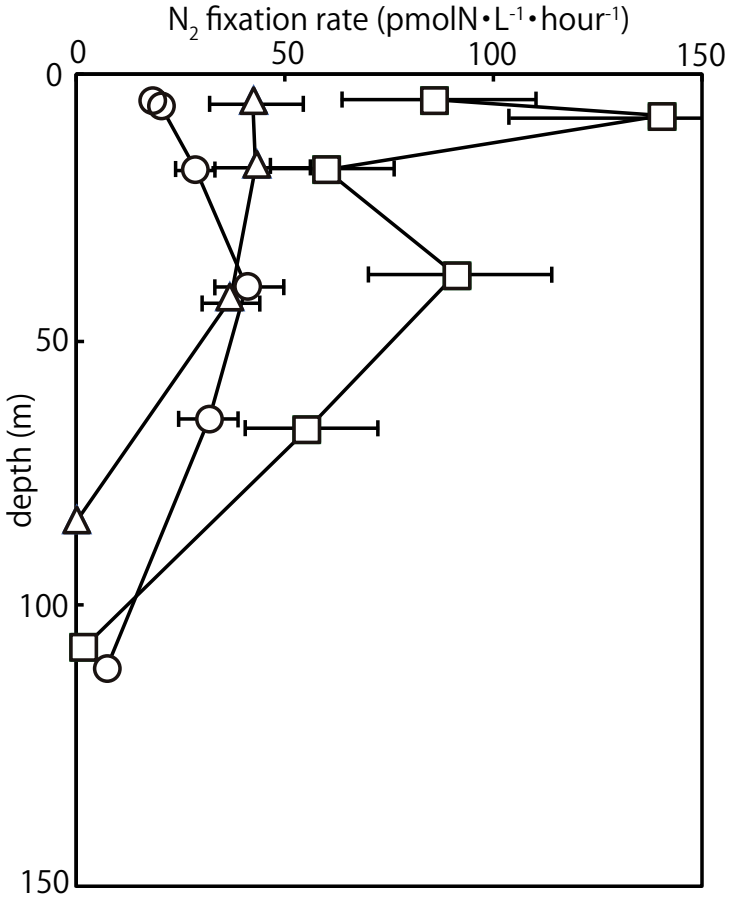

Fig. 2. Depth profiles of N_2 fixation rates in the PON fractions during the KH06-2 expedition at Stns. 1, 6, and 7 (denoted by triangles, circles, and squares, respectively). The error bars represent the standard deviations for the triplicate water samples.

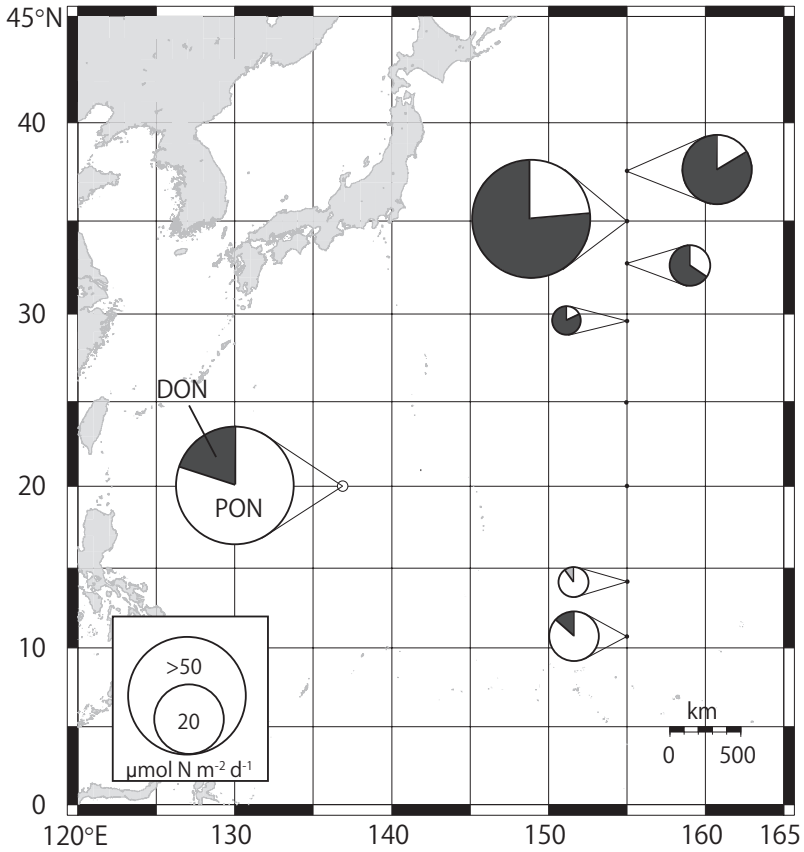

Fig. 3. Pie charts showing the distribution of N_2 fixation rates estimated for PON (>0.7 μm) and DON (<0.7 μm) fractions. The white and gray regions denote N_2 fixation in the PON and DON fractions, respectively. For the stations where the N_2 fixation rates in the DON fractions were below the detection limit, the maximum values are shown in light gray.

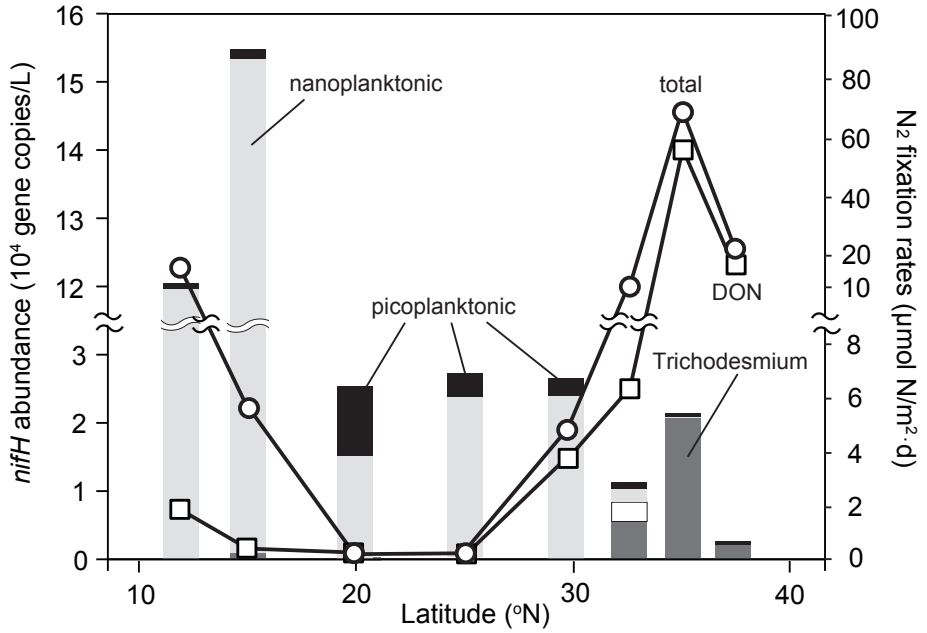

Fig. 4. Abundance of *nifH* gene copies (bar charts: left axis) and the N_2 fixation rates (line charts: right axis) during the KH08-2 expedition. The dark gray, white, light gray, and black bars denote the results for *Trichodesmium* spp., heterocystous diazotroghs, nanoplanktonic diazotrophs, and picoplanktonic diazotrophs, respectively. The circles and squares on the line charts represent the total N_2 fixation rates (μmol N m^{-2} d^{-1}), and the rates in the DON fractions, respectively.