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Determination of total N_2 fixation rates in the ocean taking into account both the particulate and filtrate fractions

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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 for ocean samples by dividing them into particulate (>0.7 µm) and filtrate (<0.7 µm) fractions. While N_2 fixation in the filtrate fraction had been ignored in previous studies, we found a significant N_2 fixation rates in the filtrate fraction in our study. The areal N_2 fixation rates in the western North Pacific Ocean estimated from the particulate fraction varied from <1 to 160 µmol N m^{-2} d^{-1}, and those rates estimated from the filtrate fraction ranged from <0.5 to 54 µmol N m^{-2} d^{-1}. Thus, N_2 fixation in the filtrate fraction accounts for on average 50% (ranging from <10% to 84%) of the total N_2 fixation rates. If these results are confirmed generally in the ocean, the new total N_2 fixation flux, which includes fixation in the filtrate fraction, possibly doubles 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 extensively studied as one of the representative nutrients that regulate 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 efflux 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). Earlier estimates have revealed the efflux of 95 ± 20 Tg N yr^{-1} through sedimentary denitrification (Gruber and Sarmiento, 1997) and the influx of 110 ± 40 Tg N yr^{-1} through oceanic N_2 fixation (Codispoti et al., 2001). These values almost balance the total fixed nitrogen budget. However, recent studies on denitrification in sediments and water columns have revealed a considerably large efflux of 275–450 Tg N yr^{-1} (Codispoti et al., 2001; Brandes and Devol, 2002) that substantially exceeds the influx. Therefore, a larger 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 acetylene (C_2H_2) reduction method. The former method measures the ^{15}N uptake rate of diazotrophs by determining the temporal variation in the nitrogen isotope compositions ($\delta^{15}N = (^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{AirN2} - 1$) of diazotrophs incubated under $^{15}N_2$ (Montoya et al., 1996). The GF/F filter (pore size = 0.7 µm) has been traditionally used to gather diazotrophs as particulate organic matter for the $^{15}N_2$ tracer method, because glass fiber filters are characterized by lower N blanks than other filters (in this paper, unless otherwise

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noted, we have used the GF/F filter and defined the border between particulate and filtrate fractions to be 0.7 µm) (e.g. Montoya et al., 1996; Zehr et al., 2001; Mulholland et al., 2006; Shiozaki et al., 2009).

Glibert and Bronk (1994) found that roughly half of recently fixed N could be released into filtrate fraction (<0.2 µm) during the incubation of *Trichodesmium thiebautii* and *T. erythreum* collected in the Caribbean Sea. Besides, several recent studies have indicated the possibility of diazotrophy in picoplanktonic organisms (Bird et al., 2005; Langlois et al., 2005; Church et al., 2008) that can pass through the GF/F filter. As a result, the estimated N_2 fixation rate determined only for the particulate fraction on the GF/F filter (e.g. Montoya et al., 1996; Zehr et al., 2001; Mulholland et al., 2006; Shiozaki et al., 2009) could be underestimated if a considerable amount of N is released into the filtrate fraction during incubation experiments (Bronk and Glibert, 1991; Glibert and Bronk, 1994) or active N_2 fixation in the filtrate fraction. Therefore, in addition to the particulate, one should determine the initial and final values of the concentration and δ^{15}N for the filtrate 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 δ^{15}N value of filtrate 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 reliability 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 the conventional $^{15}N_2$ tracer 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 over a span of a few days. Furthermore, it is impossible to accurately estimate N_2 fixation rates using the conversion factors estimated by the conventional $^{15}N_2$ tracer method based only on the $^{15}N_2$ uptake rate by diazotrophs collected on GF/F, as already presented. 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 δ^{15}N values not only for the particulate but also for the filtrate during $^{15}N_2$ tracer incubation. Recent developments in high-sensitivity δ^{15}N analysis of organic nitrogen have now enabled us to determine the δ^{15}N values of filtrate as well (Tsunogai et al., 2008). The primary objective of this study is to quantify the total N_2 fixation rates (N_2 fixation in both the particulate and the filtrate fractions) in the field to estimate the difference from those determined only for the particulate fraction. Our estimate would contribute to reassess the global oceanic fixed nitrogen budget. Further, in order to clarify the mechanisms of the N_2 fixation signal in the filtrate fraction, we quantify *nifH* gene abundances in the same field study to investigate what types of diazotrophs are dominant when significant N_2 fixation signals are detected in the filtrate fraction. If small sized (<GF/F filter) diazotrophs are dominant, direct N_2 fixation by these diazotrophs in the filtrate fraction could be the main reason, whereas recently fixed N release into filtrate fraction could be the main reason if large sized (>GF/F filter) diazotrophs are dominant. This is the first report on the estimation of the total N_2 fixation rates in the ocean, including the filtrate fraction.

2 Sampling and methods

2.1 Sample collection and incubation experiments

Both the collection and the incubation of water samples were carried out 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 in 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 atom %; Shoko Co. Ltd., Tokyo, Japan) was injected into each incubation 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 carried out 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 these 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).

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

sample I.D.	date	location	SST (° C)	salinity (psu)	chl-*a* (µg L^{-1})	NO_3^- + NO_2^- (µmol N L^{-1})	NH_4^+ (µmol N L^{-1})	PO_4^{3-} (µmol P L^{-1})	column PO_4^{3-}* (µmol P 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.

Immediately after incubation, the particulate in each incubated water sample was 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. In addition to the samples for incubation, seawater samples without $^{15}N_2$ addition were sub-sampled into 500-mL polycarbonate bottles and filtered within an hour after the sampling through the pre-combusted GF/F filter for natural organic nitrogen analysis in a manner identical to that for the samples for incubation.

The <0.7 µm filtrate was collected in a light-resistant polyethylene bottle (100 ml) and frozen until analysis. The particulate collected on the filter was further washed with filtered (using a pre-combusted GF/F filter) 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.

2.2 Geochemical data analysis

The concentrations and $\delta^{15}N$ values of organic nitrogen in both the particulate and the filtrate, including those incubated under $^{15}N_2$ addition, were analyzed using the method developed by Tsunogai et al. (2008). This method involves oxidation/reduction methods such as the oxidation of organic nitrogen to nitrate using persulfate (Knapp et al., 2005; Tsunogai et al., 2008, 2010), 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 more than 90% for the samples. The blank level was <10 nmol N for the particulate (corresponding to 0.02 µmol N L^{-1} when the filtrate volume was 500 mL) and <1.0 µmol N L^{-1} for the filtrate. All the data presented herein had already been corrected for blank contributions. The standard deviation of the sample measurements was less than 0.3‰ for samples containing more than 50 nmolN and less than 0.5‰ for those containing more than 20 nmolN. For the filtrate samples, not only organic nitrogen but also inorganic fixed nitrogen (nitrate, nitrite and ammonium) were included in the determined concentrations and $\delta^{15}N$ values. Because the concentrations of the fixed inorganic nitrogen, which were quantified by using an AutoAnalyzer (AACS II; Bran + Luebbe), were always low (mostly below detection levels) at the studied sites (Table 1), we neglected these contributions and interpreted that the determined values represented those of organic nitrogen. Even though the contributions of inorganic nitrogen were significant for the samples for ^{15}N incubation, they did not affect the final results of the N_2 fixation rates. The concentrations of particulate and filtrate nitrogen 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). These values are the typical concentrations of organic nitrogen in oligotrophic oceans (e.g. Minagawa et al., 2001; Mino et al., 2002; Meador et al., 2007).

^{15}N enrichment was clearly observed over time in most of the filtrate and particulate samples incubated under $^{15}N_2$ addition (Fig. 1). The enrichment in $\delta^{15}N$ values ranged from

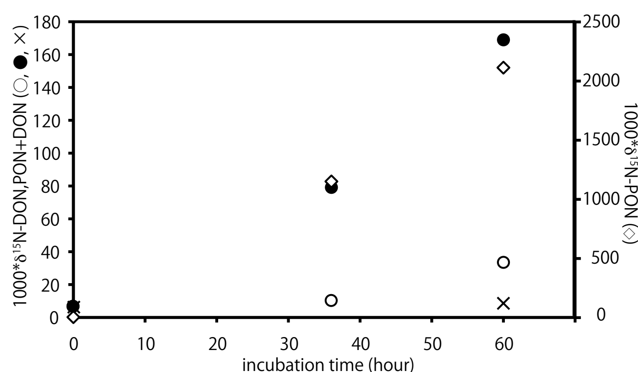

Fig. 1. Variations in δ^{15}N values for each fraction in an incubation bottle (5-m depth/KH06-2 Stn. 5) plotted as a function of the incubation period. The diamonds (◇) indicate the δ^{15}N values of particulate with $^{15}N_2$ (right axis), open circles (○) indicate filtrate with $^{15}N_2$ (left axis), closed circles (●) indicate particulate + filtrate with $^{15}N_2$ (left axis) and crosses (×) indicate filtrate without $^{15}N_2$ (left axis). All error bars were smaller than symbols.

<2 to 1090‰ in particulate fixed nitrogen and from <0.5 to 10.7‰ in filtrate fixed nitrogen. This result indicates that a part of the recently fixed nitrogen was transferred not only into particulate fraction but also into filtrate pools or that active N_2 fixation occurred in the filtrate fraction during the incubation experiment. The N_2 fixation rate was calculated for each incubation bottle using the results for both the concentration and the δ^{15}N values of the filtrate/particulate fixed nitrogen using the following equation (referred from Montoya et al., 1996):

$$\rho = ([N]_{av}/T)(A_f - A_0)/(A_{N2} - A_0)$$

where ρ denotes the N_2 fixation rate (µmol N L^{-1} d^{-1}), $[N]_{av}$ denotes the average fixed nitrogen concentration in filtrate/particulate during experiments (µmol N L^{-1}), T denotes the incubation time, A_f denotes the final abundance ratio of ^{15}N ($100 \times {}^{15}N/({}^{15}N + {}^{14}N)$) in filtrate/particulate fixed nitrogen, A_0 denotes the initial abundance ratio of ^{15}N in filtrate/particulate fixed nitrogen, and A_{N2} denotes the initial abundance ratio of ^{15}N in N_2 in the incubation bottle. As for the values of the initial concentration and δ^{15}N of organic nitrogen, we used those determined for the natural organic nitrogen samples without adding $^{15}N_2$. If the increased δ^{15}N values estimated from the 24 h incubation experiments were less than 2‰ for the particulate and 0.5‰ for the filtrate, we classified the N_2 fixation rates as less than the detection limit.

The vertical distributions of the N_2 fixation rates estimated from the particulate fraction 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 the surface to 100 m

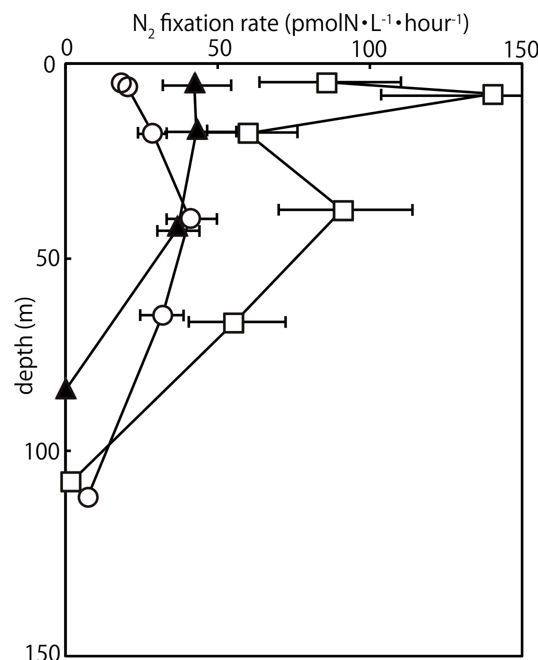

Fig. 2. Depth profiles of N_2 fixation rates in the particulate fraction during the KH06-2 expedition at Stns. 1, 6, and 7 (denoted by triangles, circles, and squares, respectively). The error bars represent the standard deviations for the triplicate water samples.

depths, assuming linear attenuation toward zero with depths of up to 100 m including the stations where the estimation was limited to the surface.

2.3 Quantification of *nifH* gene abundance

DNA extraction was performed according to the method proposed by 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

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

sample I. D.	date	concentration of paticulate N (>0.7 µm)[b] (µmol N L^{-1})	$\delta^{15}N^a$ (particulate N) (>0.7 µm)[b] (‰)	concentration of filtrate N (<0.7 µm)[b] (µmol N L^{-1})	$\delta^{15}N^a$ (filtrate N) (<0.7 µm)[b] (‰)	incubation periods (h)	N_2 fixation (particulate fraction) (>0.7 µm)[b] (µmol N m^{-2} d^{-1})	N_2 fixation (filtrate fraction) (<0.7 µm)[b] (µmol N m^{-2} d^{-1})	Contribution of N_2 fixation in filtrate fraction (% of total N_2 fixation)
KH06-2 Stn. 1	3 Jun 06	0.30	+0	ND	ND	24	104	ND	
KH06-2 Stn. 5	7 Jun 06	0.23	+0.9	6.6	+7.5	12, 36, 60[c]	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[c]	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

[a] $\delta^{15}N = (^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{AirN2} - 1$

[b] We define the size of particulate and filtrate fractions with GF/F filters as >0.7 µm and <0.7 µm, respectively.

[c] The presented data of N_2 fixation rates from KH06-2 Stns. 5 and 6 were mean values of 12–36 h and 12–60 h time course.

ND, not determined

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 particulate fraction

The areal N_2 fixation rates estimated from the particulate fraction varied from <1 to 160 µmol N m^{-2} d^{-1} during the three expeditions undertaken in the western North Pacific (Table 2). Using the conventional $^{15}N_2$ tracer method for the particulate fraction using GF/F filters, 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 particulate fraction 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 during 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 can 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) can also be attributed to the lack of nutrients in the post-blooming season because the observed column-integrated quantities (from the surface to a depth of 100 m) for 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 particulate fraction. Moutin et al. (2005) also found few direct links between the P availability and accumulation of *Trichodesmium*

spp. during the 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 the late summer when we found the direct links between the P-availability and the N_2 fixation rate. In conclusion, the estimated values of N_2 fixation rates for the particulate fraction were representative of those observed in the western North Pacific area during each season.

The $\delta^{15}N$ values of the initial particulate also supported our conclusions (Table 2). The lower $\delta^{15}N$ values of particulate (+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 a higher N_2 fixation rate in the particulate fraction 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 particulate also support the significant N_2 fixation in the particulate fraction.

3.2 N_2 fixation rates in the filtrate fraction

N_2 fixation signal was also found in the filtrate fraction. This signal could be attributed to both the active N_2 fixation into filtrate fraction (direct path) and/or the recent fixed nitrogen release into filtrate fraction (indirect path). The areal N_2 fixation rates estimated in this study for the filtrate fraction (ranging from <0.5 to 54 µmol N m^{-2} d^{-1}) accounted for on average 50% (ranging from <10 to 84%) of the total N_2 fixation rates (Table 2). Glibert and Bronk (1994) also estimated that the rates of N release by *Trichodesmium* spp. into the filtrate (DON) fraction (<0.2 µm) could account for 50% on an average of the N_2 fixation rates in the particulate (PON) fraction (>0.7 µm). Furthermore, Mulholland et al. (2006) estimated the rates of release of fixed nitrogen into the filtrate (DON + DIN; <0.7 µm) fraction 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; they found that the filtrate (DON + DIN; <0.7 µm) fraction comprised 52% (ranging from 9.1% to 81%) of the total N_2 fixation by using a theoretical conversion factor of 3 for the C_2H_2 reduction method. Both the average N_2 fixation rate for the filtrate fraction within the total N_2 fixation rate and the range of variation estimated in this study corresponded well with the estimated rate and variation in past studies. The N_2 fixation rates in the filtrate fraction 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 the 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 particulate fraction in this study (0.4–3.2 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 because 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 particulate fraction when the N_2 fixation rates in the filtrate fraction was significant, the systematic difference between the estimates obtained by the two different methods for the same region implies that the N_2 fixation rates for the filtrate fraction were almost as significant as those for the particulate fraction.

The $\delta^{15}N$ values of the initial filtrate also support our conclusions. Within the whole $\delta^{15}N$ values of the initial filtrate 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 the filtrate (+5.5‰; Stn. 16 during the KH08-2 expedition) was found at the station where the highest N_2 fixation rate in the filtrate fraction 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 the filtrate also support the significant N_2 fixation rates in the filtrate fraction. In conclusion, the estimated significant N_2 fixation rates in the filtrate fraction are representative of those in the western North Pacific region.

3.3 Mechanisms of N_2 fixation signal in the filtrate fraction

As already presented, the significant N_2 fixation rates observed in the filtrate fraction can be explained by the following two mechanisms: (1) N_2 fixation in the filtrate fraction by small unicellular plankton and (2) secondary release of recently fixed nitrogen into the filtrate fraction from the particulate fraction during incubation on board, which includes 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).

The large N_2 fixation rates in the filtrate fraction were found at high latitudes (Fig. 3). In several previous studies, it has been observed that γ-proteobacteria are more abundant in waters characterized as both cooler in temperature and richer in nutrients than waters where 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 is actively expressed in γ-proteobacterial phylotypes at stations far north up to 44° N in the north

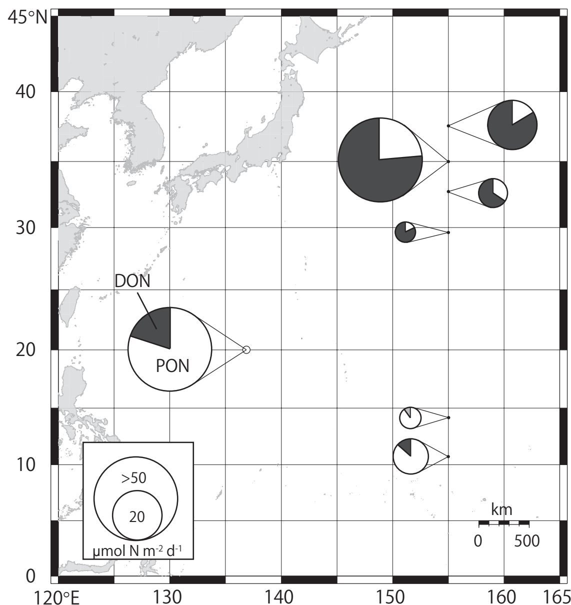

Fig. 3. Pie charts showing the distribution of N_2 fixation rates estimated for particulate (>0.7 μm) and filtrate (<0.7 μm) fractions. The white and gray regions denote N_2 fixation in the particulate and filtrate fractions, respectively. For the stations where the N_2 fixation rates in the filtrate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

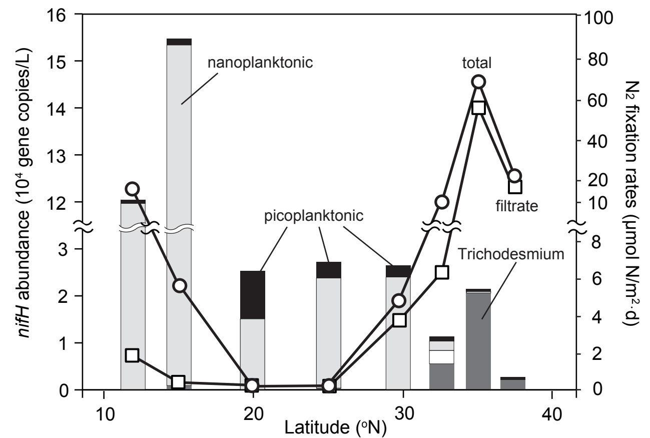

Fig. 4. Abundance of *nifH* gene copies (bar charts: left axis) and the N_2 fixation rates (line charts: right axis) during the KH08-2 expedition. The dark gray, white, light gray, and black bars denote the results for *Trichodesmium* spp., heterocystous diazotroghs, nanoplanktonic diazotrophs, and picoplanktonic 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 filtrate fraction, respectively.

Eastern Pacific region. Because bacterioplankton and/or picoplankton with sizes of ca. 0.2 to 2 μm can pass through the GF/F filter (pore size: 0.7 μm) and mix with the filtrate fraction, they can cause active N_2 fixation in the filtrate fraction.

However, the abundance of *nifH* gene copies determined by the quantified PCR method indicates that the dominant N_2 fixer for stations at high latitudes and exhibiting an active N_2 fixation signal in filtrate fraction is *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 filtrate fraction at high latitudes can be attributed to the active secondary release of N into the filtrate fraction from recently fixed nitrogen by *Trichodesmium* spp. Glibert and Bronk (1994) also found that the rates of release of N from *Trichodesmium* spp. into the filtrate fraction may account for on average 50% of the N_2 fixation rates in the particulate fraction. The release rate of N into the filtrate fraction corresponded well with our results for the natural samples. However, further studies are essential to confirm the precise mechanisms of active N_2 fixation and/or N_2 fixation signal in filtrate fraction.

3.4 Implication for total N_2 fixation flux in ocean

By using the N* parameter taking into account total N_2 fixation, the global oceanic N_2 fixation rates were estimated to be in the range of 70–150 Tg N yr^{-1} (e.g. Gruber and Sarmiento, 1997; Deutsch et al., 2007). However, Karl et al. (2002) noted that the N:P stoichiometry in the N* parameter method could not be accurately determined due to ignoring the dissolved organic matter (DON and DOP). On the other hand, the oceanic N_2 fixation rates were estimated to be in the range of 80–140 Tg N yr^{-1} on the basis of the $^{15}N_2$ tracer method (Brandes et al., 2007); however, these estimates only accounted for the N_2 fixation rates in the particulate fraction. Using the present data, we can estimate the total N_2 fixation rates more accurately by correcting the past estimates. Using a roughly average N_2 fixation signal of 50% in the filtrate fraction 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 efflux 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 efflux exceeds the influx by ca. 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 filtrate fraction can be highly variable on different temporal and spatial scales. Further studies should be conducted to estimate the N_2 fixation rate in the filtrate fraction more accurately.

4 Conclusions

We found significant N_2 fixation rates in the filtrate fraction ($<0.7\,\mu m$) from the western North Pacific region; N_2 fixation in this fraction had been ignored in previous studies. In our results, N_2 fixation rates in the filtrate fraction accounted for on average 50% (ranging from $<10\%$ to 84%) of the total N_2 fixation rates. The abundance of *nifH* gene copies determined by the quantified PCR method indicated that the large N_2 fixation rates observed in the filtrate fraction at high latitudes could be attributed to active secondary N release processes for filtrate fraction from recently fixed nitrogen by *Trichodesmium*. If these results are confirmed generally in the world's oceans, the new total N_2 fixation flux including the N_2 fixation signal in filtrate fraction possibly doubles the original estimates; therefore, the revised influx may reduce the imbalance in the global oceanic fixed nitrogen budget.

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