1 Significance of N_2 fixation in filtrate fraction

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11 **abstract**

12 Using the $^{15}N_2$ tracer method and high-sensitivity $\delta^{15}N$ analytical systems, we
13 determined N_2 fixation rates for ocean samples by dividing them into retentate (>0.7
14 μm) and filtrate (<0.7 μm) fractions. While N_2 fixation in the filtrate had been ignored
15 in previous studies, we found a significant N_2 fixation signal in the filtrate, in our study.
16 The areal N_2 fixation rates estimated from the retentate fractions varied from <1 to 160
17 μmol N m^{-2} d^{-1}, and those rates estimated from the filtrate fractions ranged from <0.5 to
18 54 μmol N m^{-2} d^{-1}. Thus, N_2 fixation in the filtrate fractions accounts for 50% (ranging
19 from <10% to 84%) of the total N_2 fixation rates, on an average. The new total N_2
20 fixation flux, which includes fixation in the filtrate fractions, possibly to doubles the
21 original estimates; therefore, the revised influx may reduce the imbalance in the global
22 oceanic fixed nitrogen budget.

23 **1 Introduction**

24 Over the last three decades, the global budget of oceanic fixed nitrogen (NO_3^-,
25 NO_2^-, NH_4^+, particulate organic nitrogen (PON), and dissolved organic nitrogen (DON))
26 has been extensively studied as one of the representative nutrients that regulate the
27 primary production in the ocean (e.g., Wada et al., 1975; Codispoti and Christensen,
28 1985; Gruber and Sarmiento, 1997; Brandes and Devol, 2002). The total fixed nitrogen
29 is predominantly controlled by the total influx of fixed nitrogen through N_2 fixation and
30 by the total outflux of fixed nitrogen through denitrification (Codispoti et al., 2001;
31 Brandes and Devol, 2002; Deutsch et al., 2004). However, the estimated values of both
32 fluxes obtained in different studies are highly variable (e.g., Wada et al., 1975; Liu,
33 1979; Codispoti and Christensen, 1985; Gruber and Sarmiento, 1997; Codispoti et al.,
34 2001; Brandes and Devol, 2002; Capone and Knapp 2007; Naqvi et al., 2008). Earlier
35 estimates have revealed the an efflux of 95 ± 20 Tg N yr^{-1} through sedimentary
36 denitrification (Gruber and Sarmiento, 1997) and an influx of 110 ± 40 Tg N yr^{-1}
37 through oceanic N_2 fixation (Codispoti et al., 2001). These values almost balance the
38 total fixed nitrogen budget. However, recent studies on denitrification in sediments and
39 water columns have revealed a considerably large outflux of 275–450 Tg N yr^{-1}
40 (Codispoti et al., 2001; Brandes and Devol, 2002) that substantially exceeds the influx.
41 Therefore, a larger influx by N_2 fixation can be expected to balance the oceanic fixed
42 nitrogen budget (e.g., Altabet, 2007; Brandes et al, 2007; Codispoti et al., 2007).

43 The two most commonly used incubation methods to estimate the N_2 fixation
44 rate are the $^{15}N_2$ tracer method and the C_2H_2 reduction method. The former method

45 measures the ^{15}N uptake rate of diazotrophs by determining temporal variation in the
46 nitrogen isotope compositions ($\delta^{15}N = (^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{AirN2} - 1$) of diazotrophs
47 incubated under $^{15}N_2$ (Montoya et al., 1996). The GF/F filter (pore size = 0.7 μm) have
48 been traditionally used to gather diazotrophs as retentate for the $^{15}N_2$ tracer method,
49 because glass fiber filter can be characterized by lower N blank than other filters (e.g.,
50 Montoya et al., 1996; Zehr et al., 2001; Mulholland et al., 2006; Shiozaki et al., 2009).

51 However, Glibert and Bronk (1994) found that on an average, about 1/3 of
52 fixed N can be released into filtrate fractions (<0.2 μm) during incubation of
53 *Trichodesmium thiebautii and T. erythreum* collected in the Caribbean Sea; they also
54 found that the rates of fixed N release into the DON fractions (<0.2 μm) accounted for
55 50% of the N_2 fixation rates in PON fractions (>0.7 μm), on an average. Recently,
56 several studies have indicated the possibility of diazotrophs in picoplanktonic sized
57 organisms (Bird et al., 2005; Langlois et al., 2005; Church et al., 2008) that can pass
58 through the GF/F filter (mesh size: 0.7 μm). As a result, the estimated N_2 fixation rate
59 determined only for the retentate on the GF/F filter (e.g., Montoya et al., 1996; Zehr et
60 al., 2001; Mulholland et al., 2006; Shiozaki et al., 2009) could be underestimated if a
61 considerable amount of N is released into the filtrate during incubation experiments
62 (Bronk and Glibert, 1991; Glibert and Bronk, 1994) or active N_2 fixation in the filtrate.
63 Therefore, in addition to the retentate, one should determine the initial and final values
64 of the concentration and nitrogen isotopic composition for the filtrate in each incubation
65 bottle in order to estimate the total N_2 fixation rates using the $^{15}N_2$ tracer method.
66 However, it is difficult to determine the $\delta^{15}N$ value of filtrate in natural samples using

67 the conventional elemental analyzer isotope ratio mass spectrometry (EA-IRMS)
68 techniques (Mulholland et al., 2004; Meador et al., 2007).

69 On the other hand, the C_2H_2 reduction method is used to measure the C_2H_4
70 production rate through the reduction of C_2H_2 by nitrogenase (Capone, 1993). However,
71 this method requires the use of a conversion factor to convert the observed C_2H_2
72 reduction rates to N_2 fixation rates. While the theoretical reduction ratio of C_2H_2:N_2 is
73 3:1 (mol:mol) (Montoya et al., 1996; Postgate, 1998), little evidence has been found to
74 support the reliability of this ratio under natural conditions (Mulholland et al., 2006;
75 Tsunogai et al., 2008). Nitrogenase-dependent H_2 evolution, which is inhibited by C_2H_2,
76 results in deviations from this theoretical stoichiometry (Robson and Postgate, 1980;
77 Mulholland et al., 2006, 2007). Therefore, the conversion factor is generally determined
78 using the same field measurements by comparing the N_2 fixation rate calculated using
79 both the C_2H_2 reduction method and the conventional ^{15}N tracer method. Although
80 Capone and Montoya (2001) recommended a conversion factor of 4, Mulholland et al.
81 (2006) showed that the factor was variable, with values ranging from 3.7 to 15.7 even in
82 experiments conducted over a span of a few days. Furthermore, as stated previously, it
83 is impossible to estimate the accurate N_2 fixation rates using the conversion factors
84 estimated by the conventional ^{15}N tracer method, based only on the ^{15}N uptake rate by
85 diazotrophs on GF/F, as already presented. Therefore, it is impossible to estimate the
86 quantitative values of the total N_2 fixation rates by the C_2H_2 reduction method.

87 The only way to solve the above mentioned problem is to determine the $\delta^{15}N$
88 values not only for the retentate but also for the filtrate during $^{15}N_2$ tracer incubation.

89 Recent developments in high-sensitivity $\delta^{15}N$ analysis of organic nitrogen have now
90 enabled us to determine the $\delta^{15}N$ values of filtrate as well (Tsunogai et al., 2008). The
91 primary objective of this study is to quantify the total N_2 fixation rates (N_2 fixation in
92 both the retentate and the filtrate) in the field and revise the global oceanic fixed
93 nitrogen budget. Further, we would like to clarify the mechanisms of N_2 fixation signals
94 in the filtrate fractions through biological assays for the same samples. This is the first
95 report on the estimation of the total N_2 fixation rates in the ocean, including the filtrate
96 fractions.
97
98 **2 Sampling and Methods**
99 **2.1. Sample collection and incubation experiments**
100 Both the collection and the incubation of water samples were carried out
101 onboard the R/V *Hakuho Maru* cruise in the western North Pacific region during the
102 KH06-2 (SNIFFS 2006) expedition in June 2006, KH07-2 expedition in September
103 2007, and KH08-2 (SPEEDS/SOLAS 2008) expedition in August–September 2008. The
104 date and locations at which the samples were collected are listed in Table 1. Water
105 samples were collected from depths between 5 and 150 m using a CTD-Carousel
106 multi-sampling system (911plus; Sea-Bird Electronics Inc.). Seawater samples were
107 sub-sampled into 250-mL Pyrex bottles (KH06-2, KH07-2, and KH08-2) or 500-mL
108 polycarbonate bottles (KH08-2) with septum caps without headspace. Then, 1.0 mL of
109 $^{15}N_2$ (99 at%; Shoko Co. Ltd., Tokyo, Japan) was injected into each bottle using a
110 gas-tight syringe. The bottles were gently shaken and then incubated in thermostatic

111 baths on a deck covered with screens to simulate the in situ temperature and light
112 intensity for periods ranging from 12 to 72 h. Although the duration of incubation was
113 variable, the incubation was mainly carried out during diurnal periods (24, 48, or 72 h)
114 to avoid the bias caused by the day-night cycle on the N_2 fixation rate. However, two
115 samples were incubated for three different periods of 12, 36, and 60 h during the
116 KH06-2 expedition and may have been biased by the day-night cycle. Therefore, we
117 corrected the bias by adopting the relative variations between 36 and 12 h
118 (corresponding to 24 h incubation), or those between 60 and 12 h (corresponding to 48
119 h incubation).

120 Immediately after incubation, the retentate in each incubated water sample
121 was collected on a pre-combusted (450 °C for 4 h) Whatman GF/F filter (pore size = 0.7
122 μm) by gentle vacuum filtration. The pressure difference was strictly controlled to be
123 <100 mm Hg to avoid the leakage of small particles from the filters. The <0.7-μm
124 filtrate was collected in a light-resistant polyethylene bottle (100 ml) and frozen until
125 analysis. The retentate collected on the filter was further washed with filtered clean
126 seawater, placed in a plastic case, frozen instantaneously, and stored in a deep freezer
127 (-80 °C) until analysis. For quantitative polymerase chain reaction (qPCR) assays
128 targeting partial *nifH* fragments, seawater (1 L) was filtered onto 25-mm Supor filters
129 (pore size: 0.2 μm, Pall Corporation) under gentle vacuum (<100 mm Hg). The obtained
130 filters were frozen in a deep freezer (-80 °C) until analysis.

131

132 **2.2. Geochemical data analysis**

133 The concentrations and $\delta^{15}N$ values of organic nitrogen in both the retentate
134 and the filtrate, including those incubated under $^{15}N_2$ addition, were analyzed using the
135 method developed by Tsunogai et al. (2008). This method involves oxidation/reduction
136 methods such as the oxidation of organic nitrogen to nitrate using persulfate (Knapp et
137 al., 2005; Tsunogai et al., 2008, 2010), reduction of nitrate to nitrite using spongy
138 cadmium, and further reduction of nitrite to nitrous oxide using sodium azide. The total
139 recovery rate of N was around 90% for the samples. The blank level was <10 nmol N
140 for the retentate (corresponding to 0.02 μmol N L^{-1} when the filtrate volume was 500
141 mL) and <1.0 μmol N L^{-1} for the filtrate. All the data presented herein had already been
142 corrected for blank contributions. The standard deviation of the sample measurements
143 was less than 0.3‰ for samples containing more than 50 nmolN and less than 0.5‰ for
144 those containing more than 20 nmolN. For the filtrate samples, not only organic
145 nitrogen but also inorganic fixed nitrogen (nitrate, nitrite and ammonium) were included
146 in the determined concentrations and $\delta^{15}N$ values. Because the concentrations of the
147 fixed inorganic nitrogen, which were quantified by using an AutoAnalyzer (AACS II;
148 Bran+Luebbe), were always low (mostly below detection levels) at the studied sites
149 (Table 1), we neglected these contributions and interpreted that the determined values
150 represented those of organic nitrogen. Even though the contributions of inorganic
151 nitrogen were significant for the samples of ^{15}N incubation, they did not affect the final
152 results of the N_2 fixation rates. The concentrations of retentate and filtrate nitrogen
153 ranged from 0.11 to 0.60 μmol N L^{-1} and from 4.0 to 7.0 μmol N L^{-1}, respectively (Table

154 2). These values are the typical concentrations of organic nitrogen in oligotrophic
155 oceans (e.g., Minagawa et al., 2001; Mino et al., 2002; Meador et al., 2007).
156 The total N_2 fixation rate was calculated for each incubation bottle using the
157 results for both the concentration and the $\delta^{15}N$ values of the filtrate/retentate (ρ = ([N]$_{av}$
158 /2T) (A_f - A_0)/(A_{N2} - A_0), ρ: N_2 fixation rate; [N]$_{av}$: average fixed nitrogen concentration;
159 T: incubation time; $A = 100 \times {}^{15}N/({}^{15}N + {}^{14}N)$: abundance ratio of ^{15}N; A_f: final
160 abundance ratio for fixed nitrogen; A_0: initial abundance ratio for fixed nitrogen; A_{N2}:
161 initial abundance ratio for N_2 in the incubation bottle). ^{15}N enrichment was clearly
162 observed over time in most of the filtrate and retentate samples incubated under $^{15}N_2$
163 addition (Fig. 1); this result indicates that a part of the recently fixed-nitrogen was
164 transferred into filtrate pools during the incubation experiment. The vertical
165 distributions of the N_2 fixation rates estimated from the retentate fractions during the
166 KH06-2 expedition are shown in Fig. 2. The profiles indicate that the N_2 fixation rates
167 at the water surface were high and that these rates linearly decreased to nearly zero at
168 depths of ca. 100 m. Therefore, we calculated the areal N_2 fixation rates by integrating
169 the N_2 fixation rates on a volume from surface to 100 m depths, assuming linear
170 attenuation toward zero with depths up to 100 m including the stations where the
171 estimation was limited to the surface. If the increased $\delta^{15}N$ values estimated from the
172 incubation experiments were less than 2‰, we classified the N_2 fixation rates as less
173 than the detection limit and presented the maximum value.
174
175 **2.3. Biological data analysis**

176 DNA extraction was performed according to the method of Short and Zehr
177 (2005) with slight modifications. In brief, 600 µl of XS buffer (1% potassium ethyl
178 xanthogenate; 100 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0; 1% sodium
179 dodecylsulfate; 800 mM ammonium acetate) and ca. 0.2 g of 0.1-mm glass beads were
180 added to the vials containing the filters. The vials were placed in a bead beater
181 (BioSpec Products) and agitated three times at 4800 rpm for 50 s. The samples were
182 incubated at 70 °C for 60 min. After incubation, the XS buffer was transferred to a
183 1.5-ml microtube, vortexed for 10 s, and placed on ice for 30 min. Cell debris was
184 removed by centrifugation at 15,000 g at 5 °C for 15 min; the supernatants were then
185 decanted into another 1.5-ml microtube with the same amount of isopropanol. The
186 samples were incubated at room temperature for 10 min, and the precipitated DNA was
187 pelleted by centrifugation at 15,000 g for 15 min at 5°C. Isopropanol was decanted,
188 and the DNA pellets were washed with 70% ethanol, vacuum dried, and resuspended
189 in 100 µl of 10 mM Tris-HCl (pH 8.5). The obtained samples were stored at -20 °C
190 until further analysis.
191 Quantitative PCR (qPCR) assays targeting partial *nifH* fragments were
192 carried out with a Thermal Cycler Dice Real Time System (TP800; TaKaRa) using
193 primers and TaqMan probes designed by Church et al. (2005); they determined five
194 *nifH* phylotypes including the cyanobacteria *Crocosphaera* spp. (termed Group B), an
195 uncultivated phylotype termed Group A that was presumed to be a unicellular
196 cyanobacterium, *Trichodesmium* spp., heterocystous cyanobacteria, and
197 g-proteobacteria in the North Pacific Ocean. For each set of primers and probes set,

198 standard curves were derived using duplicate or triplicate serial dilutions of linearized
199 pUC18 plasmids (TaKaRa) containing the positive control insert. The number of
200 molecules of a plasmid was estimated from the amount of DNA according to the
201 equation derived by Short and Zehr (2005). The PCR amplification mixture solution
202 (25 μl) contained 12.5 μl of Premix Ex Taq (Perfect Real Time, TaKaRa), 0.05 μl of
203 each primer (final conc.: 0.2 μM), 0.1 μl of the probe (final conc.: 0.4 μM), 11.3 μl of
204 sterile Milli-Q water, and 1 μl of DNA template. In each qPCR run, environmental
205 DNA and no template controls (i.e. sterile Milli-Q water) were also prepared in
206 duplicate or triplicate. The thermal cycling reactions were carried out as follows: 95 °C
207 for 10 s, and 50 cycles of 95°C for 5 s followed by 60°C for 30 s.

208

209 3 Results and Discussion

210 3.1. N_2 fixation rates in the retentate fractions

211 The areal N_2 fixation rates estimated from the retentate fractions varied from
212 <1 to 160 μmol N m^{-2} d^{-1} during the three expeditions undertaken in the western North
213 Pacific (Table 2). Using the conventional $^{15}N_2$ tracer method for the retentate fractions
214 using GF/F filters, Shiozaki et al. (2009) estimated the areal N_2 fixation rates in the
215 western North Pacific region to be 29–152 μmol N m^{-2} d^{-1} in early spring. The areal N_2
216 fixation rates estimated from the retentate fractions in the present study during the
217 early summer expedition (KH06-2 cruise; 22–160 μmol N m^{-2} d^{-1}) agreed well with
218 those reported by Shiozaki et al. (2009). On the other hand, the areal N_2 fixation rates
219 estimated during the late summer expeditions (KH07-2 and KH08-2 cruises; <20 μmol

220 N m^{-2} d^{-1}) were lower than those estimated during the early summer expeditions. The
221 concentration of the sea surface chlorophyll-a was lower on the late summer
222 expeditions (0.01 to 0.05 μg L^{-1}) than during the early summer expeditions (0.07 to
223 0.21 μg L^{-1}), indicating that the late summer expeditions coincided with the
224 post-blooming season, when nutrients are limited. Thus, the observed difference in the
225 areal N_2 fixation rates can be attributed to the seasonal variations in the N_2 fixation
226 rates (Sañudo-Wilhelmy et al., 2001; Moutin et al., 2005). The lowest observed N_2
227 fixation rates at the two stations during the late summer expeditions (Stns.19 and 20,
228 KH08-2) (Table 2) can also be attributed to the lack of nutrients in the post-blooming
229 season, because the observed column-integrated quantities (from the surface to a depth
230 of 100m) 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)
231 were the smallest for those stations during the late summer expeditions (Table 1).

232 However, the column-integrated quantities of PO_4^{3-} were also small at Stns. 5,
233 6, and 7 (KH06-2) during the early summer expedition, where active N_2 fixation (>22
234 μmol N m^{-2} d^{-1}) was observed in the retentate fractions. Moutin et al. (2005) also found
235 few direct links between the P availability and accumulation of *Trichodesmium* spp.
236 during the early summer. Therefore, explanations other than the limited availability of
237 nutrients may be required to explain active N_2 fixation during the early summer. Owing
238 to the ability to store P, *Trichodesmium* spp. is active for a few months after PO_4^{3-}
239 deficiency (Thompson et al., 1994; Moutin et al., 2005). Therefore, during early
240 summer, the N_2 fixers were using the stored P supplied during winter or spring, while
241 they may exhaust the stored P during the late summer when we found the direct links

242 between P-availability and the N_2 fixation rate. In conclusion, the estimated values of
243 N_2 fixation rates for the retentate fractions were representative of those observed in the
244 western North Pacific area during each season.

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

253

254 **3.2. N_2 fixation rates in the filtrate fractions**

255 The areal N_2 fixation rates estimated in this study for the filtrate fractions
256 (ranging from <0.5 to 54 μmol N m^{-2} d^{-1}) accounted for 50% (ranging from <10 to 84%)
257 of the total N_2 fixation rates on an average (Table 2). Glibert and Bronk (1994) also
258 estimated that the rates of N release by *Trichodesmium* spp. into the filtrate (DON)
259 fractions (<0.2 μm) could account for 50% on an average of the N_2 fixation rates in the
260 retentate (PON) fractions (>0.7 μm). Furthermore, Mulholland et al. (2006) estimated
261 the rates of release of fixed nitrogen into the filtrate (DON; <0.7 μm) fractions in the
262 ocean on the basis of the discrepancies in the N_2 fixation rates estimated by the $^{15}N_2$
263 tracer method and the C_2H_2 reduction method for the same samples; they found that the

264 filtrate (DON) fraction comprised 52% (ranging from 9.1% to 81%) of the total N_2
265 fixation by using a theoretical conversion factor of 3 for the C_2H_2 reduction method.
266 Both the average N_2 fixation rate for the filtrate fractions within the total N_2 fixation
267 rate and the range of variation estimated in this study corresponded well with the
268 estimated rate and variation in past studies. The estimated N_2 fixation rates in the filtrate
269 fractions estimated in this study may be highly reliable for estimating those in the
270 ocean.

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

285 The $\delta^{15}N$ values of the initial filtrate also support our conclusions. Within the

286 whole $\delta^{15}N$ values of the initial filtrate in surface water (ranging from +5.5‰ to
287 +8.8‰, Table 2), which agree with those reported in a previous study in the Central
288 Pacific region (Meador et al., 2007), the lowest $\delta^{15}N$ value of the filtrate (+5.5‰;
289 Stn.16 during the KH08-2 expedition) was found at the station where the highest N_2
290 fixation rate in the filtrate fractions was observed. Because the fixation of atmospheric
291 N_2 ($\delta^{15}N$ = 0‰) produces organic nitrogen with $\delta^{15}N$ values similar to atmospheric N_2
292 (Bourbonnais et al., 2009), the geographical variations in $\delta^{15}N$ of the filtrate also
293 support the significant N_2 fixation in the filtrate fractions. In conclusion, the estimated
294 significant N_2 fixation rates in the filtrate fractions are representative of those in the
295 western North Pacific region.
296
297 **3.3. Mechanisms of N_2 fixation signal in filtrate fractions**
298 The significant N_2 fixation signal observed in the filtrate fractions can be
299 explained by the following two mechanisms: (1) active N_2 fixation in the filtrate
300 fraction by small plankton such as bacterioplankton and/or picoplankton and (2) active
301 secondary release of N into the filtrate fractions from the retentate fractions during
302 incubation on board, which includes recently fixed nitrogen such as viral cell lysis
303 (Hewson et al., 2004), grazing (O'Neil et al., 1996), cell death (Berman-Frank et al.,
304 2004), or direct release of N-compounds (Glibert and Bronk, 1994).
305 In particular, we focused on the mechanisms of the large N_2 fixation rate in
306 the filtrate fractions at high latitudes (Fig. 3). In several previous studies, it has been
307 observed that γ-proteobacteria are more abundant in waters characterized as both cooler

308 in temperature and richer in nutrients than waters where usual cyanobacterial N_2 fixers
309 are dominant (Bird et al., 2005; Langlois et al., 2005; Church et al., 2008). In particular,
310 Church et al. (2008) found that the *nifH* gene is actively expressed in γ-proteobacterial
311 phylotypes at stations far north up to 44°N in the north Eastern Pacific region. Because
312 bacterioplankton and/or picoplankton with sizes of ca. 0.2 to 2 μm can pass through the
313 GF/F filter (pore size: 0.7 μm) and mix with the filtrate fractions, they can cause active
314 N_2 fixation in the filtrate fractions.

315 However, the abundance of *nifH* gene copies determined by the quantified
316 PCR method indicates that the dominant N_2 fixer for stations at high latitudes and
317 showing active N_2 fixation signal in filtrate fractions is *Trichodesmium* spp. (Fig. 4).
318 Although we could not directly compare the number of *nifH* gene copies with the N_2
319 fixation rates (Zehr et al., 2007), the large N_2 fixation rates observed in the filtrate
320 fractions at high latitudes can be attributed to the active secondary release of N into the
321 filtrate fractions from recently fixed nitrogen by *Trichodesmium* spp. Glibert and Bronk
322 (1994) also found that the rates of release of N from *Trichodesmium* spp. into the filtrate
323 fractions may account for 50% of the N_2 fixation rates in the retentate on an average.
324 The release rate of N into the filtrate fractions corresponded well with our results for the
325 natural samples. However, further studies are essential to confirm the precise
326 mechanisms of active N_2 fixation and/or N_2 fixation signals in filtrate fractions.
327

328 **3.4. Implication for total N_2 fixation flux in ocean**

329 In previous studies, the oceanic N_2 fixation rates were estimated to be in the
330 range of 80–140 Tg N yr^{-1} on the basis of the data estimated using the $^{15}N_2$ tracer
331 method (Brandes et al., 2007); however, these estimates only accounted for the N_2
332 fixation rates in the retentate fractions. Using the present data, we can estimate the total
333 N_2 fixation rates more accurately by correcting the past estimates. Using a roughly
334 average N_2 fixation of 50% in the filtrate fractions that were underestimated in previous
335 studies over oceans worldwide, the revised N_2 fixation inputs should be increased to
336 160–280 Tg N yr^{-1}. Codispoti et al. (2001) estimated the total influx and outflux of
337 fixed nitrogen to be 287 and 482 Tg N yr^{-1}, respectively, for the current global fixed
338 nitrogen budget in oceans; in this budget, the outflux exceeds the influx by ca. 200 Tg N
339 yr^{-1}. The revised influx reduces the imbalance in the global fixed nitrogen budget.
340 However, as observed during this study, the N_2 fixation rates in the filtrate fractions can
341 be highly variable on different temporal and spatial scales. Further studies should be
342 conducted to estimate the N_2 fixation rate in the filtrate fractions more accurately.
343

344 **4 Conclusions**

345 We found a significant N_2 fixation signal in the filtrate fraction (<0.7 μm)
346 from the western North Pacific region; N_2 fixation in these fractions had been ignored in
347 previous studies. In our results, N_2 fixation in the filtrate fractions accounted for 50%
348 (ranging from <10% to 84%) of the total N_2 fixation rates, on an average. The
349 abundance of *nifH* gene copies determined by the quantified PCR method indicated that
350 the large N_2 fixation rates observed in the filtrate fractions at high latitudes could be

351 attributed to active secondary N release processes for filtrate fractions from recently
352 fixed-nitrogen by *Trichodesmium*. The new total N_2 fixation flux including N_2 fixation
353 in filtrate fractions possibly doubles the original estimates; therefore, the revised influx
354 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 (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.

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

sample I.D.	date	Retentate (µmol N L^{-1})	$\delta^{15}N^{\#}$ (retentate) (‰)	filtrate (µmol N L^{-1})	$\delta^{15}N^{\#}$ (filtrate) (‰)	incubation periods (h)	N_2 fixation (retentate) (µmol N $m^{-2}d^{-1}$)	N_2 fixation (filtrate) (µmol N $m^{-2}d^{-1}$)	Contribution of N_2 fixation in filtrate fractions (% of total 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

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

ND, not determined

Figure captions

Figure 1. Variations in $\delta^{15}N$ 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 $\delta^{15}N$ values of retentate with $^{15}N_2$, filtrate with $^{15}N_2$, retentate + filtrate with $^{15}N_2$, and filtrate without $^{15}N_2$, respectively.

Figure 2. Depth profiles of N_2 fixation rates in the retentate 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.

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

Figure 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 fractions, respectively.

Figure 1

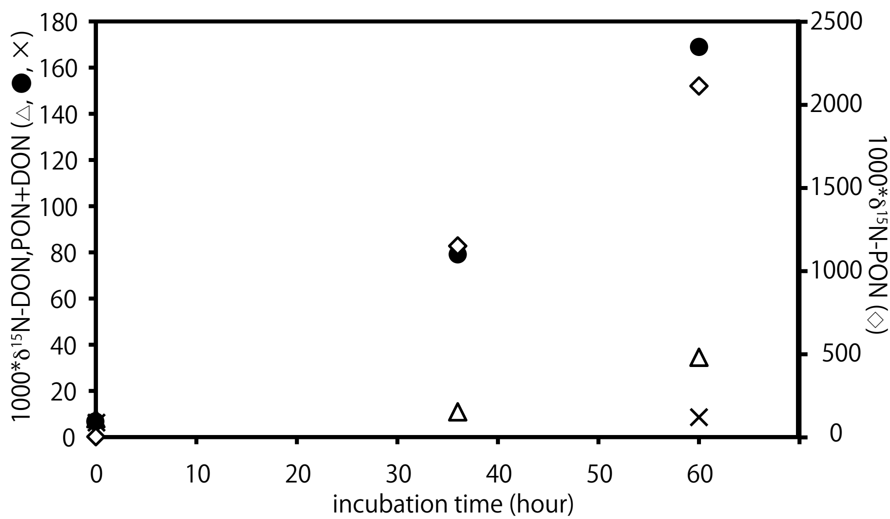

Figure 2

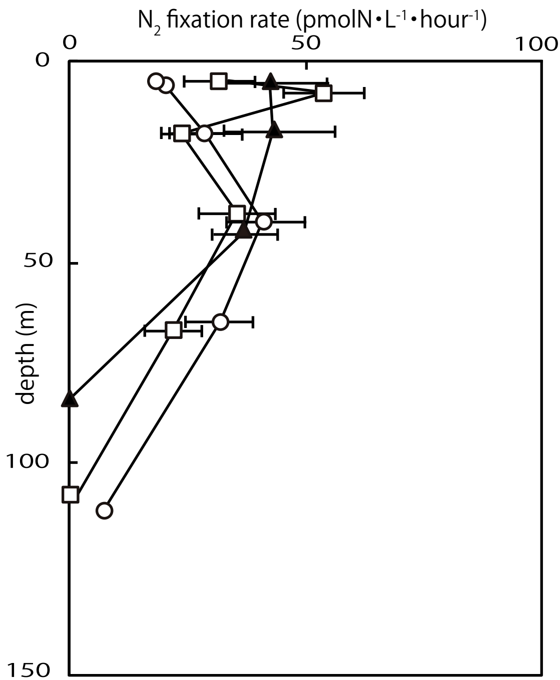

Figure 3

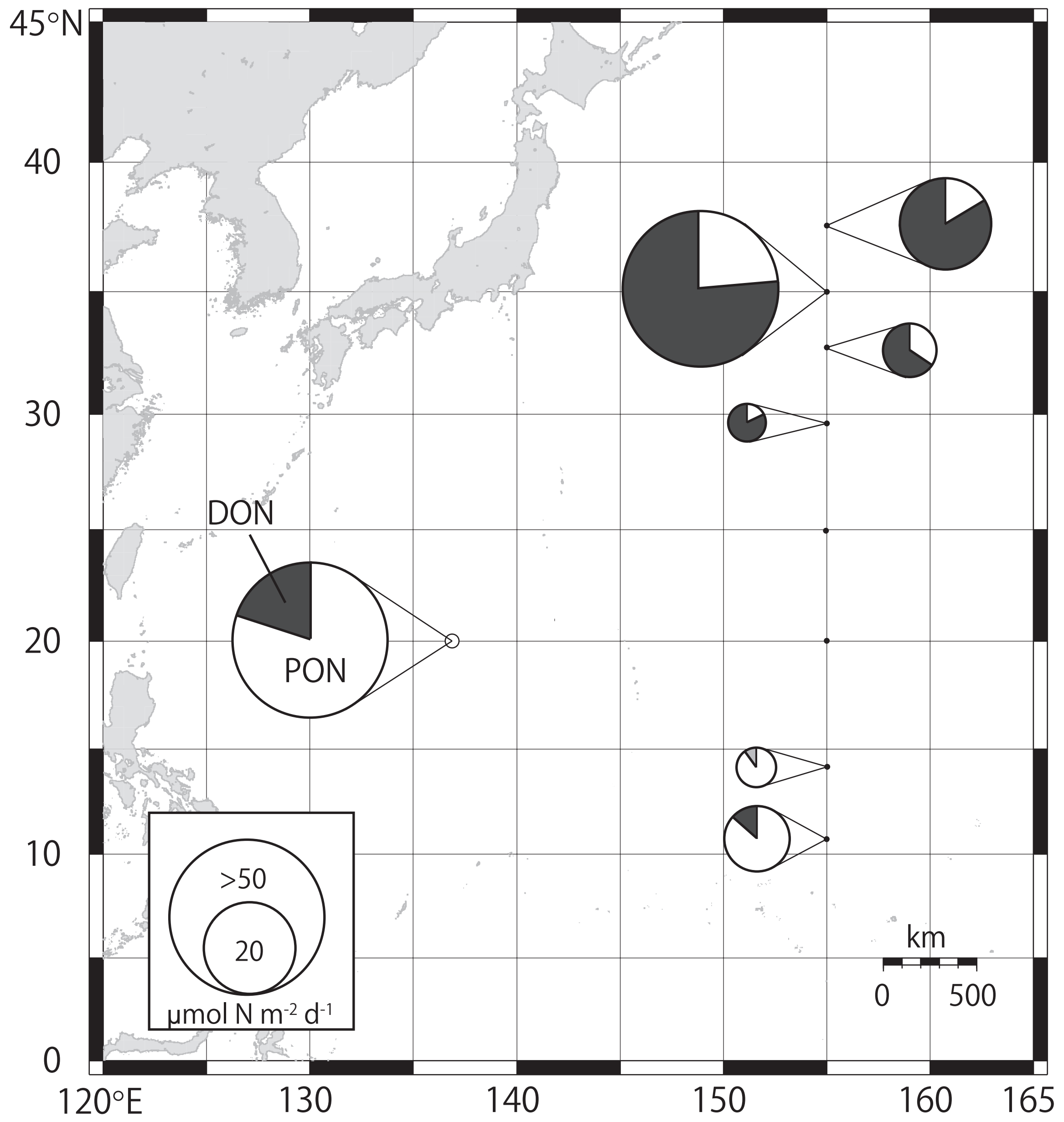

Figure 4