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Seasonal nitrogen fixation and primary production in the Southwest Pacific: nanoplankton diazotrophy and transfer of nitrogen to picoplankton organisms

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ABSTRACT: We applied a high-sensitivity dual isotopic tracer technique (^{13}C:^{15}N) to measure N_2 fixation and primary production in the total phytoplanktonic community and in 3 size fractions (>10, <10 and <3 µm) in oceanic waters around New Caledonia (Southwest Pacific). This region seemed to be favourable for diazotrophy, which is observed at significant rates over the year throughout both oceanic and lagoonal habitats. Nitrogen-fixation rates were high, but presented some spatial heterogeneity and high seasonal variability. Large phytoplankton (>10 µm, i.e. *Trichodesmium*) often fixed the bulk of available nitrogen at very high rates (up to 1.8 nmol l^{-1} h^{-1}). Elevated $^{15}N_2$ accumulation (up to 0.83 nmol l^{-1} h^{-1}) was always observed in the <10 µm fraction, representing a mean of 31 ± 20% of total N_2 fixation, up to 92% in the lagoon and 98% in the oceanic region. Direct fixation was detected in the <10 µm fraction during the day as well as during the night in the New Caledonia lagoon, indicating that unicellular nanoplanktonic cyanobacteria could be a significant source of new nitrogen. Some accumulation of $^{15}N_2$ was also detectable in the <3 µm fraction, especially in surface samples. The rates of this nitrogen accumulation were generally very low (<0.17 nmol l^{-1} h^{-1}), representing ~10% of total fixation. However, in August 2002, this ^{15}N accumulation in the <3 µm fraction contributed nearly 50% of the total nitrogen fixation. However, with the post-size-fractionation experiments it was not possible to distinguish direct N_2 fixation from picoplanktonic assimilation of organic compounds released by large cyanobacteria. Nevertheless, the results demonstrate a close coupling between larger diazotrophs and picoplanktonic populations, and show that new nitrogen could rapidly be provided for the pelagic microbial food web.

KEY WORDS: Nitrogen fixation · Nanoplankton · Picoplankton · New Caledonia

INTRODUCTION

Nitrogen is generally accepted as the most common nutrient limiting phytoplanktonic primary production throughout much of the world's oceanic surface waters. Because of this, biological fixation of gaseous nitrogen (an unlimited source from the atmosphere), also called diazotrophy, has gained recognition as an important process in supporting oceanic primary production (Lipschultz & Owens 1996, Capone et al. 1998). Due to their ability to fix N, the cyanobacteria can contribute substantially to the input of new nitrogen into nutrient-poor environments. This nitrogen pool can represent a potentially important nitrogen source for other organisms in the pelagic food web. The diazotrophy process can be carried out by a number of different types of prokaryotic organisms, but in the ocean, the process appears to be supported for the most part photoautotrophically by cyanobacteria in sunlit surface waters (Paerl 1990). The majority of studies on N_2 fixation in open ocean waters have focused on rate measurements of the colonial forms of *Trichodesmium* spp. The large size classes (*Trichodesmium* and diatoms containing endosymbiotic *Richelia*) appear to be the

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dominant diazotrophs in tropical and sub-tropical waters (Zehr & Capone 1996, Capone et al. 1997, 2005; Mulholland & Capone 2000) and are thought to be responsible for the bulk of marine N_2 fixation. Recently, however, unicellular diazotrophic cyanobacteria and bacterioplankton have been found in the nanoplankton community of the North Pacific central gyre (Zehr et al. 2001). A range of molecular and isotopic evidence suggests that these unicells could support a significant fraction of new production in oligotrophic waters (Montoya et al. 2004), even if low numeric abundance of these organisms poses challenges for quantifying their nitrogen fixation activity.

In the present study, we applied the $^{15}N_2$-tracer method, which made it possible to measure the rate of N_2 fixation directly on non-concentrated natural samples and to follow the fate of the new nitrogen into different components of the planktonic food web. We provide evidence for the existence of nanoplanktonic diazotrophic cyanobacteria (<10 µm) in the surrounding waters of New Caledonia, and noted a rapid and significant accumulation of newly fixed nitrogen in the picoplanktonic fraction (<3 µm).

MATERIALS AND METHODS

Experiments were performed around New Caledonia, Southwest Pacific (Fig. 1), between January 2002 and October 2003, during 6 Diapalis cruises on the 'Institut pour la Recherche et le Développement' (IRD) ship 'l'Alis', and in the Noumea lagoon during October 2005 (Table 1). Several stations representing different ecosystems (lagoon, open bay, open ocean) were sampled. Specifically, our study was concerned with 4 locations where size-fractionation experiments were performed: the 'Baie du Santal' (open bay, 20° 8′ S, 167° 1′ E), the 'Chenal des Loyautés' (open ocean, 21° 5′ S, 167° E), the 'Nord Ouvéa' (open ocean, 20° 00′ S, 166° 23′ E) and the Noumea lagoon (22° 20′ S, 16° 30′ E).

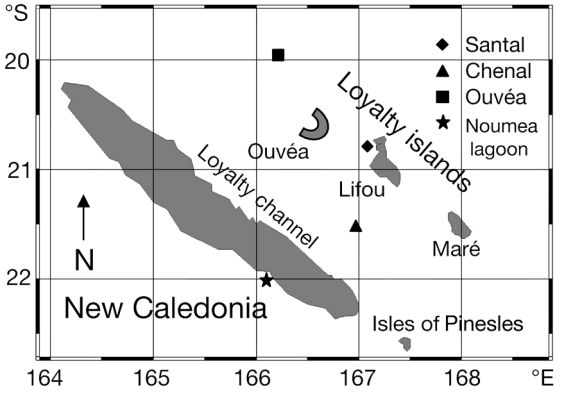

Fig. 1. Study area with sampling locations

Table 1. Dates of the Diapalis cruises and Noumea Lagoon experiments

Cruise	Date
Diapalis 3	15–22 Jan 2002
Diapalis 4	3–8 Apr 2002
Diapalis 5	21–28 May 2002
Diapalis 6	5–11 Aug 2002
Diapalis 7	3–12 Feb 2003
Diapalis 9	9–16 Oct 2003
Noumea Lagoon	20–28 Oct 2005

During Diapalis cruises, water samples were collected using a CTD-rosette (12 × 8 l Niskin bottles) at 7 different depths (0, 10, 20, 30, 40, 80 and 100 m). In the lagoon, samples were taken between 0 and 20 m depth. Nitrogen fixation rates were determined using a highly sensitive $^{15}N_2$-tracer method (Montoya et al. 1996), in parallel with primary production by the dual ^{13}C:^{15}N-labelling technique.

Polycarbonate Nalgene flasks (1200 ml) were filled directly from the Niskin bottles without any treatment, except during lagoon experiments, in which some samples were immediately filtered (by gravity) on a 10 µm polycarbonate membrane to eliminate large diazotrophs. Thus, in these pre-fractionated samples, only the nanoplanktonic fraction was incubated, isolated from the large phytoplankton, to test whether any N_2 is fixed by this nanoplanktonic fraction. Subsequent experimental and analytical procedures were the same for all samples. In each bottle, ^{13}C-labelled bicarbonate sodium ($NaH^{13}CO_3$: 6 g 250 ml^{-1} deionised water, 99 atomic% ^{13}C; Eurisotop) was added in order to obtain a 10% final enrichment (1 ml 1200 ml^{-1} seawater). The $^{15}N_2$ gas (99 atomic% ^{15}N, Eurisotop) was introduced to the bottle through a gas-tight septum (2 ml of gas 1200 ml^{-1} seawater), to achieve a tracer addition of ~10%. We added a fixed quantity of $^{15}N_2$ gas and calculated the enrichment of each bottle on the basis of its volume and the solubility of N_2. We used the equations provided by Weiss (1970) to calculate the N_2 initial concentration, assuming equilibrium with the overlying atmosphere. The $^{15}N_2$ enrichment in the incubation bottles ranged between 11 and 14%. The sample was carefully shaken to allow equilibration between $^{15}N_2$ and natural N_2. After ^{13}C and ^{15}N addition, the samples were incubated on a drifting rig at the same depth from which they were collected, from sunrise to sunset (12 ± 1 h). Night incubations were performed on the >GF/F (see explanation in following paragraph) fraction during Diapalis 5 and on the >GF/F and <10 µm pre-fractionated fractions during lagoon experiments.

After incubation, pre-fractionated samples were immediately filtered on a pre-combusted (24 h at 450°C), 25 mm Whatman GF/F filter. Size fractionation was performed on all total samples and allowed us to investigate 3 phytoplanktonic fractions in addition to the total phytoplankton community: (1) the >10 µm fraction commonly represented by diazotrophs of the genus *Trichodesmium*, (2) the <10 µm size fraction corresponding to nanoplankton (Malone 1980) and (3) the <3 µm size fraction, defined here as picoplankton (Stockner & Antia 1986). In these definitions, the nanoplankton also includes the picoplankton. First, 600 ml were directly filtered under a gentle vacuum (100 mm Hg) on a pre-combusted (24 h at 450°C), 25 mm Whatman GF/F filter; this sample is called the '>GF/F fraction'. The remaining 600 ml were treated either as the isolated <10 µm fraction or as the collected >3 µm fraction as follows: (1) To isolate the <10 µm fraction and to remove *Trichodesmium* and large phytoplankton, samples were gravity pre-filtered on a 10 µm pore-size polycarbonate filter; <10 µm filtrates were then collected on a pre-combusted (24 h at 450°C), 25 mm Whatman GF/F filter. The nitrogen fixation due to the large phytoplankton (>10 µm) was obtained from the difference between nitrogen fixation of the >GF/F fraction and the <10 µm fraction of the same incubated samples. (2) To collect the >3 µm fraction, samples were filtered on 3 µm pore-size silver pre-combusted (24 h at 450°C) Poretics filters. Nitrogen fixation in the <3 µm fraction was obtained from the difference between nitrogen fixation of the >GF/F fraction and the <3 µm fraction of the same incubated samples.

All filters were dried in a 60°C oven for 24 h and analysed as soon as possible after the cruise. The dual isotopic enrichment analysis was performed on an Integra-CN PDZ Europa mass spectrometer, calibrated with glycine references every batch of 10 to 15 samples. The accuracy of our analytical system was also regularly verified using reference materials from the International Atomic Energy Agency (AIEA, Analytical Quality Control Services). The mean values of the natural atomic percent (at.%) of ^{15}N and total nitrogen for glycine standards containing 0.2 to 10 µmol N, as well as results from standard references, are given in Table 2. These data indicated that the analysing system is highly stable and accurate. The mean at.% ^{15}N did not vary between samples from 0.5 to 10 µmol N. ^{15}N enrichment appeared slightly underestimated at the lowest nitrogen mass (0.2 µmol). Nevertheless, this variation (<0.001%) is orders of magnitude smaller than the enrichment measured during the experiments in the present study, ranging from 0.02 to 0.70%. The low background of the system allows safe analysis of samples containing low nitrogen concentrations (0.2 to 0.5 µmol), values often observed in surface oligotrophic waters. The ^{15}N isotope enrichment of a sample is reported in terms of at.% excess of ^{15}N over time in reference to the at.% ^{15}N in a non-enriched sample originating from the same phytoplankton population. Therefore, it is necessary to know the value of Time 0 enrichment is necessary; this is determined using samples (same volume as incubated sample) that are filtered immediately after isotope addition. The Time 0 value, established using 8 samples, was 0.3663 ± 0.007%. Although the Time 0 enrichment was close to natural abundance in the atmosphere (0.3663%), indicating no residual tracer on the filter after filtration and drying, we considered results with ^{15}N enrichments >0.014% (2 times the standard deviation obtained with Time 0 samples) to be significant.

Nitrogen fixation rates (ρ_{N2}) can be directly calculated from ^{15}N-excess enrichment found in the GF/F and >10 µm fractions, as well as in the >10 µm pre-fractionated fraction. But, as we cannot certify that ^{15}N-excess enrichment in the smallest fractions (<10 µm) was nitrogen fixation and not trophic transfer, calculated rates are designated nitrogen accumulation (ρ_{acN2}).

ρ_{N2} and ρ_{acN2} are calculated according to the following equation (Dugdale & Wilkerson 1986):

$$\rho_{N2} = \rho_{acN2} = [(R_{PN}/R_{n2})/T] \times PN \quad (1)$$

Table 2. Evolution of ^{15}N natural enrichment of glycine, given as ^{15}N (atomic %, at.%) and $\delta^{15}N$ (‰), as a function of quantity of nitrogen analysed, calibrated against International Atomic Energy Agency (IAEA) reference materials

Glycine samples (µmol)	Particulate nitrogen (µmol)	^{15}N (at.%)	$\delta^{15}N$ (‰)
0.2	0.21	0.3672	2.50
0.2	0.21	0.3673	2.60
0.5	0.51	0.3678	4.02
0.5	0.53	0.3678	4.19
1.0	1.02	0.3679	4.28
1.0	1.04	0.3677	3.95
2.0	2.03	0.3679	4.39
2.0	2.10	0.3681	4.84
2.5	2.58	0.3679	4.41
2.5	2.53	0.3680	4.51
5.0	5.07	0.3679	4.50
5.0	5.20	0.3680	4.59
7.5	7.04	0.3679	4.48
7.5	7.52	0.3680	4.68
10	9.79	0.3680	4.73
10	10.15	0.3681	4.91
Mean		0.3678	4.28
Standard deviation		0.0003	0.28
Reference materials			
Urea (IAEA 310A) $\delta^{15}N$ = 47‰	5.23	47.3 ± 0.27	
Urea (IAEA 310B) $\delta^{15}N$ = 244‰	4.49	243 ± 0.46	

where PN corresponds to the final particulate nitrogen concentration and T to the incubation period in hours. R_{PN} and R_{n2} are the at.% ^{15}N excess enrichments in the particulate N_2 and dissolved N_2 pool, respectively. To facilitate comparison to literature data, ^{15}N fixation rates are expressed in nmol N l^{-1} h^{-1}.

Under these experimental conditions, the detection limit for nitrogen fixation, calculated from significant enrichment (0.38%) and particulate nitrogen (0.2 µmol), is estimated with Eq. (1) to be 0.3 nmol l^{-1} 12 h^{-1}. To test the reproducibility of the procedure, 3 profiles for >GF/F nitrogen fixation (included in the results) were performed with triplicates during Diapalis 6. The coefficient of variation for each set of 3 samples varied from 7 to 20%.

Carbon fixation rates were calculated according to Slawyk & Collos (1984), with a Time 0 enrichment of 1.113 ± 0.005% (n = 8). This Time 0 value is a little higher than the natural abundance for phytoplankton (1.089), certainly due to some residual traces of ^{13}C tracer. It should be noted that ^{13}C enrichment of samples was less problematic than ^{15}N enrichment, since inorganic carbon was assimilated by all phytoplankton, and excess values ranged from 0.3 to 3.6%. ^{13}C-fixation rates, i.e. primary production, are expressed in µg C per litre per 12 h.

Concentrations of free trichomes and colonies of *Trichodesmium* were determinated during Diapalis 7 and 9, by filtering 8 l (the entire Niskin bottle) of seawater from the rosette casts onto a 10 µm pore-size, 47 mm diameter Nuclepore filter. A 47 mm filter holder Gelman Sciences containing the filter was attached to the spigot of the Niskin bottle with tubing, and the water was filtered by gravity. The filter was then collected in a polyethylene flask filled with 20 ml of 0.2 µm filtered seawater and preserved with paraformaldehyde (0.5% final concentration). Counts were made in the laboratory with a Zeiss optical microscope at 75× magnification.

RESULTS

Fig. 2 revealed the observed N_2-fixation (ρ_{N2}) rate profiles of the GF/F. ρ_{N2} values were significant for all Diapalis cruises, ranging from 0.08 to 1.8 nmol l^{-1} h^{-1}, with a maximum always observed in the surface layer (0 to 20 m). The highest rates were found in February 2003 (Diapalis 7), with a maximum of 1.8 nmol l^{-1} h^{-1} at 20 m.

The range of fixation rates varied according to the season. In April, May and August 2002, ρ_{N2} did not exceed 0.42 nmol l^{-1} h^{-1} (Fig. 2a). Intermediate values (0.4 to 0.7 nmol l^{-1} h^{-1}) characterised the Diapalis 9 cruise (October 2003), while the highest values (0.83 to 1.7 nmol l^{-1} h^{-1}) were found in February 2003, for Diapalis 7 (Fig. 2b). The night fixation rates, only measured during Diapalis 5, were not significant (Fig. 2a).

The N_2-fixation rate in the >10 µm size fraction was generally dominant, ranging between 0.08 and 1.5 nmol l^{-1} h^{-1} (Fig. 3), the highest value being obtained during Diapalis 7 (February 2003). Diapalis 9 (October 2003) showed a unique pattern, with no N_2-fixation rates measured in the >10 µm size fraction (Fig. 3d). $^{15}N_2$ accumulation (ρ_{acN2}) in the <10 µm size fraction showed different results according to the respective cruises. For Diapalis 4, 5 and 7 (Fig. 3a–c), rates <0.4 nmol l^{-1} h^{-1} were observed along the water column. The Diapalis 9 cruise (Fig. 3d) presented the highest ρ_{acN2} in the <10 µm size fraction, with a maximum of 0.75 nmol l^{-1} h^{-1} at the surface and around 0.4 nmol l^{-1} h^{-1} down to 40 m. It should be noted that at depth (80 to 100 m), ρ_{acN2} in the <10 µm size fraction was always detected and significant. Results obtained from 7 successive lagoon experiments demonstrated the existence of intense nitrogen fixation in the <10 µm fraction (Fig. 4), even when previously isolated from the largest phytoplankton species. Day and night rates were similar, ranging from 0.33 to 0.7 nmol l^{-1} h^{-1} over the water column. It should be noted that, during the same experiments, nitrogen fixation in the >10 µm fraction was very low during the day (<0.08 nmol l^{-1} h^{-1} in mean) and close to 0 during the night, indicating low activity of large diazotrophs during these experiments.

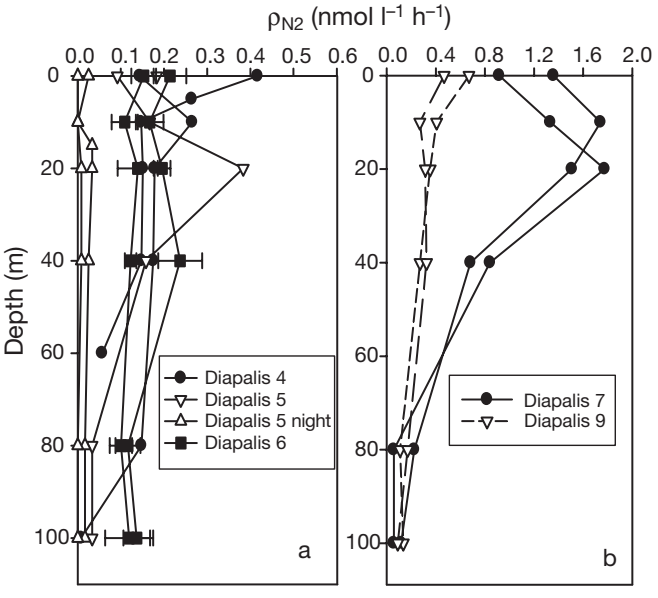

Fig. 2. Vertical profiles of nitrogen fixation (ρ_{N2}) obtained at stations where size fractionation was performed: (a) cruises with rates <0.4 nmol l^{-1} h^{-1} and (b) cruises with highest rates. Two night incubation profiles, obtained during the Diapalis 5 cruise, are included

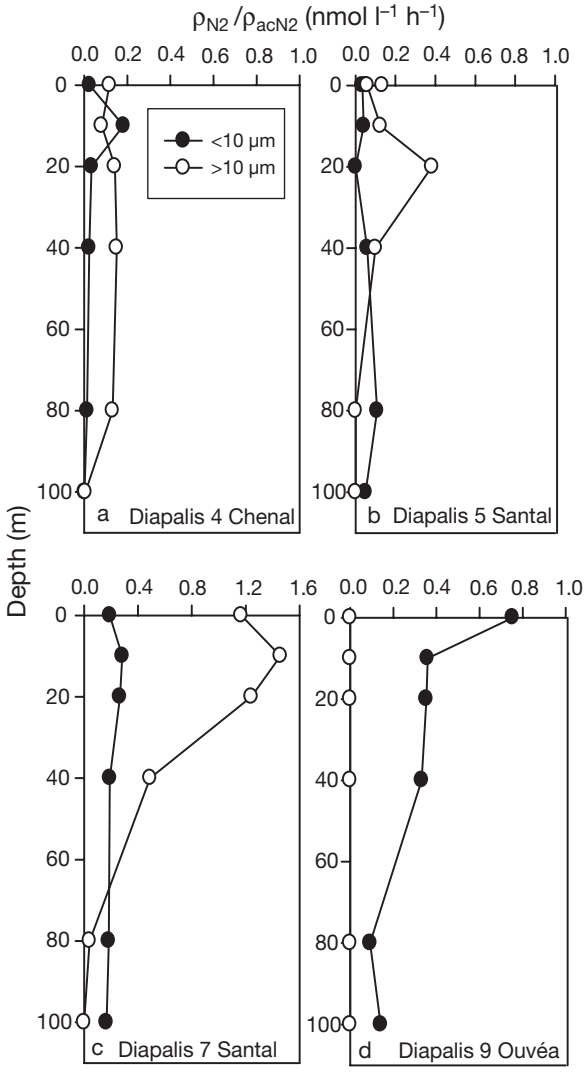

Fig. 3. Examples of vertical profiles of nitrogen fixation in the >10 µm (ρ_{N2}) and $^{15}N_2$ accumulation (ρ_{acN2}) in the <10 µm size fraction: (a) Diapalis 4 'Chenal des Loyautés' location, (b) Diapalis 5 'Baie du Santal' location, (c) Diapalis 7 'Baie du Santal' location and (d) Diapalis 9 'Nord Ouvéa' location

In addition, we investigated the ^{15}N enrichment within the picoplankton fraction (<3 µm). Fixed nitrogen accumulation (ρ_{acN2}) in the <3 µm size fraction (Fig. 5) was generally close to the detection limit. Meanwhile, significant ρ_{acN2} values were measured at some depths of the vertical profile, principally at the surface during Diapalis 4 and 5, but were always <0.08 nmol l^{-1} h^{-1}.

During Diapalis 6, we observed ρ_{acN2} values in the <3 µm size fraction equivalent to those in the >3 µm size fraction (Fig. 5c), with estimates commonly >0.08 nmol l^{-1} h^{-1}. During Diapalis 7 (Fig. 5d), when the highest total fixation rates were observed, significant ρ_{acN2} values were detected in the <3 µm size fraction, but only in subsurface samples (up to 0.17 nmol l^{-1} h^{-1}).

Pooling all available data from Diapalis 3 to 7, ρ_{acN2} in the <10 µm fraction accounted for 31 ± 20% of total nitrogen fixation and appeared to be more or less permanent and constant, while fixation in the greater fraction showed more substantial variation (Fig. 6a). In the same manner, no linear relationship was found between ρ_{N2} in the >3 µm size fraction and ρ_{acN2} in the <3 µm size fraction (Fig. 6b). Most of the time, high ρ_{N2} values were measured in the >3 µm without ^{15}N accumulation in the smaller fraction. But, in contrast, significant ρ_{acN2} values were detected in the <3 µm size fraction (>0.03 nmol l^{-1} h^{-1}), even when ρ_{N2} in the largest size fraction was very low (<0.17 nmol l^{-1} h^{-1}). In Fig. 6b, the 4 isolated points correspond to Diapalis 7 data, characterised by numerous *Trichodesmium* populations (500 to 700 trichomes l^{-1} at the surface). During Diapalis 9, very high ρ_{acN2} values were observed in the <10 µm fraction, while ρ_{N2} in the largest fraction was insignificant (Fig. 6a). Similar results were found in the lagoon with pre-fractionated and post-fractionated samples; ρ_{N2} was highly significant in the <10 µm fraction, but very low or undetectable in the largest size fraction.

Concurrently, a comparison of total primary production (^{13}C assimilation) with primary production in the <10 and <3 µm size fractions showed they were both linearly related (r^2 = 0.95, n = 63 for the <10 µm size fraction; r^2 = 0.73, n = 36 for the <3 µm size fraction).

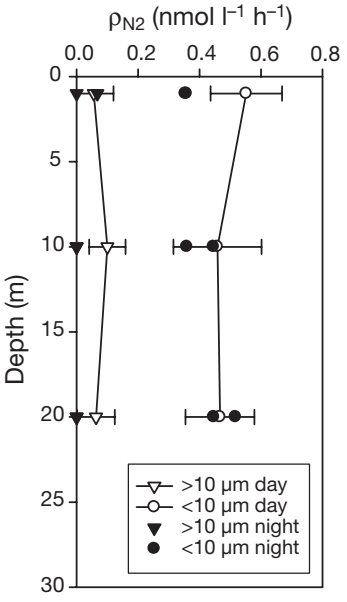

Fig. 4. Day and night lagoon vertical profiles of nitrogen fixation (ρ_{N2}) in the >10 and pre-fractionated <10 µm fraction. Mean day rates and standard deviation were obtained from 7 experiments

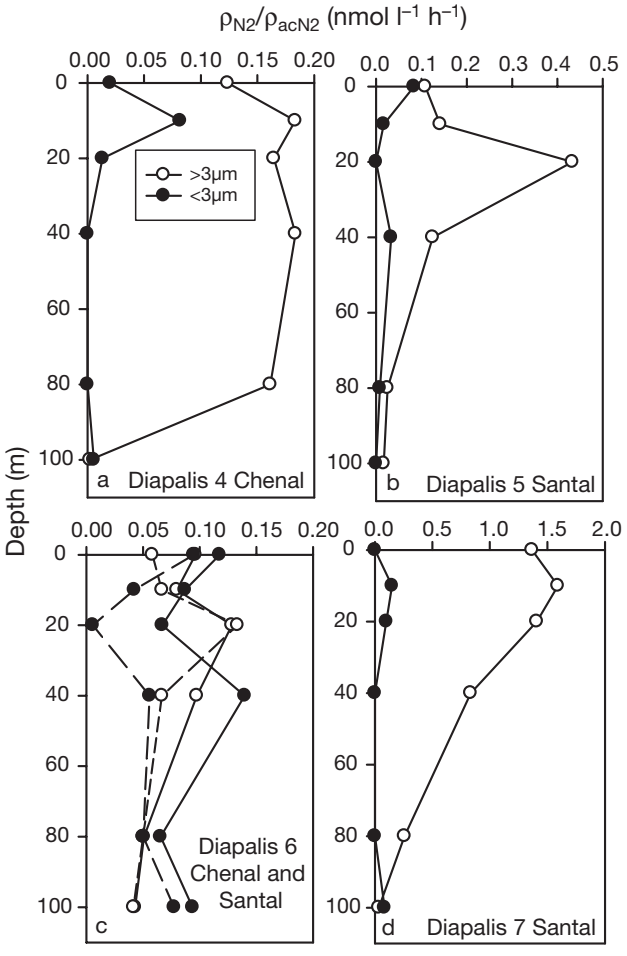

Fig. 5. Examples of vertical profiles of nitrogen fixation (ρ_{N2}) in the >3 µm and nitrogen accumulation (ρ_{acN2}) in the <3 µm size fraction: (a) Cruise Diapalis 4, 'Chenal des Loyautés' location, (b) Diapalis 5 'Baie du Santal' location, (c) Diapalis 6 'Chenal des Loyautés' (solid lines) and 'Baie du Santal' (dashed lines) locations and (d) Diapalis 7 'Baie du Santal' location

They represented, respectively, 84 and 50% of the total primary production. We noted an exception for some Diapalis 7 stations; these were characterised by a primary production of 50% carried out by >10 µm organisms. In contrast, the potential photosynthetic carbon assimilation associated with N_2 fixation (applying a C:N ratio of 6.6) in the different fractions was very low. It corresponded to 7.7 ± 8.4 and 9.5 ± 15.2% in the <10 and <3 µm size fractions, respectively. In the same way, the primary production associated with diazotrophy in the >10 µm size fraction is very weak, representing <1.1 ± 3.8% of the measured total primary production. Thus, nitrogen fixation sustained a very small part of primary production, indicating that all phytoplankton fractions used other nitrogen forms for growth. *F*-ratio values generally obtained in such oceanic regions (0.1 to 0.2), however, indicate that nitrogen fixation may account for ~50% of new production.

To facilitate comparison with literature data, we calculated mean areal values of diel rates for our experiments (Table 3). We summed day and night rates (expressed as d^{-1}) for lagoon samples. But, due to the lack of significant nitrogen fixation during the night (see Fig. 2), we assumed the 12 h rates to be daily rates at oceanic sites. This assumption could lead to some underestimation of daily integrated rates, if some oceanic diazotroph organisms, not detected in the present experiments, were able to fix nitrogen during the night. It should be noted that only Falcòn et al.'s (2004) data were obtained from experiments of isolated <10 µm organisms, and can be compared to our results of nanoplanktonic nitrogen fixation obtained in the lagoon (ρ_{N2}). All other literature data came from post-fractionated samples and, as such, must be considered as ρ_{acN2}, potentially including some trophic transfer of ^{15}N-tracer and not only direct N_2 fixation. In the Noumea Lagoon, mean areal rates obtained between 0 and 20 m depth on the isolated <10 µm fraction were high (214 µmol m^{-2} d^{-1}), representing 91% of the total daily (24 h) nitrogen fixation. This value is slightly higher than values given by Falcòn et al. (2004) (62 to 167 µmol m^{-2} d^{-1}). Mean areal ρ_{N2} values in the >10 µm size fraction calculated for all Diapalis cruises, ranging between 6 and 474 µmol m^{-2} d^{-1}, are similar to data obtained in oligotrophic areas for experiments conducted with isolated *Trichodesmium*. The mean areal N_2-fixation rate obtained during Diapalis 7 (474 µmol m^{-2} d^{-1}), when high densities of *Trichodesmium* populations were observed, is the maximum rate observed during the experimentation period and is also a higher value than normally measured in oceanic areas. In contrast, the Diapalis 9 cruise (October 2003) is characterised by the absence of *Trichodesmium* at the sampled locations, and our observations here reveal a very low ρ_{N2} in the >10 µm size fraction (6 µmol m^{-2} d^{-1}). This result is surprising when considering the high value of total nitrogen fixation measured at the same time (306 µmol m^{-2} d^{-1}). The nitrogen fixation in the >10 µm size fraction often represented >50% of the total fixation (Table 3), but never reached 100%, even when total nitrogen fixation was high, such as during Diapalis 7 (February 2003). Moreover, ρ_{N2} in the >10 µm size fraction can account only for a small part of the total nitrogen fixation (2% during Diapalis 9 and 9% in the lagoon). During these experiments, *Trichodesmium* populations were never observed.

DISCUSSION

The ability to measure high-precision isotope ratios, combined with the sensitive field tracer method (Montoya et al. 1996), permitted the 'direct' diazotrophy

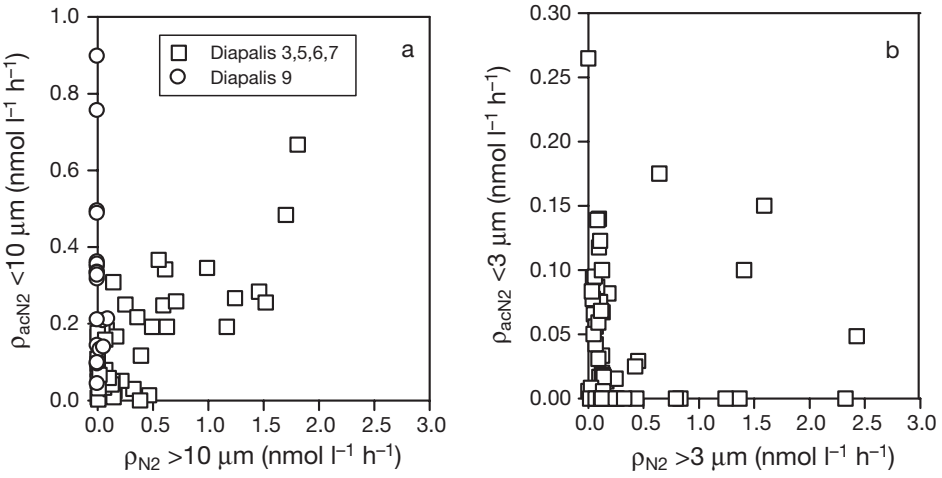

Fig. 6. Comparisons of nitrogen accumulation rates between (a) the <10 and >10 µm size fraction and (b) the <3 and >3 µm size fraction in the various Diapolis cruises

Table 3. Summary of N_2 fixation (ρ_{N2}) and N_2 accumulation (ρ_{acN2}) (mean areal rates in µmol m^{-2} d^{-1}) in different size fractions measured in the present study and from literature data. Values in brackets represent the percent of the GF/F

Location	Date	N fixation (ρ_{N2})			N accumulation (ρ_{acN2})		Source
		>GF/F (total)	>10 µm	<10 µm	<10 µm	<3 µm	
New Caledonia Diapalis	4 Apr 2002	151	111 (73%)		40 (27%)	13 (9%)	Present study
New Caledonia Diapalis	5 May 2002	169	96 (57%)		73 (43%)	21.5 (13%)	Present study
New Caledonia Diapalis	6 Aug 2002	176				87 (49%)	Present study
New Caledonia Diapalis	7 Feb 2003	703	474 (67%)		229 (33%)	52 (7%)	Present study
New Caledonia Diapalis	9 Oct 2003	306	6 (2%)		300 (98%)		Present study
Noumea Lagoon	Oct 2005	235	21 (9%)	214 (91%)			Present study
Tropical N Atlantic (7–12° N)	2001–2002		62–167	37–47			Falcon et al. (2004)
N Pacific (near ALOHA)	2002		84	2.2			Falcon et al. (2004)
ALOHA region	2000–2001				66		Montoya et al. (2004)
Hawaii region	2000–2002				24		Montoya et al. (2004)
Eastern N Pacific gyre	Jul 2002				50		Montoya et al. (2004)
North Australia Coral Sea	Nov 1999				126		Montoya et al. (2004)
Arafura Sea (Australia)	Nov 1999				3955		Montoya et al. (2004)
ALOHA region	2000–2001				20–35		Dore et al. (2002)
ALOHA station	2000				92		Zehr et al. (2001)
BATS (Bermuda)	1995–1997		41				Orcutt et al. (2001)
Arabian Sea	May 1995		35–99 (bloom)				Capone et al. (1998)
N Atlantic	1995–2003		239				Capone et al. (2005)
ALOHA	1990–1992		84				Karl et al. (1997)
Caribbean Sea		161					Carpenter & Price (1977)
Southwestern N Atlantic	Nov 1964		41				Goering et al. (1966)
NW Mediterranean Sea	2003–2004	40–100					Garcia et al. (2006)

measurements carried out in the present study; such measurements are very rarely made without concentrated natural seawater or pre-concentrated phytoplankton. Marine waters around New Caledonia seem to be a favourable region for diazotrophy, which can be observed at significant rates throughout the year in both oceanic and lagoonal habitats. The nitrogen fixation rates obtained were high and presented some spatial heterogeneity and high seasonal variability. The reason for this variability is not easy to determine, even though several nutrient conditions have been pointed out as a possible source (Van den Broeck et al. 2004). Results from size fractionation clearly demonstrate that large phytoplankton (>10 µm) are not always the predominant pool for accumulation of newly fixed N_2, as might be inferred from the preponderance of studies previously conducted on *Trichodesmium* and diatoms with cyanobacterial endosymbionts.

However, in most of our experiments, post-size-fractionation results do not allow us to distinguish between

direct nanoplanktonic $^{15}N_2$ fixation and $^{15}N_2$ transferred from large diazotrophs to small organisms via recently released organic nitrogen. Glibert & Bronk (1994) have shown that a high level of N_2 fixed (a mean of 50%) may be released as dissolved organic nitrogen (DON) by *Trichodesmium*. The released compounds, even if relatively minimal when compared with ambient seawater concentrations of DON, can be a significant source of new nitrogen for the non-nitrogen-fixing organisms. Indeed, Ohlendieck et al. (2000) showed that $^{15}N_2$ enrichment measured in the <3 µm size fraction (5 to 10% of the total $^{15}N_2$ fixed) is entirely due to assimilation of organic nitrogen recently released by large diazotrophic phytoplankton. Paerl (1984) found a significant amount of ^{15}N fixed in cultures of *Anabaena*, which appear to have associated bacteria after a short incubation time. Nevertheless, when *Trichodesmium* populations were missing (as observed during Diapalis 9), as well as in pre-fractionated samples, the <10 µm organisms were found to be solely responsible for diazotrophic activity, at very high integrated rates (200 to 300 µmol m^{-2} d^{-1}). The range of <10 µm N_2 fixation values is close to those previously reported in the literature (see Table 3), with the exception of the extremely high rate measured in the Arafura Sea of Australia (Montoya et al. 2004). Rates are equivalent or higher than many values previously given for *Trichodesmium*. Our study shows that, regardless of the period and of the presence of large diazotrophic populations, nanoplanktonic nitrogen fixation can be a real and significant process (up to 98% of the total mean areal N_2 fixation) in lagoon waters. Since pre-fractionated experiments were not performed during the Diapalis cruises, this result must be confirmed for oceanic waters.

The nanoplanktonic organisms responsible for the high daily and night rates remained unidentified in the present study. Zehr et al. (2001) found small unicellular cyanobacteria (2 to 3 µm in diameter) expressing the gene *nifH* in the subtropical North Pacific; these were associated with significant nitrogen accumulation (0.008 to 0.016 nmol l^{-1} h^{-1}) in the <10 µm fraction. Mitsui et al. (1986) isolated a marine strain from the benthos that could fix nitrogen in the dark in a chemostat culture. Two larger marine coccoid strains (3 to 4 µm in diameter) that fix N_2 at night, *Cyanothece* spp. (Reddy et al. 1993) and *Erytthrospera marina* (Waterbury et al. 1988), have also been isolated. In the present study, the significant dark fixation obtained from the isolated <10 µm fraction tends to confirm the existence of such small unicellular nocturnal diazotrophs, at least in the Caledonian Lagoon. If all natural nanoplanktonic diazotrophs act like cyanobacteria in cultures, the impact of these organisms on oceanic nitrogen cycling would be even more important.

The large accumulation of $^{15}N_2$ in the <3 µm size fraction demonstrates a tight coupling between larger diazotrophs and picoplanktonic populations, and shows that newly fixed nitrogen could rapidly provide for the pelagic food web. During all experiments, even if total fixation rates were low, $^{15}N_2$ accumulation (ρ_{acN2}) in picoplankton ranged from 7 to 13% of the total nitrogen fixation. These percentages are equivalent to those found by Ohlendieck et al. (2000) due to trophic transfer (5 to 10%). Diapalis 6 results show more important nitrogen accumulation in the <3 µm size fraction. The ρ_{acN2} integrated rates ranged between 13 and 87 µmol m^{-2} 12 h^{-1}, which corresponds to 52% of the total fixed nitrogen. These values are of the same order of magnitude as Zehr et al.'s (2001) data (92 µmol m^{-2} 12 h^{-1}), calculated from surface nitrogen fixation rates and concentrations of *nifH*-containing unicellular cyanobacteria over the water column. This observation allows us to pinpoint a previously hypothesised, but still undocumented process, i.e. picoplanktonic nitrogen fixation (Capone 2001, Zehr et al. 2001). Unfortunately, post-fractionation experiments did not offer the opportunity to distinguish direct N_2 fixation from assimilation of ^{15}N-labeled organic compounds by heterotrophic picoplankton. Nevertheless, these experiments highlight new information regarding the accumulation of recently fixed nitrogen in picoplankton and its trophic transfer through the microbial food web. This finding poses a serious challenge to our understanding of the nitrogen cycle. Thus, the microbial loop, generally considered to be driven only by nitrogen regeneration, could also be an important way for new nitrogen to enter the system, either by short-term trophic transfer from large cyanobacteria to small heterotroph organisms or by direct nitrogen fixation by nanoplanktonic cyanobacteria. However, this last process needs to be confirmed through future studies which should take into account all size populations (and not only *Trichodesmium*), in order to correctly quantify diazotrophy rates in pelagic waters and identify the associated organisms. It will then be essential to focus on size-fractionation experiments with pre-filtered seawater coupled with organism examination using microscopy and molecular techniques. Moreover, the considerable temporal and local variations in nitrogen fixation found in the present work indicate that broader studies in time and location are needed to assess the contribution of N_2 fixation in different size classes to the nitrogen budget in this region of the Pacific Ocean.

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