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High abundance of diazotrophic picocyanobacteria (<3 µm) in a Southwest Pacific coral lagoon

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ABSTRACT: In the present study we detected unicellular diazotrophic cyanobacteria along a nutrient gradient in New Caledonia's coral lagoon (24 000 km^2) using whole-cell hybridization of specific Nitro821 probes (TSA-FISH, Tyramide Signal Amplification of Fluorescent *In Situ* Hybridization). The specificity of this probe was confirmed on cultures and in the natural environment. Surprisingly, the community of Nitro821-targeted cells was numerically dominated by picocyanobacteria (97%, 1 to 1.5 µm). These organisms were either free living (63%), recovered with the 0.2 to 3 µm size fraction or associated (37%) to particles or planktonic dinoflagellates from larger size fractions (3 to 10 µm and >10 µm). Diazotrophic picocyanobacterial abundance ranged from 3 to 140 cells ml^{-1} along the nutrient gradient and was highest at the oligotrophic lagoonal station. These cells may contribute to N_2 fixation from the <10 µm size fraction (4.4 to 8 nmol N^{-1} 24 h^{-1}), which was 4 times higher than the >10 µm size fraction (0 to 1.9 nmol N^{-1} 24 h^{-1}). In addition, in the middle of the lagoon daylight $^{15}N_2$ fixation could explain 78% of $^{15}N_2$ fixation over 24 h. The results presented in the present study reveal for the first time a significant concentration of photosynthetic diazotrophs within the marine picoplankton community.

KEY WORDS: Diazotrophs · Cyanobacteria · Picoplankton · FISH · Pacific · Coral lagoon · Symbiosis

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INTRODUCTION

Marine diazotrophs are a functional group of heterotrophic prokaryotes and cyanobacteria, which are able to fix dissolved dinitrogen (N_2) from the atmosphere. This function offers them an important ecological advantage compared to other microorganisms living in nitrogen-depleted waters. In oceanic and coastal oligotrophic waters, up to 50% of new primary production can be explained by diazotrophic cyanobacteria (Karl et al. 1997). Highest rates of N_2 fixation were detected on tropical coastal areas from cyanobacterial mats, but their restricted colonized areas do not make them significant contributors to total N_2 fixation (Herbert 1999). Since the discovery of diazotrophy in the ubiquitous filamentous cyanobacteria *Trichodesmium* sp., it was believed that this genus was responsible for the majority of N_2 fixation in tropical and subtropical oceanic waters as it can form massive blooms (Capone et al. 1997). Other oceanic cyanobacteria that are known to significantly contribute to N_2 fixation are intracellular symbionts of diatoms (*Richelia* sp.) (Carpenter et al. 1999).

Many recent biogeochemical studies have concluded that previous rates of global ocean N_2 fixation have been significantly underestimated and cannot be reconciled with observed *Trichodesmium* sp. and *Richelia* sp. abundance (Gruber & Sarmiento 1997, Capone & Carpenter 1999). It has been recently recognized that unicellular pico- and nanoplanktonic organisms (<10 µm) can support a significant amount of N_2 fixation (Montoya et al. 2004, Garcia et al. 2007). These organisms have been identified as unicellular nanoplanktonic cyanobacteria and heterotrophic Bacteria

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and Archaea, which are either free living or associated with other organisms (Zehr et al. 2001, Falcón et al. 2004b, Foster et al. 2006a). Some of these nanoplanktonic cyanobacteria are maintained in culture collections (e.g. *Cyanothece* sp. ATCC51142 and *Crocosphaera watsonii* WH8501), while others have not been observed or cultivated so far, such as Group A. Groups A, B and C represent clusters of mainly unidentified *nif*H sequences, which are closely connected to *Crocosphaera* sp. and *Cyanothece* sp. for Groups B and C, respectively, when group A is weakly affiliated to Group C (Zehr et al. 2001). Although these cells seem to have a significant effect on nitrogen fluxes, they are 2 or 3 orders of magnitude less abundant than non-diazotrophic marine *Synechococcus* sp. and *Prochlorococcus* sp. (Campbell et al. 2005, Zehr et al. 2007). Another peculiar characteristic is that Group A has been recently suspected to fix N_2 during the day, while the other unicellular cyanobacteria fix N_2 during the night (Church et al. 2005, Zehr et al. 2007). This feature is very surprising within unicellular diazotrophic cyanobacteria, as it is known that nitrogenase, which catalyses N_2 fixation, is irreversibly inhibited by O_2 (Burgess & Lowe 1996). As such, diazotrophic cyanobacteria have segregated in time and/or space the O_2-generating photosynthesis from nitrogen fixation. While most filamentous cyanobacteria have differentiated cells in heterocyst to fix N_2, unicellular cyanobacteria show photosynthetic activity during the day and diazotrophic activity at night (Zehr et al. 2007).

The Southwest Pacific harbors regular massive blooms of *Trichodesmium* sp., which share or alternate in space with *Crocosphaera*-like cells (Campbell et al. 2005). Dominant southeast trade winds drive these oceanic waters into Noumea's coral lagoon (New Caledonia), and after 3 to 11 d of residential time they are washed through the coral reef passes (Jouon et al. 2006). New Caledonia's coral lagoon is one of the largest in the world (24 000 km^2 and 25 m deep on average). It is mainly oligotrophic (0.3 µg chl *a* l^{-1}), but an important concentration of dissolved nitrogen is regularly discharged from Noumea city (132 000 habitants), which increases its chl *a* concentration 10-fold (3 µg chl *a* l^{-1}) (Jacquet et al. 2006) and maintains detectable nutrient concentrations (0.9 µg DIN l^{-1}, 0.15 µg DIP l^{-1}, Jacquet et al. 2006). Filamentous cyanobacteria (*Trichodesmium* sp. and *Richelia* sp), as well as planktonic N_2-fixation activity have been regularly detected inside and outside the lagoon both from >10 µm and <10 µm fractions (Garcia et al. 2007). However, unicellular planktonic diazotrophs have never been observed.

To determine the relative contribution of these various groups of unicellular diazotrophs to global oceanic N_2 fixation, it is important to estimate their abundance while simultaneously expressing their specific diazotrophic activity. So far 2 groups of techniques have been used on marine diazotrophs to identify and quantify cells while expressing their N_2-fixing activities. The first one uses quantitative PCR (qPCR) on *nif*H genes and transcripts, which code for nitrogenase (Zehr et al. 2001, Falcón et al. 2004a), and the second one uses transmission electronic microscopy (TEM) associated with nitrogenase immunolocalization (Falcón et al. 2004b, Foster et al. 2006a). Recently Mazard et al. (2004) designed a PCR 16S rDNA primer (Nitro821R coupled with a general cyanobacteria primer Cya359F) to specifically target a group of unicellular diazotrophic cyanobacteria (i.e. UCN_2-Fix lineage). This was possible as *nif*H is one of the oldest functional genes and has coevolved with 16S rDNA (Hennecke et al. 1985). Therefore phylogenetic trees based on 16S and *nif*H genes have strong similarity (Zehr et al. 2003). With this novel approach in the field of unicellular diazotrophs, Mazard et al. (2004) successfully detected and quantified the targeted cells along an oceanic transect in the Arabian Sea.

In the present study the use of Nitro821R primer was first tested as a fluorescent probe on unicellular cyanobacterial cultures and environmental samples using the TSA-FISH (Tyramide Signal Amplification of Fluorescent *In Situ* Hybridization) technique. In order to find out if the Nitro821-targeted cells could be active diazotrophs, cell abundance was compared with $^{15}N_2$-fixing activity from different size fractions. A second aspect of our work was to investigate whether the nutrient gradient from Noumea's lagoon influences the abundance and distribution of unicellular diazotrophic cyanobacteria. Finally, by processing different size fractions, we described whether the cells were free living or associated with particles or organisms. Two size fractions were collected, >10 µm and 3 to 10 µm, for filamentous and nanoplanktonic cells, respectively. The filtrates from the 3 µm filter were also collected on a 0.2 µm pore size filter as a negative control for the Nitro821 probe, as this probe was first suspected to target only nanoplanktonic unicellular cyanobacteria.

MATERIALS AND METHODS

Strains, cultivation, study site and sampling. To test probe and primer specificity, 3 non-axenic cyanobacterial strains — *Cyanothece* sp. (PCC 8801), *Gleocapsa* sp. (PCC 73106) and *Synechocystis* sp. (PCC 6803) — were grown in culture media BG11, at 28°C, under a light:dark cycle of 12:12 h. Natural samples were collected with 5 l Niskin bottles within 1 wk (from 21 to 27 October 2005) inside and outside Noumea's coral

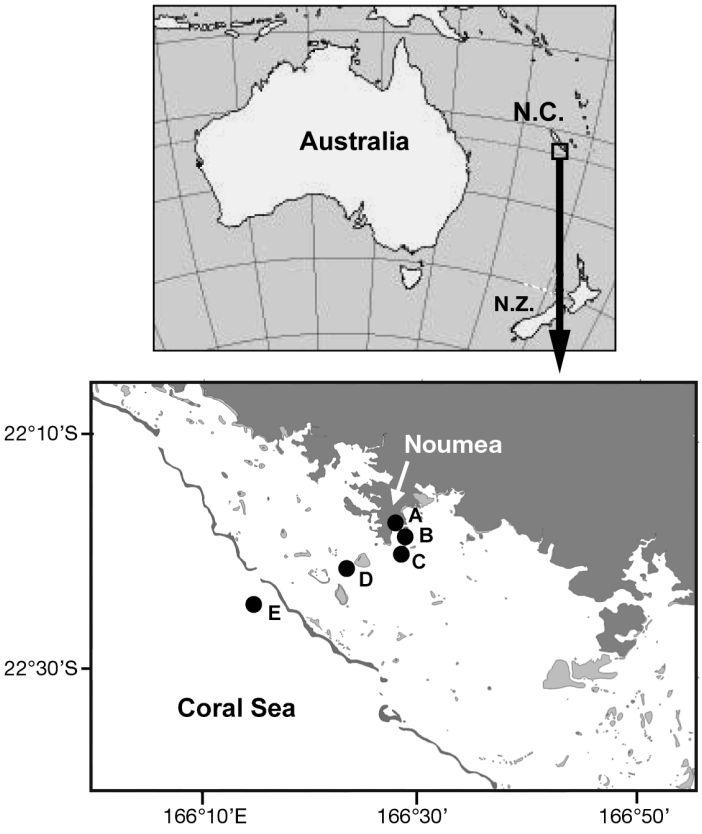

Fig. 1. Sampling stations (A to E) in Noumea's southwest coral lagoon (New Caledonia, N.C., France, Southwest Pacific; N.Z.: New Zealand). Dark grey means permanently emerged coral reef or land and light grey shows the presence of totally or temporarily immersed coral reef

lagoon (New Caledonia, France, Southwest Pacific) at 5 different stations (Fig. 1): A (22° 17′ S, 166° 28′ E), B (22° 19′ S, 166° 28′ E), C (22° 20′ S, 166° 28′ E), D (22° 21′ S, 166° 23′ E) and E (22° 22′ S, 166° 13′ E), following a nutrient and chl *a* gradient ranging from 0.3 to 0.03 µmol NH_4^+ l^{-1} and 3 to 0.3 µg chl *a* l^{-1} (Jacquet et al. 2006). The most eutrophic Stns A and B are located in the middle of Sainte Marie's bay, 1.5 km away from land, and just outside the bay, respectively. Sainte Marie's bay is not influenced by any direct release of sewage, but receives small polluted rivers and city runoff waters. Temperature and salinity were measured with a profiler (CTD SBE 19, Seabird) and were ranged along the nutrient gradient from 23 to 25 and 35.6 to 35.9°C, respectively. For Stns D and E, CTD profiles were homogeneous throughout the water column, when weak stratifications were observed at Stns A, B and C.

For TSA-FISH experiments (Figs. 2, 3 & 4), water samples were collected at 3 different depths when possible (0.5, 10 or 20 m, Fig. 4). For N_2-fixation experiments over 24 h (Fig. 5), samples were taken at Stns B, C, D and E at 3 different depths (0.5, 10 and 20 m), apart from Stn E where only subsurface water was sampled (0.5 m). For additional N_2-fixation experiments over the daylight period (12 h), samples were taken only at Stns C and D at 3 depths (Fig. 5). Apart from the 12 h incubation experiment on Stn D all other samples for both TSA-FISH and $^{15}N_2$ experiments were collected simultaneously.

TSA-FISH. Before hybridization, cyanobacterial cultures were collected on 0.2 µm pore-size ISOPORE™ 47 mm diameter membrane filters (Millipore), and water samples were size fractionated. According to oligotrophic status, filters were more or less quickly saturated and different volumes of waters were filtered through the 10 or 3 µm membranes by gravity. The amount of seawater to be filtered was defined by changes of flow through membranes. Between 400 ml and 4 l were filtered by gravity through 10 µm ISOPORE™ 47 mm filters. Between 200 ml and 1 l of filtrate were then collected by gravity on 3 µm ISOPORE™ 47 mm filters. Finally, 200 ml of filtrate were collected under 200 mm Hg vacuum on 0.2 µm ISOPORE™ 47 mm filters. Once collected, cells were fixed with 1% paraformaldehyde (pH 7.2, buffered with PBS and clarified by filtration, Sigma-Aldrich) at room temperature for 15 min. Cells were subsequently dehydrated with 100% EtOH molecular grade at room temperature for 10 min and stored at −80°C or in liquid nitrogen until further analysis.

Hybridizations were done according to Biegala et al. (2002) with slight modifications. Probes (Table 1) were purchased HRP (Horse Radish Peroxidase) labeled (Thermo Electron Corporation). Before hybridization, filters were thawed and quickly dipped in warm 0.07% agarose (1500 g cm^{-2}, Eurogentec) and dried at 37°C for 10 min to avoid cell loss. Cells on filters were either stored back at −80°C or further processed. To allow

Table 1. Oligonucleotide probes used in the present study

Probe	Target	Sequence (5′–3′)	Source
Nitro821	16S rRNA in unicellular cyanobacterial diazotroph	CAA GCC ACA CCT AGT TTC	Mazard et al. (2004)
Cya664	16S rRNA in most marine cyanobacteria	GGA ATT CCC TCT GCC CC	Schönhuber et al. (1999)
Eub338	16S rRNA in most bacteria	GCT GCC TCC CGT AGG AGT	Amann et al. (1990)

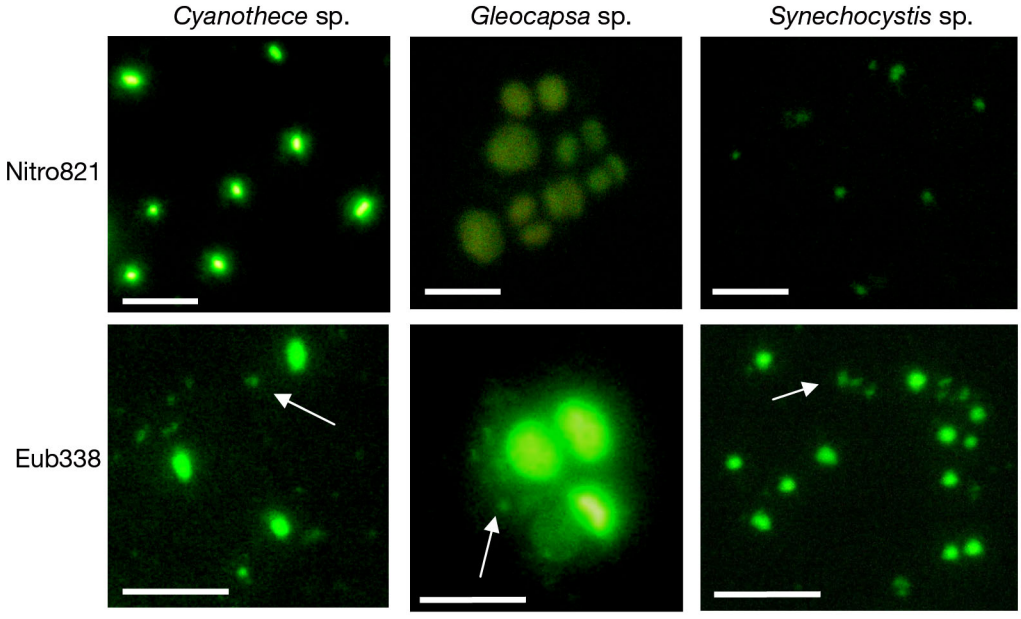

Fig. 2. Cyanobacterial cultures *Cyanothece* sp. (PCC 8801), *Gleocapsa* sp. (PCC 73106) and *Synechocystis* sp. (PCC 6803) hybridized with either the Nitro821 probe, specific for unicellular diazotroph cyanobacterial cluster, or with the general bacterial probe Eub338 (Table 1). Hybridized cells were stained with FITC (green fluorescence) using the TSA-FISH technique. Arrows point to hybridized heterotrophic bacteria from non axenic cultures. Scale bars = 10 µm

HRP-labeled probes to reach their 16S rRNA target, cells were perforated by successive enzymatic incubations: in 10 mg ml^{-1} lysozyme (47 000 U mg^{-1}, Sigma-Aldrich) at 37°C for 1 h and in 60 U ml^{-1} acromopeptidase (3200 U mg^{-1}, Sigma-Aldrich) at 37°C for 30 min. Each enzyme treatment was followed by 3 rinses in Milli-Q sterile water, 10 min in 100% EtOH and drying at 37°C. At the latter step, cells could be either stored back at –80°C or processed for hybridization steps and TSA reactions. To check empiric Nitro821 probe specificity, a range of stringent hybridization conditions was tested on cyanobacteria cultures (hybridization temperature and salt concentrations were kept constant, when formamide concentration varied from 20 to 65%, data not shown). The best compromise between fluorescence intensity from positive hybridization and probe specificity was achieved at 50% of formamide concentration in the hybridization buffer. This stringent condition was then used for hybridizations on cultures and natural samples (Figs. 2 & 3).

Microscopy and cell counts. Images were acquired with an epifluorescence BX61 microscope (Olympus Optical) equipped with a mercury lamp (HBO 100W/2, Osram), 2 objectives — 10× (NA 0.3 N Plan Fluor DT: 10 mm, Olympus) and 40× (NA 0.75 N Plan Fluor PH2 DT: 0.51 mm, Olympus) — excitation (ex.) and emission (em.) dichroïc filters (360 ± 20 ex., 410 ± 5 em. for the DAPI [blue fluorescence], 480 ± 20 ex., 510 long pass em. for the FITC associated with Nitro821 probe [green fluorescence] and for the remaining chlorophyll fluorescence [orange fluorescence], 540 ± 15 ex., 605 ± 25 short pass em. for the CY3 associated with Cya664 probe [red fluorescence]) and a digital camera (Coolpix 5400, Nikon, Figs. 1 & 2). Differences between FITC-labeled cells and remaining autofluorescence of phytoplanktonic cells were checked under a different dichroïc filter (480 ± 20 ex., 535 ± 40 short pass em.), which removed the entire orange- or red-emitted wavelength and allowed only the green stain to be seen.

The entire surface of each filter portion was counted, corresponding to 20–25 microscopic fields. According to oligotrophic conditions, filter surfaces corresponded to 7–12 ml of water for the 0.2 to 3 µm size fraction, 9–56 ml for 3 to 10 µm and 27–250 ml for >10 µm. For all 3 size fractions, diazotroph counts were acquired with the 40× objective. A second count was done with the 10× objective on the 3 to 10 µm and >10 µm size fractions to count only nanoplanktonic unicellular diazotrophs. At that magnification picoplanktonic cells were not seen. Picoplanktonic cell counts were obtained by subtracting counts (Fig. 4).

Nitrogen fixation. Nitrogen-fixation rates were determined on prefiltered (<10 µm or >10 µm) seawater using the high-sensitive $^{15}N_2$ tracer method (Fig. 5) as described by Garcia et al. (2007). The detection limit of the $^{15}N_2$ tracer method was estimated at 0.3 nmol l^{-1} 24 h^{-1}. The day and night incubations were

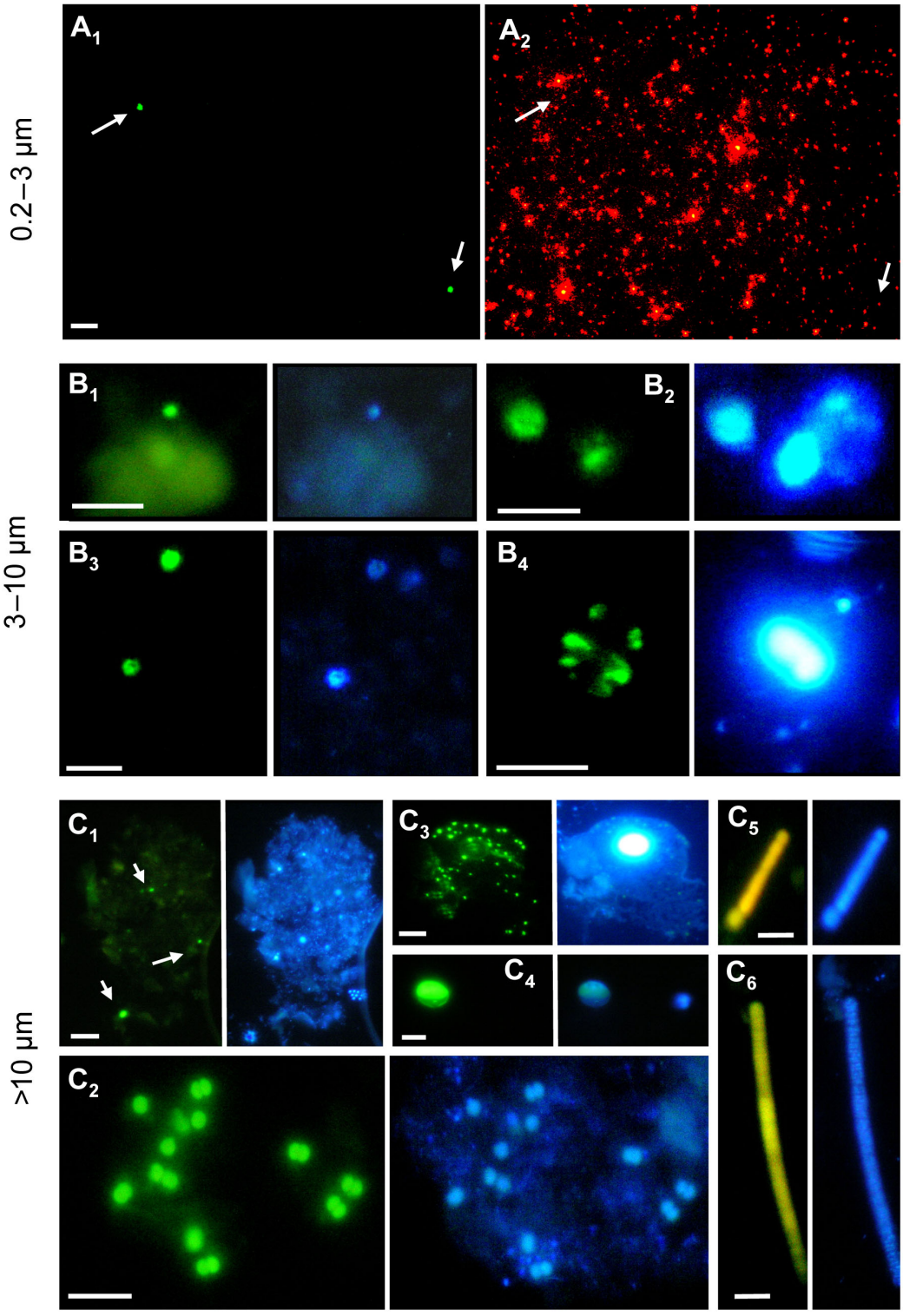

Fig. 3. Different size-fractionated plankton hybridized with the Nitro821 probe, specific for unicellular cyanobacterial diazotroph clusters (A_1, B_1 to C_4) (Table 1), or with the general cyanobacterial probe (A_2: Cya664) (Table 1). (C_5, C_6) Orange autofluorescence from filamentous cyanobacteria *Richelia* sp. (C_5) and *Trichodesmium* sp. (C_6), not hybridized by Nitro821. Hybridized cells were labeled with FITC (green fluorescence) or CY3 (red fluorescence) using the TSA-FISH technique. Corresponding micrographs in blue fluorescence showing cells DNA-stained with DAPI. Unicellular diazotrophs were either free living (A_1, B_2, B_3 & C_4), associated with inert particles (B_1, C_1, C_2) or intracellular of dinoflagellates (B_2 cell on the right; B_4, C_3). Arrows point to picoplankton cyanobacteria diazotrophs either free living (A_1, A_2) or attached to inert particles (C_1). Scale bars = 10 µm

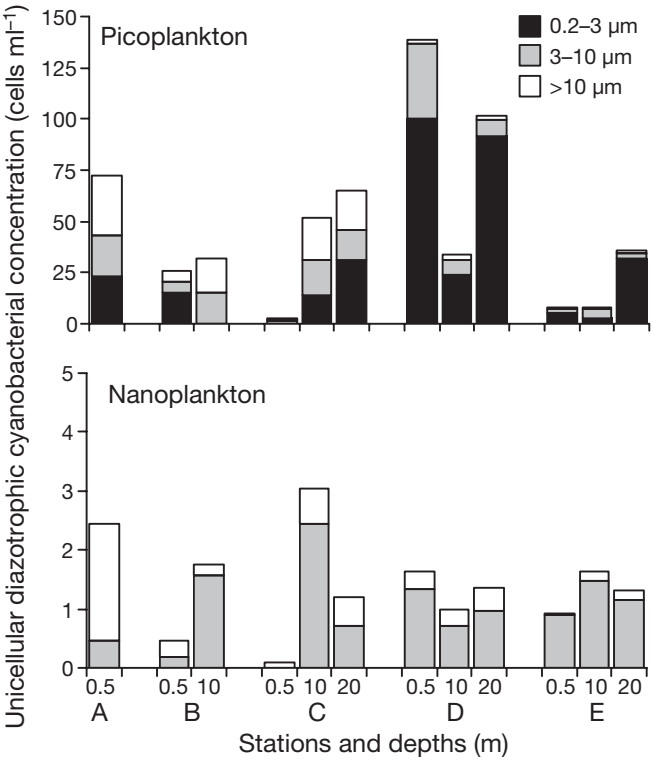

Fig. 4. Cell concentration of Nitro821-targeted unicellular cyanobacterial diazotrophs from pico- or nanoplanktonic sizes. Counts were recovered from 3 size fractions (0.2 to 3 µm, 3 to 10 µm and >10 µm) and 3 depths (0.5, 10 and 20 m), when possible, along a nutrient gradient from Noumea's coral lagoon (see Fig. 1)

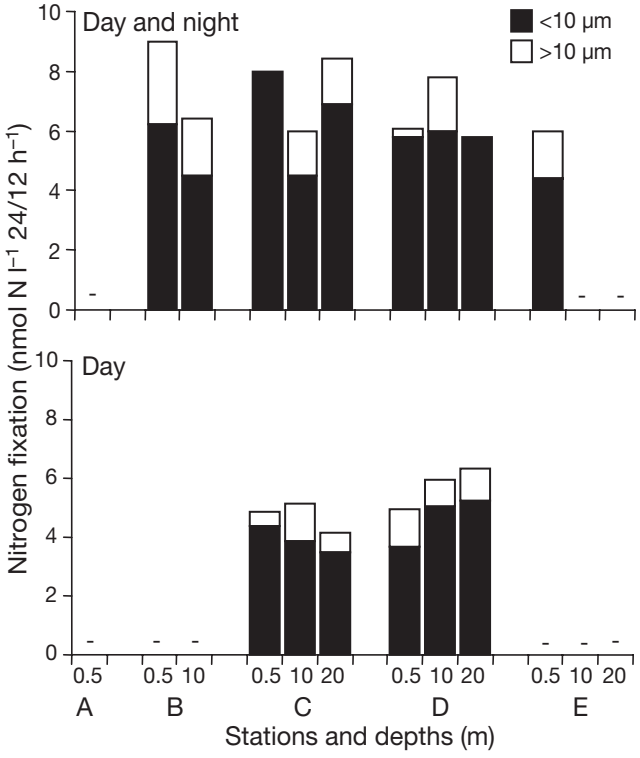

Fig. 5. Nitrogen fixations measured within different size fractions (<10 µm and >10 µm) at Stns B, C, D and E (see Fig. 1) from different depths (0.5, 10 and 20 m). Incubations were done over 24 h (day and night) for all stations and also during the daylight period (12 h, from sunrise to sunset) for Stns C and D. –: depths not sampled

done over 24 h, when the daylight experiment was run for 12 h from sunrise to sunset. Incubations were performed in simulated environmental conditions in an opaque incubator equipped with a light screen which allowed 50% light penetration.

RESULTS

Probe specificity

To test the specificity of the Nitro821 probe (Table 1), which targets unicellular cyanobacterial diazotrophs, 3 non-axenic unicellular cyanobacteria cultures (*Cyanothece* sp., *Gleocapsa* sp. and *Synechocystis* sp.) were used. Among the 3 strains, only the *nif*H containing *Cyanothece* sp. was successfully hybridized by the Nitro821 probe (Fig. 2). A positive control for hybridization was done using the general Bacteria probe Eub338 (Table 1). All 3 cyanobacterial strains, as well as heterotrophic bacteria, showed a positive signal with the Eub338 probe (Fig. 2).

In the natural environment the Nitro821 probe hybridized to unicellular cyanobacteria from an unexpectedly wide range of cell sizes (1 to 1.5 µm and up to 13 µm, Fig. $3A_1$–C_4), while the filamentous cyanobacteria *Richelia* sp. and *Trichodesmium* sp. showed only remnants of natural orange autofluorescence as they were not hybridized by the Nitro821 probe (Fig. $3C_5$,C_6). To confirm that the Nitro821 probe targets were cyanobacteria, a double hybridization experiment was done on 3 size fractions, with the general Cya664 probe, specific for cyanobacteria (Table 1). Double hybridization was positive for every Nitro821 probe target (data shown only for the picoplankton size fraction 0.2 to 3 µm, Fig. $3A_1$,A_2).

Cell abundance

In Noumea's coral lagoon, the abundance of Nitro821 probe targets ranged from 3 to 140 cells ml^{-1} along the nutrient gradient (Fig. 4), while hardly any larger filamentous diazotrophs (*Trichodesmium* sp. and *Richelia* sp.) were observed (Fig. $3C_5$,C_6). Picoplanktonic (<3 µm) cyanobacteria largely dominated the targeted community, representing 97% of the total abundance. These small cells were recovered in all

size fractions investigated. The remaining 3% were nanoplanktonic (>3 µm), two-thirds of them were in the 3 to 10 µm fraction and one-third in the >10 µm fraction (Fig. 4). Among the picoplankton cyanobacterial diazotrophs, 63% were free living and 37% were associated to inert particles (Fig. $3B_1$,C_1) or intracellular of eukaryotes (Fig. $3B_2$,B_4,C_3). These organisms were mainly dinoflagellates, which were easily recognized by their high DNA content (DAPI micrographs in Fig. $3B_2$,B_4,C_3) and condensed chromosomes (data not shown). Within dinoflagellates, targeted cells had different shapes and sizes, either grouped in clusters around the nucleus (Fig. $3B_4$) or spread all over the eukaryote's cytoplasm (Fig. $3C_3$). Up to 60 cells were detected within a dinoflagellate. The distribution of picoplanktonic cyanobacterial diazotrophs was heterogeneous in the water column and along the nutrient gradient, reaching a maximum and a minimum in the middle and outside Noumea's lagoon, respectively (Stns D and E, Figs. 1 & 4).

Diazotrophic activity

Results from the ^{15}N-tracer experiments showed significant nitrogen fixation in the <10 µm fraction, which was 4 times higher than the >10 µm fraction (Fig. 5). This was true whether the rates were measured over 24 h incubation or over 12 h, from sunrise to sunset (Fig. 5). Although the 12 and 24 h incubations were done simultaneously only for Stn C and with 1 d delay for Stn D, N_2 fixation by day was high (average 5.3 nmol N l^{-1} 12 h^{-1}, n = 12) and could explain 78% of the average activity measured over 24 h (6.8 nmol N l^{-1} 24 h^{-1}, n = 12). Along the nutrient gradient, slightly higher rates were measured over 24 h at nutrient-rich Stns B and C (4.5 to 8 nmol N l^{-1} 24 h^{-1}) compared to oligotrophic Stns D and E (4.4 to 6 nmol N l^{-1} 24 h^{-1}). Diazotrophic activity did not correlate with the Nitro821-targeted cell number from both the <10 µm and >10 µm size fractions.

DISCUSSION

In the present study we confirmed that the Nitro821 probe was specific to aerobic unicellular diazotrophic cyanobacteria, such as *Cyanothece* sp. PCC 8801 (also called *Synechococcus* sp. RF–1), and to other *Crocosphaera*-like cells recovered from the natural environment (Zehr et al. 2001, Fig. $3B_3$,C_2). As predicted, the Nitro821 probe did not label cultures of pico- and nanoplanktonic non-diazotrophic cyanobacteria (*Gleocapsa* sp. and *Synechocystis* sp., Fig. 2), nor filamentous diazotrophic cyanobacteria from Noumea's lagoon (*Trichodesmium* sp. and *Richelia* sp., Fig. $3C_5$,C_6). Furthermore, the general cyanobacterial probe Cya664 double labeled the Nitro821-targeted cells (Fig. $3A_1$,A_2). Since Mazard et al. (2004)'s first description, the specificity of the Nitro821 probe was checked over the entire 16S and 18S rDNA GenBank database and remained stable. The probe showed several mismatches with heterotrophic Bacteria or Archaea and marine filamentous cyanobacteria. As such, it is unlikely that Nitro821-labeled picocyanobacteria would be spores or baeocytes from benthic filamentous cyanobacteria or heterotrophic picodiazotrophs.

The population of cells targeted by the Nitro821 probe, specific for the unicellular diazotrophic cyanobacteria, belonged essentially to the picoplanktonic size fraction. This result was surprising as the *nif*H gene has never been recovered from the marine picocyanobacteria *Prochlorococcus* sp. or *Synechococcus* sp. (Palenik et al. 2003, Dufresne et al. 2005). Both genera are known to be abundant in the Southwest Pacific oligotrophic waters (Campbell et al. 2005, Jacquet et al. 2006). To support the presence of pico- and nanoplanktonic unicellular diazotrophs in Noumea's coral lagoon, a significant N_2 fixation was measured in the <10 µm fraction (Fig. 5). Similar rates of N_2 fixation from the <10 µm fraction have previously been measured in adjacent oceanic waters at similar times of the year (Garcia et al. 2007), from the Northern Pacific, Arafura Sea and Northern Atlantic (Falcón et al. 2004a, Montoya et al. 2004, Zehr et al. 2007). These rates were assigned to diazotrophic unicellular cyanobacteria from Groups A, B or C and to a lesser extent heterotrophic Proteobacteria. In addition, it is interesting to stress that 78% of the $^{15}N_2$ fixation was done during the daylight period, at least for Stns C and D (Fig. 5). However, all known nanoplanktonic diazotrophic cyanobacteria affiliated to Groups B or C are diazotrophically active at night in the natural environment, when Group A and heterotrophic bacteria have been shown to express their *nif*H at day (Church et al. 2005, Foster et al. 2006b). As the newly discovered Nitro821-labeled picocyanobacteria numerically dominated the <10 µm fraction (Fig. 4), it is probable that they contributed to the daylight N_2 fixation from this small fraction. However, changes in N_2 fixation and Nitro821-targeted cell abundance were not correlated. This may reveal either the presence of organisms not targeted by the Nitro821 probe, such as Proteobacteria, and/or important single-cell variability in N_2-fixing activity. This latter hypothesis is probable as a lack of correlation between *nif*H copy and transcript numbers have been shown previously within cyanobacterial Groups A and B (Zehr et al. 2007). The high numbers of Nitro821-labeled picocyanobacteria in all size fractions

and the significant daylight $^{15}N_2$ fixation allow us to hypothesize that the newly discovered picocyanobacteria from the present study could be affiliated to Group A. Group A diazotrophic cyanobacteria were recently suspected to belong to the picoplanktonic size fraction and to have their 16S rRNA amplified by Nitro821 and Cya359 primers (Mazard et al. 2004, Zehr et al. 2007).

The picoplanktonic Nitro821-targeted cells reached a maximum of 10^2 cells ml^{-1}, which makes them difficult to detect among the non-diazotrophic picocyanobacteria which are major contributors to the lagoon chl *a* (10^4 to 10^5 cells ml^{-1}, Figs. $3A_2$ & 4) (Jacquet et al. 2006). The concentration of fluorescently labeled picocyanobacteria agrees with the concentration of *nif*H copy numbers of unicellular cyanobacteria from various oceanic and coastal waters, including Australian coral lagoon (Mazard et al. 2004, Zehr et al. 2007, Hewson et al. 2007). Although picocyanobacteria were numerically dominating the diazotrophic cyanobacterial community at every station, their highest concentration was detected at the most oligotrophic station from the lagoon (Stn D, Figs. 1 & 4), where DIN and chl a concentrations were typically 0.2 µmol l^{-1} and 0.25 µg l^{-1}, respectively (Jacquet et al. 2006). This high concentration was expected as diazotrophs can sustain their nitrogen needs by fixing dissolved N_2 from the atmosphere, when other plankters will be limited by these oligotrophic conditions. However, similar oligotrophic conditions are present outside the reef barrier (Stn E, 0.2 µmol DIN l^{-1} and 0.2 µg chl *a* l^{-1}) (Jacquet et al. 2006), but low concentrations of diazotrophic picocyanobacteria were detected. Both stations can receive sporadic nutrient inputs either from Noumea city and nearby river discharge (Stn D) or from local barrier reef upwelling (Stn E). These sources of enrichment are mainly driven by wind force and direction and do not necessarily occur simultaneously (Jouon et al. 2006). These inputs might carry phosphorus, which is important for diazotrophic cyanobacterial growth (Mills et al. 2004). Beside a bottom-up control of the unicellular cyanobacterial diazotrophs, a top-down control of this community by predation activity may contribute to local changes in Nitro821-targeted cell concentration.

Among the diazotrophic picocyanobacteria we show that one-third of their concentration was associated with inert particles or intracellular of dinoflagellates within the 3 to 10 µm and >10 µm size fractions. Most of these associations were recovered in the nutrient-rich Stns A, B and C (Fig. 4). This is probably because these stations have a higher concentration of particulate organic matter than the most oligotrophic ones (Jacquet et al. 2006). The cells are either passively trapped on inert particles (Fig. $3B_1$,C_1) or may be chemically attracted towards these microenvironments as they may have found essential nutrients for growth, such as phosphorus and biologically available iron. Both elements have been shown to control diazotrophic activity (Mills et al. 2004). Nitro821-labeled cells have also frequently been observed intracellular of dinoflagellates, with up to 60 targeted cells spread over a eukaryote cytoplasm (Fig. $3C_3$). Based on the fact that a high fluorescent signal was recovered with the rRNA-targeting Nitro821 probe, it is argued that cells were in a good physiological state at the time of sampling and were probably cyanobionts. Similar signal intensity has been previously detected in eukaryotes harboring intra-cytoplasmic or intra-nuclear prokaryote symbionts (Alverca et al. 2002, Biegala et al. 2005). Non-thecate dinoflagellates are abundant in Noumea's lagoon (Jacquet et al. 2006) and cyanobionts from pico- and nanoplanktonic sizes were recently revealed in *Histoneis* sp., a tropical non-thecate dinoflagellate (Foster et al. 2006a). Foster et al. (2006a,b) demonstrated that nitrogenase was expressed at night at least in one nanoplanktonic cell, which looked like *Cyanothece* sp. ATCC51142. The present study provides the first evidence of potential N_2-fixing picoplanktonic cyanobionts (Fig. $3C_3$).

In conclusion, the use of whole-cell hybridization of specific Nitro821 fluorescent probes proved to be a useful technique in the field of marine nitrogen fixation by revealing for the first time the presence of diazotrophic picocyanobacteria. In Noumea's lagoon these picocyanobacteria were numerically dominating the community of cyanobacterial diazotrophs. They were mainly free-living organisms but also associated to particles and probably symbionts of non-thecate dinoflagellates. In addition, they were present at high concentrations over a large range of nutrient levels during austral spring 2005 when other planktonic diazotrophs were not abundant. Further studies will be necessary to prove the identity of these Nitro821-targeted picocyanobacteria and quantify their specific contribution to global N_2 fixation.

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LITERATURE CITED

Alverca E, Biegala IC, Kennaway GM, Lewis J, Franca S (2002) *In situ* identification and localization of bacteria associated with *Gyrodinium instriatum* (Gymnodiniales, Dinophyceae) by electron and confocal microscopy. Eur J Phycol 37:523–530

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 56: 1919–1925

Biegala IC, Kennaway G, Lennon JF, Vaulot D, Simon N (2002) Identification of bacteria associated with dinoflagellates (Dinophyceae) *Alexandrium* spp. using tyramide signal amplification-fluorescent *in situ* hybridization and confocal microscopy. J Phycol 38:404–411

Biegala IC, Cuttle M, Mary I, Zubkov MV (2005) Hybridization of picoeukaryotes by eubacterial probes is widespread and may introduce bias in biodiversity researches or reveal intimate relationship between both types of organisms. Aquat Microb Ecol 41:293–297

Burgess BK, Lowe DJ (1996) Mechanism of molybdenum nitrogenase. Chem Rev 96:2983–3011

Campbell L, Carpenter EJ, Montoya JP, Kustka AB, Capone DG (2005) Picoplankton community structure within and outside a *Trichodesmium* bloom in the southwerstern Pacific Ocean. Vie Milieu 55:185–195

Capone DG, Carpenter EJ (1999) Nitrogen fixation by marine cyanobacteria: historical and global perspectives. In: Charpy L, Larkum A (eds) Marine cyanobacteria. Institut Océanographique, Monaco, p 235–256

Capone DG, Zehr JP, Paerl HW, Bergman B, Carpenter EJ (1997) *Trichodesmium*, a globally significant marine cyanobacterium. Science 276:1221–1229

Carpenter EJ, Montoya JP, Burns J, Mulholland MR, Subramaniam A, Capone DG (1999) Extensive bloom of a N_2-fixing diatom/cyanobacterial association in the tropical Atlantic Ocean. Mar Ecol Prog Ser 185:273–283

Church MJ, Short CM, Jenkins BD, Karl DM, Zehr JP (2005) Temporal patterns of nitrogenase gene (*nif*H) expression in the oligotrophic North Pacific Ocean. Appl Environ Microbiol 71:5362–5370

Dufresne A, Garczarek L, Partensky F (2005) Accelerated evolution associated with genome reduction in a free-living prokaryote. Genome Biol 6:R14

Falcón LI, Carpenter EJ, Cipriano F, Bergman B, Capone DG (2004a) N_2 fixation by unicellular bacterioplankton from the Atlantic and Pacific Oceans: phylogeny and *in situ* rates. Appl Environ Microbiol 70:765–770

Falcón LI, Lindwall S, Bauer K, Bergman B, Carpenter EJ (2004b) Ultrastructure of unicellular N_2 fixing cyanobacteria from the tropical North Atlantic and subtropical North Pacific Oceans. J Phycol 40:1074–1078

Foster RA, Carpenter EJ, Bergman B (2006a) Unicellular symbionts in open ocean dinoflagellates, radiolarians, and tintinnids: ultrastructural characterization and immunolocalization of phycoerythrin and nitrogenase. J Phycol 42: 435–463

Foster RA, Collier JL, Carpenter EJ (2006b) Reverse transcription PCR amplification of cyanobacterial symbiont 16S rRNA sequences from single non-photosynthetic eukaryotic marine planktonic host cells. J Phycol 42:243–250

Garcia N, Raimbault P, Sandroni V (2007) Nanoplanktonic diazotrophy and trophic transfer of newly fixed nitrogen in oceanic and lagoonal waters off Southwest Pacific (New Caledonia). Mar Ecol Prog Ser 343:25–33

Gruber N, Sarmiento JL (1997) Global patterns of marine nitrogen fixation and denitrification. Global Biogeochem Cycles 11:235–266

Hennecke H, Kaluza K, Thöny B, Fuhrman W, Ludwig W (1985) Concurrent evolution of nitrogenase genes and 16S ribosomal-RNA in *Rhizobium* species and other nitrogen fixing bacteria. Arch Microbiol 1142:342–348

Herbert RA (1999) Nitrogen cycling in coastal marine ecosystems. FEMS Microbiol Rev 23:563–590

Hewson I, Moisander PH, Morrison AE, Zehr JP (2007) Diazotrophic bacterioplankton in a coral reef lagoon: phylogeny, diel nitrogenase expression and response to phosphate enrichment. ISME J 1:78–91

Jacquet S, Delesalle B, Torréton JP, Blanchot J (2006) Response of phytoplankton communities to increased anthropogenic influences (south-western lagoon, New Caledonia). Mar Ecol Prog Ser 320:65–78

Jouon A, Douillet P, Ouillon S, Fraunié P (2006) Calculations of hydrodynamic time parameters in a semi-open coastal zone using a 3D hydrodynamic model. Cont Shelf Res 26:1395–1415

Karl D, Letellier R, Tupas L, Dore J, Christian J, Hebel D (1997) The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. Nature 388:533–538

Mazard SL, Fuller NJ, Orcutt KM, Bridle O, Scanlan D (2004) PCR analysis of the distribution of unicellular cyanobacterial diazotrophs in the Arabian sea. Appl Environ Microbiol 70:7355–7364

Mills MM, Ridame C, Davey M, La Roche J, Geider RJ (2004) Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. Nature 429:292–294

Montoya JP, Holl CM, Zehr JP, Hansen A, Villareal TA, Capone DG (2004) High rates of N_2 fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. Nature 430:1027–1031

Palenik B, Brahamsha B, Larimer FW, Land M and others (2003) The genome of a motile marine *Synechococcus.* Nature 424:1037–1042

Schönhuber W, Zarda B, Eix S, Rippka R, Herdman M, Ludwig W, Amann R (1999) *In situ* identification of cyanobacteria with horseradish peroxidase-labeled, rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 65:1259–1267

Zehr JP, Waterbury JB, Turner PJ, Montoya JP and others (2001) Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. Nature 412:635–638

Zehr JP, Jenkins BD, Short S, Steward GF (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ Microbiol 5:539–554

Zehr JP, Montoya JP, Jenkins BD, Hewson I and others (2007) Experiments linking nitrogenase gene expression to nitrogenase fixation in the North Pacific subtropical gyre. Limnol Oceanogr 52:169–183

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