Biogeosciences, 8, 827–840, 2011
www.biogeosciences.net/8/827/2011/
doi:10.5194/bg-8-827-2011

Biogeosciences

Intriguing diversity among diazotrophic picoplankton along a Mediterranean transect: a dominance of rhizobia

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Received: 22 November 2010 – Published in Biogeosciences Discuss.: 2 December 2010
Revised: 21 March 2011 – Accepted: 22 March 2011 – Published: 29 March 2011

Abstract. The Mediterranean Sea is one of the most oligotrophic marine areas on earth where nitrogen fixation has formally believed to play an important role in carbon and nitrogen fluxes. Although this view is under debate, the diazotrophs responsible for this activity have still not been investigated in the open sea. In this study, we characterised the surface distribution and species richness of unicellular and filamentous diazotrophs across the Mediterranean Sea by combining microscopic counts with size fractionated in situ hybridization (TSA-FISH), and 16S rDNA and *nifH* genes phylogenies. These genetic analyses were possible owing to the development of a new PCR protocol adapted to scarce microorganisms that can detect as few as 1 cell ml^{-1} in cultures. Low concentrations of diazotrophic cyanobacteria were detected and this community was dominated at 99.9% by picoplankton hybridized to the Nitro821 probe, specific for unicellular diazotrophic cyanobacteria (UCYN). Among filamentous cyanobacteria only 0.02 filament ml^{-1} of *Richelia* were detected in the eastern basin, while small (0.7–1.5 µm) and large (2.5–3.2 µm) Nitro821-targeted cells were recovered at all stations with a mean concentration of 3.5 cell ml^{-1}. The affiliation of the small Nitro821-targeted cells to UCYN-A was confirmed by 16S and *nifH* phylogenies in the western Mediterranean Sea. In the central and the eastern Mediterranean Sea no 16S rDNA and *nifH* sequence from UCYN was obtained as cells concentration were close to, or below PCR detection limit. *Bradyrhizobium* sequences dominated *nifH* clone libraries from picoplanktonic size fractions. A few sequences of γ-proteobacteria were also detected in the central Mediterranean Sea. While low phosphate and iron concentrations could explain the absence of *Trichodesmium* sp., the factors that prevent the development of UCYN-B and C remain unknown. We also propose that the dominating picoplankters probably developed specific strategies, such as associations with protists or particles, and/or photosynthetic activity, to acquire carbon for sustaining diazotrophy.

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1 Introduction

The Mediterranean Sea has long been recognized for the strong oligotrophy of its waters, with a clear decrease in nutrient concentration and primary productivity from west to east (Moutin and Raimbault, 2002). Deep Mediterranean waters are characterized by unusually high N:P ratios compared to Redfield (Béthoux and Copin-Montégut, 1986; Krom et al., 1991). The processes that induce such atypical ratios are under debate, and two hypotheses have been proposed, involving either significant diazotrophic activity, or low denitrification rates in combination with external inputs of nutrients (Béthoux and Copin-Montégut, 1986; Krom et al., 2010). Direct N_2 fixation measurements conducted in the eastern and western Mediterranean basins have shown mainly low diazotrophic activity with punctual peaks at different sites or seasons (0.01–129 nmol N L^{-1} d^{-1}, Rees et al., 2006; Sandroni et al., 2007; Ibello et al., 2010). This heterogeneity in rates of N_2-fixation implies the need of high frequency surveys to fully integrate, over the long term, the role of diazotrophy in Mediterranean biogeochemical cycles. However, the importance of diazotrophy has been argued to be inconsistent with the known phosphate-starved conditions found in the Mediterranean Sea (Krom et al., 2004, 2010), as diazotrophic activity was demonstrated to be controlled by phosphate in areas dominated by *Trichodesmium* sp. (Saňudo-Wilhelmy et al., 2001; Moutin et al., 2005). *Trichodesmium* sp., a filamentous cyanobacterium, has been

regarded for a long time as the main marine N_2 fixer (Falkowski, 1997). This view has now changed because N_2-fixation measurements within small (<10 µm) and large (>10 µm) planktonic size fractions suggest that unicellular diazotrophs fix equally or more nitrogen than filamentous species (Montoya et al., 2004; Biegala and Raimbault, 2008). Due to their higher surface/volume ratios, small cells have been recognised to be better adapted to phosphate-limiting conditions than larger ones (Smith and Kalff, 1982; Falcón et al., 2005). This is coherent with past and recent Mediterranean observations (Trégouboff, 1957; Bar Zeev et al., 2008) which reported only low concentrations of filamentous cyanobacteria, while smaller diazotrophs were hypothesized to be the main planktonic N_2-fixers in this environment (Béthoux and Copin-Montégut, 1986). Acquiring information on the size, the diversity and the spatio-temporal distribution of diazotrophs is thus essential to improve our understanding of Mediterranean biogeochemical cycles.

To date significant diversity has been recovered among marine planktonic diazotrophs. While filamentous organisms include essentially *Trichodesmium* sp. and the diatom symbiont *Richelia intracellularis*, unicellular diazotrophs are highly diverse and embrace Cyanobacteria, Proteobacteria, and Archaea. So far, three groups of unicellular diazotrophic cyanobacteria (UCYN) have been reported in the literature, UCYN-A, B, and C (Zehr et al., 2001; Foster et al., 2007). While UCYN-B and C are nanoplanktonic cells (2 to 10 µm) closely affiliated to the cultivated strains *Crocosphaera watsonii* and *Cyanothece* sp., respectively (Church et al., 2005a; Foster et al., 2007), UCYN-A are of picoplanktonic size (0.7–1.5 µm, Biegala and Raimbault, 2008; Goebel et al., 2008) and so far uncultivated. In addition to being free-living, UCYN have also been suggested to produce mucilage, to attach to inert particles or to live in association with planktonic eukaryotes (Biegala and Raimbault, 2008; Bonnet et al., 2009). These observations imply that UCYN could also contribute to nitrogen fixation of the large size fraction, thus reinforcing their role in global diazotrophic activity.

In the Mediterranean Sea, only two coastal studies have revealed the presence of unicellular diazotrophs (Man-Aharonovich et al., 2007; Le Moal and Biegala, 2009). Organisms affiliated to UCYN-A, Proteobacteria, and Archaea were recovered in the south-eastern basin and were expressing their *nifH* gene, which encodes the dinitrogenase reductase enzyme involved in nitrogen fixation process (Man-Aharonovich et al., 2007). In the north-western Mediterranean Sea, putative pico and nanoplanktonic UCYN were detected and hypothesized to belong to UCYN-A and UCYN-B or C, respectively (Le Moal and Biegala, 2009). Their concentrations were low along a seasonal cycle (4.6 cell ml^{-1}) compared to abundance estimates from other oceanic basins (reviewed in Le Moal and Biegala, 2009; Moisander et al., 2010), except for summer abundances that reached 1900–5300 cell ml^{-1}. While UCYN and filamentous diazotrophic cyanobacteria have been investigated in coastal waters, their spatial distributions have never been characterized in the open Mediterranean Sea.

Different molecular approaches have been used in the literature to study (i) the concentrations and (ii) the species richness of diazotrophs. First, concentrations have been determined either with the help of the quantitative polymerase chain reaction (qPCR) of *nifH* copies or by direct microscopic counts of fluorescently labeled UCYN, using a specific 16S rDNA probe combined with tyramide signal amplification-fluorescence in situ hybridization (TSA-FISH, Church et al., 2005a; Biegala and Raimbault, 2008). It was possible to design a specific 16S oligonucleotide for UCYN (Nitro821, Mazard et al., 2004) as the co-evolution of both *nifH* and 16S rDNA genes has resulted in similar phylogenies, especially in the phylum Cyanobacteria (Rosado et al., 1998; Zehr et al., 2003). Compared to qPCR, the whole cell hybridization technique allows characterizing UCYN size and provides some information on their ecology such as their free living or associated life styles (Biegala and Raimbault, 2008). However, visual discrimination among UCYN-B and -C is not possible and requires complementary species richness analysis. Therefore, the species richness can be investigated in a second time using PCR and phylogenetic techniques on 16S rDNA and *nifH* genes. Such information is tricky to acquire for diazotrophs less concentrated than 10 cell ml^{-1}(Kirshtein et al., 1993; Mazard et al., 2004) as their DNA may be diluted too much during the different extraction steps. To increase the amount of target within extracted DNA, a nested approach using the *nifH* gene was introduced (Zani et al., 2000). However, several studies still reported the inability to detect low concentrations of diazotrophs at the DNA level (e.g. Zani et al., 2000; Man-Aharonovich et al., 2007), thus illustrating the need to develop an adapted PCR protocol for scarce microorganisms in this field of research.

Planktonic diazotrophic activity has long been attributed to cyanobacteria, as they can sustain this energetically expensive process via photosynthesis (Stewart, 1971). Conversely, non-cyanobacterial diazotrophs are considered unable to acquire an autonomous carbon source through photosynthesis, and have thus been neglected in biogeochemical studies (Madigan, 1995; Tyrell, 1999). However, this metabolic function has also recently been missing within UCYN-A (Zehr et al., 2008), although these picocyanobacteria can actively express *nifH* gene and can reach high concentrations (Church et al., 2005b; Moisander et al., 2010). Similar *nifH* expression patterns were measured among planktonic proteobacteria, for which phototrophy has not been discovered (Zehr et al., 2007; Riemann et al., 2010). These results underline the importance to characterize the entire diazotrophic community including Proteobacteria and Archaea to better understand their role into global nitrogen fixation.

In this study we used a combination of approaches (i) to assess the distribution of diazotrophic cyanobacteria using epifluorescence microscopy for filamentous cells and

size-fractionated TSA-FISH technique for the UCYN; (ii) to identify the species richness of UCYN as well as those of potential other non-cyanobacterial diazotrophs, by specific 16S and general *nifH* phylogenetic analyses; and (iii) to develop a protocol for PCR amplifying scarce organisms necessary for phylogenetic analyses.

2 Material and methods

2.1 Natural environment sampling and cultures

Two types of samples were used in this study, environmental samples to determine diazotroph distributions and species richness across the Mediterranean Sea, and a combination of cultures and environmental samples to develop a protocol for PCR amplifying scarce diazotrophs.

Mediterranean samples were collected during the oceanographic BOUM transect (Biogeochemistry of Oligotrophic to Ultra-Oligotrophic Mediterranean) in June–July 2008 onboard the R/V Atalante. For TSA-FISH assays, ten liters of water were sampled using Niskin bottles at 5 m depth from 13 stations across the oligotrophic gradient (Fig. 1). At stations A, B, and C (Fig. 1), two nutrient contrasted depths were sampled: an oligotrophic one at 12.5 m and a nutrient rich one at the upper deep chlorophyll maximum (DCM^+) at 80 m, 120 m, and 100 m for each of the three stations, respectively. Cells collected at 12.5 m from stations A, B, and C were also used as DNA template for PCR reactions necessary for phylogenetic studies.

For methodological development, the strain *Crocosphaera watsonii* WH8501 was used to define the detection limit of 16S rDNA PCR amplification on unicellular diazotrophic cyanobacteria. The *C. watsonii* strain was graciously provided by T. Shi and was grown under 12/12 h light/dark conditions on a modified YBCII medium (Chen et al., 1996). Two milliliters of culture were fixed with 1% paraformaldehyde (PFA) and stored at −80 °C until further analyses inculding serial dilutions, counts by flow cytometry and PCR amplification. Environmental samples collected in different marine areas, such as samples M (Mediterranean Sea, 43° N; 5° E) and samples P (Pacific Ocean, 22° S, 166° E) were stored for one to five years at −80 °C to test the stability of PCR efficiency with time. Two types of DNA samples were used, either DNA from entire cells collected on filters following the same protocol as the one for TSA-FISH experiments (see below for TSA-FISH) or extracted DNA (see below PCR and cloning).

2.2 TSA-FISH and microscopy

Before TSA-FISH experiments, plankton from sea water samples were collected from three size fractions (0.2–3 µm, 3–10 µm, and >10 µm). Depending on the degree of oligotrophy, 1.3–8.2 liters were filtered by gravity through 10 µm

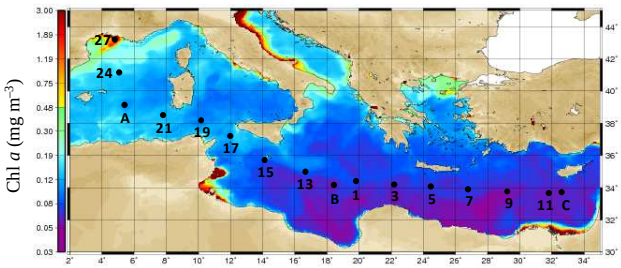

Fig. 1. Position of sampled stations during the BOUM transect across the Mediterranean Sea. Numbers (1–27) represent stations where surface TSA-FISH analyses were done and letters (A, B, and C) represent stations where TSA-FISH analyses were done at 12.5 m and DCM^+ as well as 16S and *nifH* phylogenies at 12.5 m.

ISOPORE^{TM} (Millipore, France) 47 mm filters, and 0.8–3.4 liters of the remaining filtrate were collected by gravity on 3 µm ISOPORE^{TM} (Millipore, France) 47 mm filters. Then, 200 mL of the <3 µm filtrate were collected under 200 mmHg vacuum on 0.2 µm ISOPORE^{TM} (Millipore, France) 47 mm filters. Cells were subsequently fixed with buffered 1% PFA for 15 min at room temperature (RT), dehydrated with 100% ethanol for 10 min at RT, and stored at −80 °C until analyses, according to Biegala and Raimbault (2008). Prior to hybridizations, filters containing fixed cells were covered with agarose to avoid cell loss during hybridization steps, according to Biegala and Raimbault (2008), with slight modification in Le Moal and Biegala (2009), where 0.4% agarose was used instead of 0.1% for the >10 µm size fraction. Hybridizations were done according to the protocol of Biegala et al. (2002), modified in Biegala and Raimbault (2008). Briefly, unicellular diazotrophic cyanobacteria were hybridized with the Horse Radish Peroxidase-labeled 16S rDNA Nitro821 probe (Thermo, Germany, 5′-CAA GCC ACA CCT AGT TTC-3′) which is specific for the UCYN lineage (Mazard et al., 2004), and subsequently stained in green with FITC (Fluorescein IsoThioCyanate, TSA-Kit Perkin Elmer, France). DNA from all prokaryotic and eukaryotic cells were counterstained with the specific blue DAPI dye (Sigma-Aldrich, France).

Photomicrographs and cells counts were done according to Biegala and Raimbault (2008). Briefly, entire surfaces of each filter portion (approx. 40 microscopic fields, 0.5 cm^2) were counted. These surface corresponded in mean to 250, 100 and 16 mL of water for the >10, the 3–10 and the 0.2–3 µm size fractions, respectively. This filtration protocol allows to detect either UCYN concentration as low as 0.08 to 0.004 cell ml^{-1} depending on size fractions, or filamentous cyanobacteria concentration >0.004 cell ml^{-1}. The homogeneity of Nitro821-hybridized cells on filters was confirmed by the low standard deviation obtained on triplicate counts done for station A, B, and C in the picoplanktonic size fraction (4.6 ± 0.6, 2.3 ± 0.4 and 2.3 ± 0.6 cell ml^{-1}

respectively). This homogeneity allowed us to define that the count of an entire filter portion was representative of the whole population for each sample station, and, only one replicate of entire filter portion was counted for each sample. Sizes of Nitro821-targeted cells were determined with the help of 1 µm calibration beads (Apogee Flow Systems, UK) according to Le Moal and Biegala (2009).

2.3 Flow cytometry

Before PCR assays, PFA fixed *C. watsonii* (0.5–800 cell ml^{-1}) were counted and isolated with the help of a MoFlo cell sorter (Beckman Coulter, Florida, USA) using a 488 nm laser for phycoerythrin and chlorophyll excitations and 580 ± 15 nm band pass and a 640 nm long pass filters for orange and red emission wavelengths of these pigments. Prior to flow cytometry analysis, cells were centrifuged to remove culture medium and subsequently diluted in MilliQ sterile water to provide a range of concentrations for the test of PCR detection limit. After flow cytometry, cells were collected directly in PCR tubes, in 27 µl, and stored at $-80\,^{\circ}C$ until the addition of PCR mixture. The detection limit of 1 cell ml^{-1} of *C. watsonii* corresponds to 12 cells per PCR reaction, according to UCYN counts realised on 0.2 µm pore-size filter portions from natural samples after TSA-FISH experiments.

2.4 PCR and cloning

DNA template used for PCR was not acquired through extraction but directly from entire fixed cells collected for TSA-FISH assays on 0.2–3 µm size fraction filters. The reason for this modification was the sparse concentration of environmental UCYN (2.3–4.6 cell ml^{-1}), which was two to three times lower than the detection limit of 7 cell ml^{-1} defined for techniques using DNA extraction (Mazard et al., 2004). Filter portions were incubated five times on polysulfone filter support (Millipore, France) in 200 µl of MilliQ sterile water for 5 min at room temperature. Between incubations water was discarded using a vacuum pump (200 mm Hg). The aim of these washing steps was to clean the fixed cells from a white film made of PFA, ethanol and sea salt. For long term stored samples (P5 and M4) dedicated to test the stability of PCR efficiency with time, extracted DNA was also used as PCR template, by filtering 4 L of waters on 0.2 µm pore size Supor filter (Pall, France) under 200-mmHg vacuum and subsequently following the DNA extraction protocol defined in Zehr and Turner (2001).

Diazotroph species richness was investigated by targeting both 16S rDNA and *nifH* genes. The 16S UCYN specific primer Nitro821 (Eurogentec, France, Table 1) was used in combination with Cya359 (Eurogentec, France, Table 1), according to the original protocol from Mazard et al. (2004), with a slight modification: 0.1 U of Taq polymerase (Promega, France) was used in PCR mixtures instead of 0.650 U. For *nifH* amplification, nested PCR was used with N3/N4 primers (Table 1) followed by N1/N2 primers (Eurofins MWG, Germany, Table 1), according to Zehr and Turner (2001). All PCR reactions were carried out in 50 µl volumes. The first PCR used fixed cells on filters as DNA template and the second PCR used 1 µl of DNA product from the first PCR. A filter with no cell was used as negative control for each environmental sample, to check the absence of contamination among samples, while all labware used to manipulate filters and PCR tubes were previously treated with a combination of bleach, UV radiation, and DNAse away (Molecular BioProducts, Mexico).

PCR products (10 µl or 25 µl of 50 µl) were resolved by gel electrophoresis (Mupidex, France) on a 1.5% gel agarose (Sigma, France) on 135 V. DNA was stained by ethidium bromide (Euromedex, France, one drop per agarose gel) and amplicons of approximately 460 and 360 bp for 16S rDNA and *nifH* genes respectively, were visualized by a UV-transilluminator (Geldoc UVITEC, France). Amplicons were excised and purified using Wizard® SV Gel and PCR Clean-up system (Promega, France). They were then cloned into a pGEM-T vector (Promega, France), and transformed within strain DH5α (Invitrogen, France) according to the manufacturer's instructions. Plasmid DNA from ten to twelve positive colonies was purified (Wizard Plus SV Minipreps DNA Purification system, Promega, France) and sequenced using ABI3730XL capillary systems (ABI, Macrogen, Korea). Sequences were deposited in EMBL under accession numbers HQ630785–HQ630811, HQ630813–HQ630820.

2.5 Phylogenetic analyses

Phylogenetic analyses were performed using the ARB program package (Ludwig et al., 2004). 16S rDNA and *nifH* databases were download from SILVA (http://www.arb-silva.de/, last access: June 2010) and Marine Microbiology Laboratory from the University of California (J. P. Zehr laboratory, http://pmc.ucsc.edu/~wwwzehr/research/database/, last access: June 2010) websites, respectively. 16S and *nifH* phylogenetic trees were constructed by the neighbour-joining method with Jukes-Cantor correction. 16S rDNA sequences longer than 1200 base pair (bp) or all nucleotide positions (approx. 360 bp) between N1 and N2 primers (including those obtained in this study) were used. Analyses were bootstrapped 1000 times to evaluate the robustness of tree branches. 16S rDNA partial sequences from this study (approx. 460 bp) were added to the tree by using the maximum-parsimony option from ARB.

Table 1. 16S rDNA and *nifH* primers used in this study.

Primer	Target	Sequence (5' to 3')	Reference
Nitro821	16S rDNA of UCYN	CAA GCC ACA CCT AGT TTC	Mazard et al. (2004)
Cya359	16S rDNA of cyanobacteria	GGG GAA TYT TCC GCA ATG GG	Nübel et al. (1997)
N1	*nifH* of prokaryotes	TGY GAY CCN AAR GCN GA	Zehr and McReynolds (1989)
N2	*nifH* of prokaryotes	ADN GCC ATC ATY TCN CC	Zehr and McReynolds (1989)
N3	*nifH* of prokaryotes	ATR TTR TTN GCN GCR TA	Zani et al. (2000)
N4	*nifH* of prokaryotes	TTY TAY GGN AAR GGN GG	Zani et al. (2000)

M: A/C; N: A/T/G/C; R: A/G; Y: C/T; D: A/T/G.

3 Results

3.1 Tests of PCR amplification for the detection of scarce microorganisms

The detection limit of PCR amplification was successfully decreased from 7 to 1 cell ml^{-1} by using entire fixed UCYN cell (*Crocosphaera watsonii*) instead of extracted DNA. 1 cell ml^{-1} corresponds to 12 *Crocosphaera* cells per PCR reaction (Fig. 2a), and to the lowest number of 12 UCYN per filter portion detected by TSA-FISH technique in this study.

DNA amplification from entire fixed cells was achieved with success on samples stored at −80 °C for up to three years and originated from different marine waters (Fig. 2b$_1$ and b$_2$). However, beyond two or three years of storage, environmental DNA could not be amplified and filters must undergo additional washing steps before their introduction into PCR tubes to clean the fixed cells from a white film made of PFA, ethanol and sea salt (Fig. 2b$_2$).

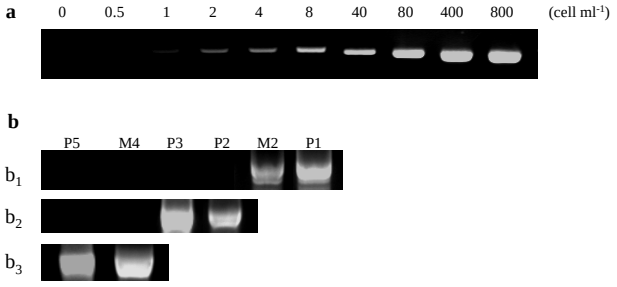

Fig. 2. Different PCR amplification tests for the detection of scarce microorganisms. **(a)** Definition of PCR detection limit of UCYN using a range of concentrations of *C. watsonii* with Nitro821/Cya359 16S primers. **(b)** Test of stability of PCR efficiency with time on different types of DNA template collected in the Pacific Ocean (P) or in the Mediterranean Sea (M) and stored at −80 °C for 1 to 5 yr. Amplifications were done with the general 16S primers 27F/1518R using as DNA template **(b$_1$)** PFA fixed cells collected on filter, **(b$_2$)** PFA fixed cells collected on filter and washed with MilliQ sterile water before amplification, and **(b$_3$)** extracted DNA.

3.2 Diazotrophic cyanobacteria distribution

Low concentrations of putative diazotrophic cyanobacteria were detected in Mediterranean surface waters using the TSA-FISH technique (Fig. 3). The community was dominated at 99.9% by picoplanktonic cells hybridized with the UCYN specific probe Nitro821 (Fig. 3a, b, d, e; Fig. 4a–d). Two Nitro821-hybridized cell types were detected, small ones from 0.8–1.5 µm (Fig. 4a) and large ones from 2.5–3.2 µm (Fig. 4b, c). Small cells dominated at 92% the Nitro821-targeted cells community (Fig. 3). Among these small cells, 75% were free living-organisms recovered in the 0.2–3 µm size fraction (Fig. 3a, d), while 25% were associated with nonthecate dinoflagellates in the 3–10 and >10 µm size fractions (Fig. 3a, d; Fig. 4d). Dinoflagellates were identified with their typical condensed chromosomes when stained with DAPI (data not shown) and associated Nitro821-hybridized cells were essentially concentrated around the dinoflagellate nucleus where their concentration ranged from 1 to 30 cells (Fig. 4d). Large picocyanobacteria represented only 8% of the Nitro821-targeted cells (Fig. 3b, e), and were recovered merely as free living cells either in the 0.2–3 µm size fraction (Fig. 4b) or in the one of 3–10 µm (Fig. 4c). Nitro821-targeted cells were 5.5 times less abundant at DCM$^+$ depth than at 12.5 m (Fig. 3b, c). Their cumulated concentrations ranged from 1–6 cell ml^{-1} with an average of 3.5 cell ml^{-1}.

Among filamentous cyanobacteria, only 0.02 filament ml^{-1} of *Richelia intracellularis* were detected in the eastern basin at 3 stations (Fig. 3c, f, Fig. 4e), while *Trichodesmium* spp. was absent or under detection (<0.004 filament ml^{-1}) all through the transect.

3.3 Diazotrophs species richness

UCYN specific 16S rDNA amplification was obtained only from station A and all the sequences were affiliated to UCYN-A (Fig. 5, Table 2). The presence of UCYN-A was confirmed in station A by *nifH* phylogenetic analyses, where they accounted for 43% of the sequences (group 1′, Figs. 6, 7). In addition to UCYN-A, four diazotroph groups of proteobacteria were recovered by *nifH* phylogenetic analyses.

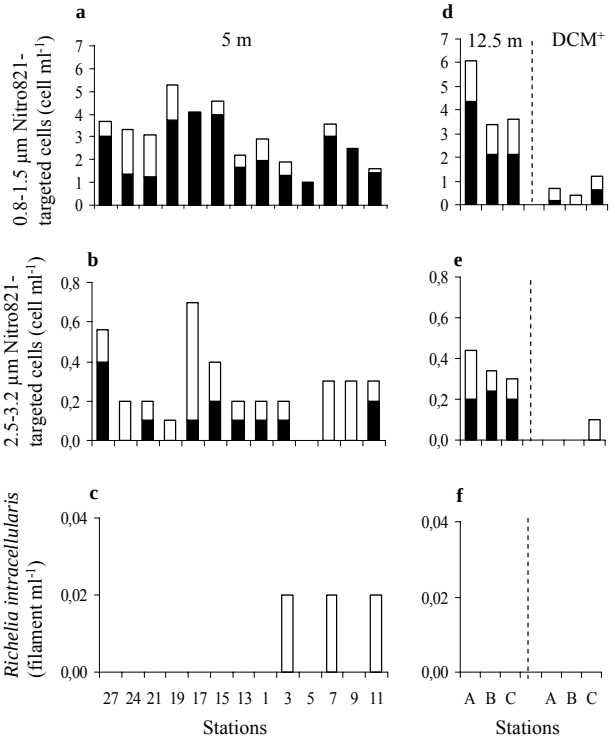

Fig. 3. Epifluorescent microscopic counts of Nitro821-targeted UCYN and filamentous diazotrophic cyanobacteria across the Mediterranean Sea within the 0.2–3 µm (black bars) and the 3–10 µm combined with >10 µm (white bars) size fractions. Counts were done at 5 m depth **(a, b, c)** and at two nutrient contrasted depths **(d, e, f)** including 12.5 m (left of dotted line) and upper deep chlorophyll maximum (DCM^+, right of dotted line). Counts were done on small Nitro821-targeted cells **(a, d)**, on large Nitro821-targeted cells **(b, e)**, and on *Richelia intracellularis* **(c, f)**.

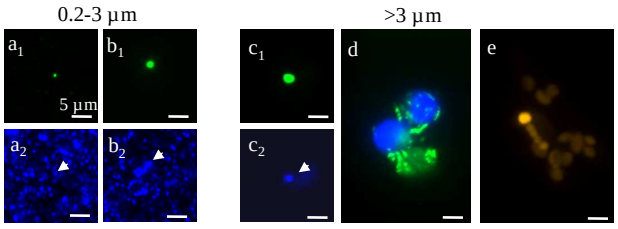

Fig. 4. Epifluorescent photomicrographs from Nitro821-targeted UCYN and filamentous diazotrophic cyanobacteria detected in the Mediterranean Sea. Nitro821-targeted UCYN were labelled with FITC (green fluorescence, $\mathbf{a_1}$, $\mathbf{b_1}$, $\mathbf{c_1}$, **d**) using TSA-FISH technique. **($\mathbf{a_2}$), ($\mathbf{b_2}$), ($\mathbf{c_2}$)** and **(d)** showed corresponding microphotograph of DAPI-stained DNA (blue fluorescence) from all prokaryotic and eukaryotic cells. Arrow heads point to the DNA from the Nitro821-targeted ones. The heterocycstous *Richelia intracellularis* was detected owning to it natural orange fluorescence **(e)**. Scale bar = 5 µm.

Bradyrhizobium sequences dominated *nifH* clone libraries from station A, B, and C, where they represented 57%, 56%, and 75% of the sequences, respectively (Figs. 6, 7, Table 2). Among them, the new marine group 2′ of *Bradyrhizobium* was recovered at the three stations, while the group 3′ was detected only at station A. Others rhizobia sequences from group 4′ were detected at station C, when γ-proteobacteria from group 5′ was detected at station B, accounting for 25% and 44% of the *nifH* clones libraries, respectively (Figs. 6, 7, Table 2).

4 Discussion

4.1 Distribution of diazotrophic cyanobacteria along a Mediterranean transect

Diazotrophic cyanobacteria have long been considered as the main contributors of N_2-fixation in the marine plankton, since they can provide through photosynthesis an autonomous source of organic carbon necessary for this energetically expensive process (Stewart, 1971). This first Mediterranean basin-wide study revealed a very low concentration of diazotrophic cyanobacteria, which were dominated at 99.9% by picoplankton. While Nitro821-targeted UCYN were detected at all stations with a mean concentration of 3.5 cell ml^{-1}, filaments of *Trichodesmium* sp. were under detection limit (<0.004 filament ml^{-1}) all through the transect and only 0.02 filament ml^{-1} of *Richelia intracellularis* were recovered in the eastern basin. The scarce distribution of these filamentous cyanobacteria was confirmed by plankton net haul data from the same transect (Crombet et al., 2011) as well as by past and recent studies (Trégouboff, 1957; Bar-Zeev et al., 2008). Although *Trichodesmium* is known to form massive blooms (10^3 filament ml^{-1}) detectable from space in other seas and oceans (Capone et al., 1998; Dupouy et al., 2000), such phenomena have never been observed in the Mediterranean Sea (C. Dupouy, personal communication, 2007). Similarly as for filamentous cyanobacteria, the concentration of UCYN was low in this study and in the same range as the ones previously quantified all through the year in coastal north Mediterranean waters (4.6 cell ml^{-1} in mean, Le Moal and Biegala, 2009). However, sporadic blooms of UCYN have been reported to reach 10^2 to 10^3 cell ml^{-1} in the same coastal Mediterranean environment, or in Pacific and Atlantic Oceans (reviewed in Table 1 in Le Moal and Biegala, 2009; Moisander et al., 2010).

Among Nitro821-targeted picocyanobacteria, two cell types were detected in this study, small and large ones (0.8–1.5 µm; 2.5–3.2 µm). While the small cell type has been recently discovered in the Pacific Ocean (Biegala and Raimbault, 2008) and confirmed to belong to UCYN-A (Goeble et al., 2008), UCYN-B and C are known to be either picoplanctonic or nanoplantonic cells (2–10 µm, Zehr et al., 2001; Ohki et al., 2008). This latter morphological information suggests that the larger Nitro821-targeted cells may

Table 2. Phylogenetic affiliation of the 16S rDNA and *nifH* sequences.

Gene	Phylogenetic groups	No. of clones	A representative clone	Closest relative (accession number)*	Identity (%)
16S rDNA	Group 1	9	BOUM_A1	Cyanobacterium UCYN-A (CP001842)	98
				Cyanothece sp. WH 8902 (EU249123)	96
nifH	Group 1′	3	BOUM_A4	Uncultured marine bacterium clone HT70A1_T7 (DQ118201)	100
				Endosymbiont of Rhopalodia gibba (AY728387)	85
	Group 2′	15	BOUM_C1	Uncultured soil bacterium clone DN18 (DQ987562)	95
				Bradyrhizobium sp. strain ORS391 (FJ347449)	94
	Group 3′	3	BOUM_A1	Uncultured bacterium clone GYMC-52B (AJ716286)	96
				Bradyrhizobium sp. TSA27s (AB542352)	95
	Group 4′	3	BOUM_C2	Uncultured bacterium clone NTC9 (GU196843)	99
				Rhizobium sp. W3 (GQ241353)	95
	Group 5′	4	BOUM_B2	Uncultured microorganism clone H05_DNA_E10 (EF568515)	98
				Denitrovibrio acetiphilus DSM 12809 (CP001968)	77

* Non-italic represents non-cultivated species; italic represent cultivated species.

be affiliated to UCYN-B or C. However, flow cytometric Mediterranean studies have never reported nanoplanktonic cyanobacteria and the only UCYN detected by phylogenetic analysis belonged to group A (Man Aharanovich et al., 2007).

4.2 UCYN species richness recovery

The affiliation of small Nitro821-targeted cells to UCYN-A was confirmed in the western Mediterranean Sea (station A) with both 16S and *nifH* phylogenies (Figs. 5 and 6). It is the first time that UCYN-A are detected at the DNA level in the Mediterranean Sea, thanks to the use of a new PCR protocol dedicated to scarce microorganisms. Entire paraformaldehyde fixed cells collected on a filter serves as PCR DNA template instead of extracted DNA. This allows lessening the detection limit defined by Mazard et al. (2004) from 7 cell ml^{-1} to 1 cell ml^{-1} in cultures. In a previous Mediterranean study, UCYN-A were recovered off Israeli coast, but only at the transcript level (Man-Aharonovich et al., 2007), likely because these picocyanobacteria were in too low concentration to be recovered after DNA extraction steps. Similar lacks of amplification using extracted DNA have been reported for UCYN-A and other diazotrophs in both marine and freshwater environments (Zani et al., 2000; Hewson et al., 2007). The use of entire cell as DNA PCR template has been previously proposed to reduce the volumes of filtered marine samples down to 25 µl including 10^4 bacteria per PCR reaction (Kirchman et al., 2001). These approaches also avoid DNA extractions which are time consuming and generate occasional PCR inhibitors. Although preservatives such as formalin and PFA have been tested on PCR DNA template in past studies, this step was not recommended because it inhibits amplification and generates PCR artefacts (Degiorgi et al., 1994; Kirchman et al., 2001). In this study we were successful with PCR amplification using preservative procedure before PCR assays, similar as the one for TSA-FISH assay, as long as samples were collected within the last four years. Beyond this age inhibitions are shown to increase with time (Fig. 2b). The chemical mixture composed of PFA, ethanol and sea salt is suspected to inhibit subsequent PCR reactions by modifying the salt concentration, and thus the stringence conditions, during the hybridization step between primers and cellular DNA template. Consequently for long term stored samples extracted DNA has to be used as PCR template (Fig. $2b_3$) and direct use of fixed cells as PCR DNA template proved to be a quick and simple protocol to assess the diversity of scarce populations.

Although the PCR protocol dedicated to scarce microorganisms was applied on samples from station B and C, no UCYN were recovered either by *nifH* or 16S UCYN specific phylogenies. We suspect the cell concentrations to be close to, or below the PCR detection limit. Small and large Nitro821-targeted cells from station B and C were 2 to 20 times less concentrated than small cells identified as UCYN-A at station A. Thus, except UCYN-A at station A, diazotrophic cyanobacteria were under detection limit at station A, B, and C, suggesting that the weak N_2 fixation rates measured in the surface layer of the water column (Bonnet et al., 2011; Ridame et al., 2011) can be attributed to other organisms than cyanobacteria.

4.3 Non-cyanobacterial diazotrophs richness recovery

In order to search for these organisms, *nifH* phylogenetic analyses were done. The dominant *nifH* sequences from the picoplanktonic size fraction of all three clone libraries were *Bradyrhizobium*, affiliated to α-proteobacteria that has so far never been detected in the marine environment (group 2′, Figs. 6, 7). In addition to *Bradyrhizobium*, two other

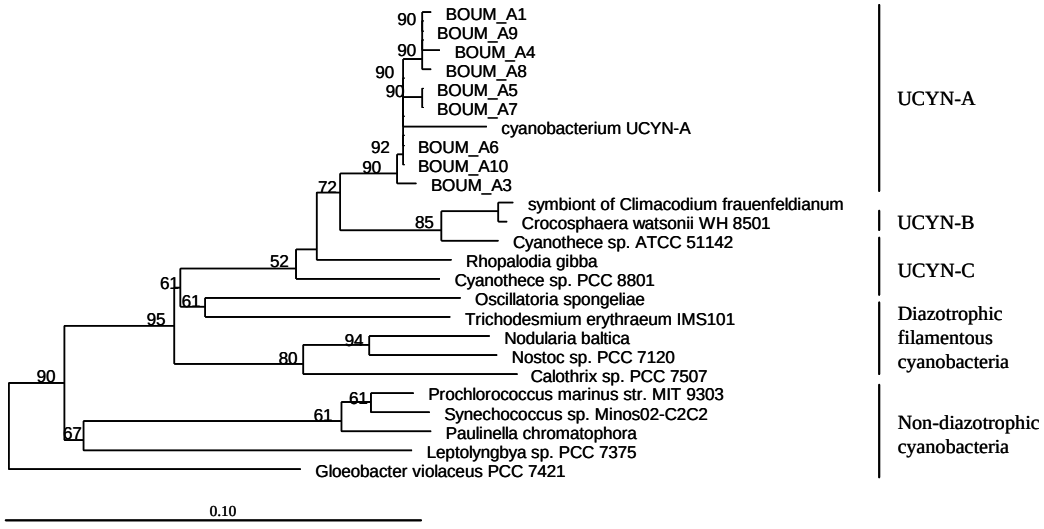

Fig. 5. Phylogenetic tree of 16S rDNA sequences from Cyanobacteria. Sequences obtained in this study are referred to (i) by the oceanographic transect BOUM, (ii) the station at which they were sampled (A, B, or C), and (iii) their clone number. Bootstrap values >50% are indicated at the nodes. Scale bar = 0.1 substitution per nucleotides.

groups of rhizobia were identified at stations A and C (group 3′ and 4′, Fig. 6), while γ-proteobacteria sequences (group 5′, Fig. 6) were recovered from station B. UCYN-A, γ-proteobacteria and distantly related α-proteobacteria have all been shown to express their *nifH* gene in the Mediterranean Sea and Pacific or Atlantic Oceans (Falcón et al., 2004; Man Aharanovich et al., 2007; Zehr et al., 2007). Consequently, N_2-fixation from the picoplanktonic size fraction (0.05–0.1 nmol N L^{-1} d^{-1}; Bonnet et al., 2011) could be attributed to a mix of rhizobia in station C, when UCYN-A and γ-proteobacteria were likely additional contributors to this activity in station A and B, respectively.

Conversely to free living diazotrophic cyanobacteria, availability of organic carbon may limit non-cyanobacterial planktonic diazotrophs to acquire sufficient energy for nitrogen fixation (Paerl et al., 1987). This explains why in terrestrial and freshwater environments many symbioses have been developed between diazotrophic bacteria and higher plants (Masson-Boivin et al., 2009). These diazotrophic symbionts all cluster within the polyphyletic group of rhizobia, which includes α and β-proteobacteria. Thanks to these associations, rhizobia are considered as the most efficient heterotrophic N_2-fixers as they receive carbon fixed by their photosynthetic host (Evans and Barber, 1977; Van Rhijn and Vanderleyden, 1995). In the marine environment it is well known that diazotrophic bacteria have developed symbiotic relationships with seagrass (Capone, 1983). Diazotrophic bacteria associated with *Posidonia oceanica*, a seagrass spread all around the Mediterranean Sea, have been estimated to contribute to two-thirds of total nitrogen fixation (Béthoux and Copin-Montégut, 1986). Consequently, it is tempting to speculate that the three groups of rhizobia that have been discovered in this study are free living stages of seagrass symbionts. An additional interesting discovery from this study concerns the widely distributed group 2' of *Bradyrhizobium*, whose closest relatives is the freshwater strain ORS391 (Fig. 6, Table 2). ORS391 belongs to the same *Bradyrhizobium* species than ORS278 and ORS285 strains that, together with *Azorhizobium caulinodans*, are the only rhizobia known to be able to fix nitrogen extra-plantae (Dreyfus et al., 1988; N. Nouwen, personnal communication, 2010). This metabolic activity is probably related to their photosynthetic capacity, a common feature among *Bradyrhizobium* cluster (Giraud and Fleischman, 2004). Despite being diazotrophic, phototrophic free-living bacteria have never been discovered to date in the marine environment (Madigan, 1995; Riemman et al., 2010). We hypothesize though that similar to their closest relatives, the Mediterranean *Bradyrhizobium* detected in this study are able to acquire an independent source of energy through photosynthesis. Such metabolic capacity may help them to sustain planktonic nitrogen fixation.

4.4 Factors controlling the distribution of diazotrophic cyanobacteria

The reasoning developed above proposes hypotheses for the presence of diverse rhizobia community all through the Mediterranean Sea. However it is puzzling that so little diversity (species richness and abundance) of diazotrophic cyanobacteria was recovered, when their presence could be expected in such subtropical oligotrophic Mediterranean waters. Among environmental parameters mentioned to limit diazotrophic cyanobacterial development, temperature, iron

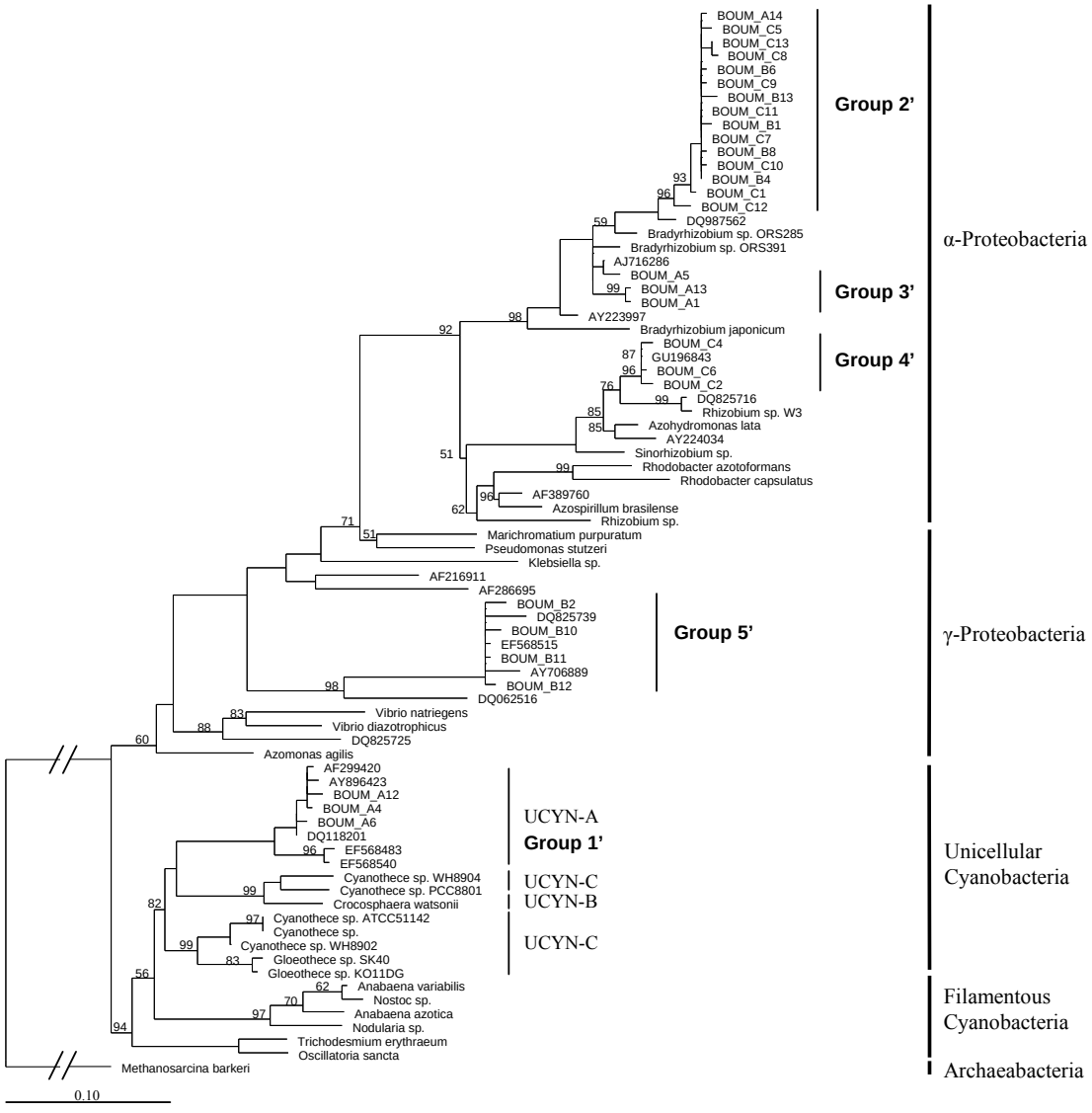

Fig. 6. Phylogenetic tree of *nifH* sequences. Sequences obtained in this study are referred to (i) by the oceanographic transect BOUM, (ii) the station at which they were sampled (A, B, or C), and (iii) their clone number. Bootstrap values >50% are indicated at the nodes. Scale bar = 0.1 substitution per nucleotides.

and phosphate are the most regularly cited (Mague, 1974; Pearl et al., 1994; Breibarth et al., 2007). High temperature induces stratification of the water column which segregates deep mesotrophic water masses from upper oligotrophic ones. In these warm nutrient deprived waters, diazotrophic cyanobacteria are considered to be at an ecological advantage compared to other phytoplankton species, as they can acquire nitrogen directly from dissolved atmospheric N_2 (Tyrell, 1999). Upper mediterranean nitrate-deprived waters were confirmed to be more favorable than deeper and nitrate-enriched ones for the development of diazotrophic cyanobacteria, as small Nitro821-targeted UCYN-A were 15 times more abundant at 12.5 m than at DCM^+ at station A (Fig. 3d; Pujo-Pay et al., 2010). Nevertheless, even in these upper waters the diversity of diazotrophic cyanobacteria was low, indicating they were limited by some elements.

Across the Mediterranean Sea, filamentous cyanobacteria were likely inhibited in surface waters by iron and inorganic phosphate, whose concentrations were two orders of magnitude lower than those reported to limit their growth (Mulholland et al., 2002; Berman-Frank et al., 2007; Pujo-Pay et al., 2010; Ternon et al., 2010). Moreover, the phosphate turnover time, which is considered as the first indicator of phosphate availability in oligotrophic marine waters, is 5 times lower in surface Mediterranean waters than the one defined to prevent *Trichodesmium* spp. growth (Moutin et

Table 3. Putative phosphorus scavenging genes present in the UCYN-A genome*.

Category	IMG gene object identifier	Gene name	Putative fonction	% coverage**	% similarity**	Reference
Response regulator	646530709	phoB	Response regulator	98	66	*Synechococcus* sp. WH8102
High affinity phosphate transport	646530204	phoS	phosphate binding	98	64	*Cyanothece* sp. ATCC 51142
	646530201	pstA	phosphate permease	100	67	*Crocosphaera watsonii* WH8501
	646530202	pstC	phosphate permease	100	70	*Crocosphaera watsonii* WH8501
	646530148	pstC	phosphate permease	97	68	*Synechococcus* sp. WH8102
	646530199	pstB	ATPase component	99	64	*Synechocystis* sp. PCC6803
	646530200	pstB	ATPase component	100	66	*Synechocystis* sp. PCC6803
Hydrolysis of phosphate esters	646530491	phoA	Alkaline phosphatase	94	57	*Prochlorococcus marinus* CCMP1986
	646529940	–	Mettalophosphoesterase	96	51	*Trichodesmium erythaeum* IMS101
	646529952	–	Mettalophosphoesterase	97	54	*Cyanothece* sp. PCC 7424
	646530014	5ND	5'-nucleotidase	97	61	*Synechococcus* sp. WH8102
Polyphosphate metabolism	646530144	ppk	Polyphosphate kinase	96	53	*Synechococcus* sp. WH8102
	646530179	ppa	Inorganic pyrophosphatase	97	65	*Nostoc punctiforme* PCC73102
Phosphonate transport	646530663	phnD	ATPase and permease component	100	62	*Synechococcus* sp. WH8102

* Publicly available on the Integrated Microbial Genome (IMG, http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

** Only genes that demonstrated >50% identity over 80% of each protein sequence are presented.

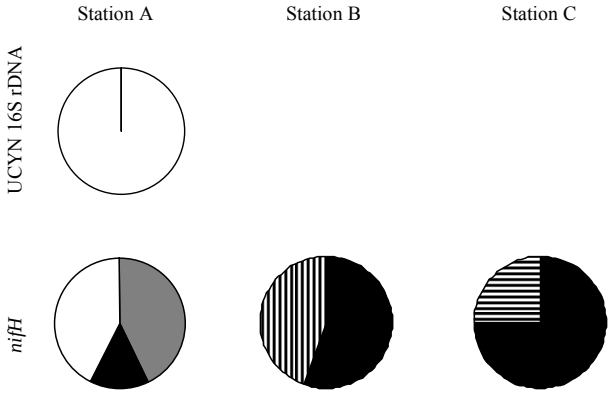

Fig. 7. Percentage of sequences from UCYN specific 16S rDNA and *nifH* clone libraries from station A, B, and C. Colors indicate phylogenetic groups from Figs. 5 and 6 in which sequences were recovered: UCYN-A group 1′ (white); *Bradyrhizobium* group 2′ (black); *Bradyrhizobium* group 3′ (grey); rhizobia group 4′ (horizontal lines); γ-proteobacteria group 5′ (vertical lines).

al., 2005, 2008; Mauriac et al., 2011). Conversely, UCYN have been shown to grow in deprived iron waters and to reach 10^2 to 10^3 cell ml^{-1} in marine areas using similar or lower inorganic phosphate concentrations (Berman-Frank et al., 2007; see review in Le Moal and Biegala, 2009). The requirements of these different cell types towards phosphate are not reflected by their genomic potential (Dyhrman and Haley, 2006; Orchard et al., 2009). Both *Trichodesmium* and UCYN-B possess a broad spectrum of genes encoding (i) a high affinity transport system to acquire inorganic phosphate and (ii) for the scavenging of phosphomonoesters, the dominant form of organic phosphate in the marine environment (75%, Kolowith et al., 2001). In addition, we provide in this study (Table 3) an analysis of the UCYN-A genome with respect to P which, despite its reduced size, has a similar P genetic toolbox to that of other picoplanktonic cyanobacteria (Scanlan et al., 2009; Tripp et al., 2010). Interestingly, *Trichodesmium* seems to be the only marine cyanobacterium that has the potential to scavenge phosphonates, the second major component of organic phosphate (25%, Kolowith et al., 2001; Dyhrman et al., 2006). Despite this unique genetic equipment *Trichodesmium* cannot grow efficiently in phosphate deprived waters. Phosphate uptake has been demonstrated to be cell size dependent (Smith and Kalff, 1982; Falcón et al., 2005), hence the low surface/volume ratio of filamentous cyanobacteria seems an obvious disadvantage compare to UCYN to efficiently grow in oligotrophic environment.

While iron and phosphate seem to control the development of *Trichodesmium* and *Richelia*, temperature has recently been suspected to limit UCYN (Moisander et al., 2010). At stations where species richness was characterised, temperature was 25 °C (Moutin et al., 2011), being slightly under or above the optimal temperature range for UCYN-A (23–24 °C) and UCYN-B and C (26–29 °C, reviewed by Le Moal and Biegala, 2009; Moisander et al., 2010). Thus other factors than temperature must co-limit or prevent their development. Still, UCYN-A limiting factors seem slightly relaxed in the less oligotrophic western basin and at coastal station off Israel, where they were recovered (Man-Aharanovich et al., 2007). We suspect carbon to be the controlling factor for UCYN-A development. UCYN-A has been recently

demonstrated to lack photosystem II, the complex which allows CO_2 reduction (Zehr et al., 2008), making it probably dependent on an unknown organic source of carbon, a unique feature among planktonic diazotrophic cyanobacteria. The origin of this carbon puzzles the scientific community, and UCYN-A has been suggested to obtain it from dead particles or other organisms (Tripp et al., 2010). Very interestingly, picoplanktonic Nitro821-targeted cells have been regularly observed, including at station A in this study, in association with dead particles and numerous nonthecate dinoflagellates (Fig. 4d; Biegala and Raimbault, 2008; Bonnet et al., 2009; Le Moal and Biegala, 2009).

5 Conclusions

In this first Mediterranean basin wide study, small picoplanktonic cells hybridized with a UCYN specific probe were recovered across the entire Mediterranean Sea, and were confirmed to belong to UCYN-A in the western part of this area. *Bradyrhizobium* was the most widely distributed diazotroph from the offshore Mediterranean waters, when other diazotrophs were recovered only in one location, such as gamma-proteobacteria in the central Mediterranean Sea, and *Richelia intracellularis* in addition to rhizobia in the eastern Mediterranean Sea. The photosynthetic diazotrophs *Trichodesmium* sp., UCYN-B and C were absent or under detection limit, although these free living organisms show a significant degree of nutritional independence towards carbon and nitrogen. While low phosphate and iron concentrations could explain the absence of *Trichodesmium* sp., limiting factors for the development of UCYN-B and C remain unknown. We hypothesize that the presence of diverse planktonic diazotrophs such as UCYN-A, *Richelia intracellularis*, rhizobia and gamma-proteobacteria, may be explained by the use of alternative strategies to acquire essential nutrients: (i) similar to their terrestrial counterparts, rhizobia may form punctual symbioses with marine higher plants such as seagrass; (ii) *Bradyrhizobium*, the most widely distributed diazotroph in this Mediterranean study, could also acquire carbon through photosynthesis; (iii) UCYN-A probably developed associations and/or symbiosis with inert particles or eukaryotes to acquire organic carbon necessary for their development. To answer these hypotheses, further work will be necessary to characterize the different strategies developed by picoplanktonic diazotrophs to acquire carbon in oligotrophic environments.

Acknowledgements. We thank the crew of the R/V Atalante for its help collecting samples. We thank Aude Barrani and the PRECYM (Plate-forme Régionale de Cytométrie pour la Microbiologie) for *Crocosphaera* cell counts by flow cytometry, and Anne-Flore Deton for PCR amplification tests realised on Pacific filters. We also thank two anonymous reviewers for their helpful comments on this manuscript. This is a contribution of the BOUM (Biogeochemistry from the Oligotrophic to the Ultraoligotrophic Mediterranean) experiment (http://www.com.univ-mrs.fr/BOUM) of the french national LEFE-CYBER program, the European IP SESAME and the inter25 national IMBER project. This work was supported by a grant from Conseil Régional Provence-Alpes-Côte d'Azur.

Edited by: C. Jeanthon

The publication of this article is financed by CNRS-INSU.

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