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Intriguing diversity among diazotrophic picoplankton along a Mediterranean transect: from the origin of plastids to the dominance of rhizobia

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Abstract

The Mediterranean Sea is one of the most oligotrophic marine areas on earth where nitrogen fixation has been 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 phylogenies. These genetic analyses were possible owning to the development of a new PCR protocol adapted for scarce microorganisms (0.2 cell ml^{-1}). Low concentrations of diazotrophic cyanobacteria were detected and this community was dominated at 99.9% by picoplankton hybridized with 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 and averaged 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. Surprisingly, the larger hybridized cells were not belonging to UCYN-B and C but to plastids of picoeukaryote. NifH gene was not recovered among picoeukaryotes, when rhizobia sequences, including the ones of *Bradyrhizobia*, were dominating nifH clone libraries from picoplanktonic size fractions. Few sequences of γ-proteobacteria were also detected in 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 photosynthetic activity to acquire carbon for sustaining diazotrophy. Among UCYN further work will be necessary to understand their suggested role in plastid evolution.

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's one (Béthoux and Copin-Montégut, 1986; Krom et al., 1991). The processes that induce such atypical ratios are under debate, and two hypothesises 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 picks 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 massive 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. Still, the importance of such process was argued to be inconsistent with Mediterranean phosphate-starved conditions (Krom et al., 2004, 2010), as diazotrophic activity was demonstrated to be control by phosphate in areas dominated by *Trichodesmium* sp. (Saudo-Wilhelmy et al., 2001; Mills et al., 2004). *Trichodesmium* sp., a filamentous cyanobacteria, has been regarded for a long time as the main marine N_2 fixer (Falkowski et al., 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 large one (Smith and Kalff, 1982). This is coherent with past and recent Mediterranean observations (Tregouboff, 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 dia-
5 zotrophs. 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 nanoplank-
10 tonic 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 of being free living, UCYN have been suggested to develop mucilage, to attach to inert particles or to live in association with
15 planktonic eukaryotes (Biegala and Raimbault, 2008; Bonnet et al., 2009). These observations imply that UCYN could also contribute to nitrogen fixation from the large size fraction, thus reinforcing their role into 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).
20 Organisms affiliated to UCYN-A, Proteobacteria, and Archaea, were recovered in the south-eastern basin and were expressing their nifH gene, which encodes nitrogenase enzyme responsible for nitrogen fixation (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
25 Biegala, 2009). Their concentrations were low (4.6 cell ml^{-1} in mean) all along a seasonal cycle, except over a summer month when they reached 1900–5300 cell ml^{-1}. While UCYN and filamentous diazotrophic cyanobacteria have been investigated in coastal waters, their spatial distribution have never been characterized in the open Mediterranean Sea.

Different molecular approaches have been used in the literature to study the concentrations and the species richness of diazotrophs. Concentrations have been determined either with the help of quantitative polymerase chain reaction (qPCR) technique on nifH gene, or direct microscopic counts of fluorescently labeled UCYN, using a specific 16S rDNA probe combined with tyramide signal amplification-fluorecence 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 on the Cyanobacteria phylum (Rosado et al., 1998; Zehr et al., 2003). Compared to qPCR, which quantify nifH gene per milliliter on extracted DNA, 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). The general UCYN specific probe was first tested in combination with the visual hybridization TSA-FISH technique to investigate for the hypothetic picoplanktonic UCYN (Biegala and Raimbault, 2008). However, discrimination among the different nanoplanktonic groups is not possible and requires specific phylogenetic analysis. Such information is tricky to acquire for diazotrophs less concentrated than $10\,\mathrm{cell\,ml^{-1}}$ when using extracted DNA as PCR template (Kirshtein et al., 1993; Mazard et al., 2004), as low concentrated targets may be too much diluted during the different extractions steps. To increase the amount of target within extracted DNA, nested approach on nifH gene has been introduced but remained partially unsuccessful, thus illustrating the need to develop an adapted PCR protocol for scarce microorganism in this field of research (e.g. Zani et al., 2000; Man-Aharonovich et al., 2007).

Diazotrophic activity in plankton has long been attributed to cyanobacteria, as they can sustain this energetically expensive process owning to photosynthesis (Stewart, 1971). Conversely, non-cyanobacterial diazotrophs are considered unable to acquire autonomous carbon source through photosynthesis, and have thus been neglected in biogeochemical studies (Madigan, 1995; Tyrell et al., 1999). However, this metabolic

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function has been recently proven to lack 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 pattern 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 diazotrophs community including Proteobacteria and Archaea to better understand their role into global nitrogen fixation.

To improve our understanding on the role of diazotrophy in Mediterranean biogeochemical cycles, it is crucial to characterize the whole community of diazotrophs all through the Mediterranean Sea. We propose in this study an original 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 diazotrophs distribution 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
5 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 hours light/dark conditions on a modified YBCII medium (Chen
10 et al., 1996). Two milliliters of culture were fixed with 1% paraformaldehyde (PFA) and stored at −80 °C until their serial dilutions, their counts by flow cytometry and their 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 from one to five years at −80 °C to test the stability of PCR efficiency with
15 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
20 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 ISOPORE™ (Millipore, France) 47 mm filters, and 0.8–3.4 liters of the remaining filtrate were collected by gravity on 3 µm ISOPORE™ (Millipore, France) 47 mm filters. Then, 200 mL of the <3 µm filtrate were collected under 200 mmHg vacuum on 0.2 µm ISOPORE™ (Mil-
25 lipore, France) 47 mm filters. According to Biegala and Raimbault (2008), 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. 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 counterstaining with the specific blue DAPI dye (Sigma-Aldrich, France).

Microphotographs and cells counts were done according to Biegala and Raimbault (2008). Briefly, entire surfaces of each filter portion (approx. 40 microscopic fields, $0.5\,\text{cm}^2$) 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 distribution on filters was confirmed by the low standard deviation obtained on triplicated 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 allow 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* (0–800 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

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640 nm long pass filters for orange and red emission wavelenghts 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 °C until the addition of PCR mixture.

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 extract (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 RT. Between incubations water was discarded by 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 mm Hg vacuum and subsequently following DNA extratction protocol defined in Zehr and Turner (2001).

Diazotroph species richness was investigated by targeting both 16S rDNA and nifH genes. Nested PCR were used for both genes. The general primers 27F/1518R were used for the first PCR, followed by the 16S UCYN specific primers Nitro821/Cya359 (Eurogentec, France, Table 1) for the second PCR. The original protocol from Mazard et al. (2004) was modified by introducing a nested approach because of the low Nitro821-targeted UCYN concentrations detected in this study. For nifH nested PCR, the N3/N4 primers were used for first PCR followed by the N1/N2 primers (Eurofins MWG, Germany, Table 1) for the second PCR, according to Zehr and Turner (2001). All PCR

reactions were carried out in 50 µl mix. The first PCR used fixed cells on filter as DNA template and the second PCR used 1 µl of product from the first PCR.

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
5 ethidium bromide (Euromedex, France, one drop per agarose gel) and amplicons of 460 and 359 bp for 16S rDNA and nifH genes, respectively, were visualized by a UV-transilluminator (Geldoc UVITEC, France). Amplicons were then 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
10 (Invitrogen, France) according to manufacturer's instruction. 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).

2.5 Phylogenetic analyses

15 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
20 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. 480 bp) were added to the tree by us-
25 ing the maximum-parsimony option from ARB, similarly as for partial 16S sequences from the three CCMP strains and *Imantonia rotunda* (approx. 900 bp).

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 down 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), which is well below the lowest number of 25 UCYN per filter portion detected by TSA-FISH technique in this study. Furthemore, the 16S rDNA nested approach allowed to lessen the detection limit from 1 cell ml^{-1} down to 0.2 cell ml^{-1} on samples from the Mediterranean transect primarily quantified by TSA-FISH technique.

It is possible to amplify DNA from entire fixed cells on samples stored at −80 °C for up to three years and originated from different marine waters (Fig. 2b$_{1,2}$). 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$_2$).

3.2   Diazotrophic cyanobacteria distribution

Low concentrations of putative diazotrophic cyanobacteria were detected in Mediterranean surface waters using 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 dinoflagellates nucleus where their concentration ranged from 1 to 30 cells (Fig. 4d). Large picocyanobacteria

represented only 8% of the Nitro821-targeted cells community (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$^+$ depht 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

High species richness was detected among the picoplanktonic 16S rDNA clones libraries (Fig. 5). 90% of the sequences from station A were affiliated to UCYN-A (group 1, Fig. 5). One sequence from station A, and all the ones from stations B and C were affiliated to plastid sequences. They grouped within 8 distinct phylotypes showing less than 95% similarity between them. When plastids sequences from groups 2, 3 and 5 clustered within known groups of Heterokontophyta or Haptophyta, groups 4, 6, 7, and 8 were new phylotypes spreading among Haptophyta and Chlorophyta (Table 2). Group 9 showed low similarity either with Rhodophyta and Chlorophyta, and thus occupied an uncertain position in the 16S rDNA phylogenetic tree.

NifH phylogenetic analyses among picoplanktonic size fractions revealed a high species richness as five distinct groups were detected (Fig. 6). Clones libraries were dominated at 90% by sequences belonging to diazotrophic proteobacteria, while the remaining 10% clustered with the UCYN-A (group 1′). 60% of the proteobacterial sequences belonged to a new marine phylotype affiliated to *Bradyrhizobium* (group 2′, Table 2), which was found at the three stations. Others rhizobia sequences from groups 3′ and 4′ were detected at station A and C, respectively and accounted each for 12% of the proteobacterial sequences. The remaining 16% sequences were affiliated to the γ-proteobacteria group 5′ which was detected at station B only.

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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 the very low concentration of diazotrophic cyanobacteria, which were dominated at 99.9% by picoplankton. While Nitro821-targeted UCYN were detected at all stations and averaged 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 were confirmed by plankton net hawls data from the same transect (Crombet et al., 2010) 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 (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 (4.5 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 later morphological information suggests that the larger Nitro821-targeted cells may 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 were belonging to group A (Man Aharanovich et al., 2007).

4.2 UCYN specific richness recovery on 16S rDNA phylogeny

The affiliation of small Nitro821-targeted cells to UCYN-A was confirmed in the western Mediterranean Sea with both 16S and nifH phylogenies (Figs. 5 and 6). Surprisingly, large hybridized cells were not affiliated to UCYN-B or C clusters but to plastids. In addition, small hybridized cells from central and eastern Mediterranean Sea were likely plastid sequences as the picoplanktonic UCYN-A was not detected in these areas and all the sequences belonged to plastids. The discovery of plastids was not expected because Nitro821 specificity is still confirmed in world-wide data-bases (on the 2010/06/01, Genbank), despite the deposition of more than one million of new 16S sequences since the design of the UCYN specific oligonucleotide (Mazard et al., 2004; and http://www.arb-silva.de/fileadmin/graphics_general/statistics/silva_102/SILVA_dbgrowth_jan10.png). These results illustrate the need to check first *in silico* the specificity of oligonucleotides (Biegala et al., 2005), while additional phylogenetic analysis are required when working in a new environment.

As illustrated in this study, phylogenetic analyses unveiled the presence of five new phylotypes among eukaryotic organelles (Fig. 5). This astonishing result contrasts with a previous study which reported difficulties to enriched clone libraries with plastid sequences (Fuller et al., 2006). One of the reasons to explain this striking discovery of organelles was the use of a new PCR protocol dedicated to scarce microorganisms which concentrations could be as low as $0.2\,\mathrm{cell\,ml^{-1}}$ (Figs. 2 and 3). Entire paraformaldehyde (PFA) fixed cells collected on a filter serves as PCR DNA template instead of extracted DNA, and general 16S amplification precedes UCYN specific one by nested procedure. This allows lessening the detection limit defined by Mazard et al. (2004) from $7\,\mathrm{cell\,ml^{-1}}$ to $0.2\,\mathrm{cell\,ml^{-1}}$. Such 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. $2b_3$). Direct use of fixed cells as PCR DNA template is a simple protocol which may be of great interest to better assess the diversity of scarce populations, such as plastids of picoeukaryotes. This technique could complete, or replace, concentration procedures that have been lately proposed, such as flow cytometry cell sorting (Marie et al., 2010).

Another reason which could explain the recovery of so many new plastid sequences from diverse picoeukaryotes (Fig. 5) is the ancestral relation between UCYN and plastids, as recently supported by biochemical and phylogenetic studies (Deschamps et al., 2008; Falcón et al., 2010). When glycogen is a well conserved polysaccharide form of storage recovered within most of living eukaryotes and bacteria, including cyanobacteria, photosynthetic eukaryotes acquired the ability to synthesize starch, a different form of polysaccharide. Starch was suggested to originate from a common cyanobacterial ancestor, who evolved in plastid since the primary endosymbiotic event (Patron and Keeling, 2005). Recently, an interesting discovery has shown the presence of starch within the modern *Cyanothece* sp., a UCYN-C (Deschamps et al., 2008). Supporting this biochemical evidence, 16S and rbcL phylogenies have shown that plastids

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are most closely related to UCYN than any other cyanobacteria (Falcón et al., 2010). A third line of evidence that many plastids are likely ancient UCYN may be provided by the punctual recovery of Nitro821 sequences among all the divisions of marine pi-coeukaryotes: Chlorophyta, Heterokontophyta and Haptophyta, (Fig. 5; Vaulot et al., 2008). Although lateral gene transfer cannot be excluded, Nitro821 sequences could originate from the common cyanobacterial ancestor which gave rise to these divisions before their diversification (Battacharya et al., 2003; Keeling and Palmer, 2008). To fur-ther support this statement, dinoflagellates and diatoms, two marine phytoplanktonic groups, have developed association with UCYN-B and C (Carpenter and Janson, 2000; Prechtl et al., 2004; Foster et al., 2006). Among these associations, one has been rec-ognized to be at a transitional organelle stage, resulting from a recent endosymbiosis between *Rhopalodia gibba* and a UCYN-C (Kneip et al., 2008). The *R. gibba* symbiont has conserved nifH gene and its ability to fix N_2 (Prechtl et al., 2004; Kneip et al., 2008), while in this study nifH gene was not amplified from any plastids or eukaryotic sequences (Fig. 6). This result is coherent with the literature that assigned the N_2-fixing activity exclusively to prokaryotes (Young et al., 1992; Zehr et al., 2003). If we consid-ered that only nifH-containing cells are able of fix dinitrogen, the weak N_2 fixation rates measured all through the Mediterranean Sea (Bonnet et al., 2010) must be attributed to UCYN-A in station A and other organisms than photosynthetic picoeukaryotes in station B and C.

4.3 Diazotrophs richness recovery on nifH phylogeny

In order to search for these organisms, nifH phylogenetic analyses were done. The dominating nifH sequences from the picoplanktonic size fraction of all three clone li-braries were belonging to a group of Bradyrhizobia, affiliated to α-proteobacteria that has so far never been detected in the marine environment (group 2′, Fig. 6). In addition of Bradyrhizobia, two other 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 recov-ered from station B. UCYN-A, γ-proteobacteria and distantly related α-proteobacteria

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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., 2010) could be attributed to a mix of rhizobia in station C, when UCYN-A and γ-proteobacteria where 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 fresh water 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 rizhobia, 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, has been estimated to contribute for tow third 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 remarkable discovery from this study concerns the widely distributed group 2′ of Bradyrhizobia, which closest relatives is the fresh water strain ORS391 (Fig. 6). 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., 1998; Nico Nouwen, personal communication, 2010). This metabolic activity is probably related to their photosynthetic capacity, a common feature among Bradyrhizobia cluster (Giraud and Fleischman, 2004). Despite diazotrophic phototrophic free living bacteria have never been discovered to date in

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the marine environment (Madigan, 1995; Riemman et al., 2010), we hypothesize that, similarly as their closest relatives, the Mediterranean Bradyrhizobia detected in this study are able to acquire 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 hypothesises 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 cyanobacteria development, temperature, iron and phosphate are the most regularly cited (Mague, 1974; Pearl et al., 1994; Breibarth and LaRoche, 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 in ecological advantage compare to other phytoplankton species, as they can acquire nitrogen directly from dissolved atmospheric N_2 (Tyrell et al., 1999). Upper mediterranean nitrate-deprived waters were confirmed to be more favorable than deepest 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. The nitrogenase that catalyses N_2-fixation is a cluster of metal rich enzymes which reaction centre are all iron dependant and require sixteen adenosine-triphosphate to produce ammonium, the biologically assimilable form of nitrogen (Seefeldt et al., 2009). This imply that diazotrophic cells may require higher iron and phosphate concentrations than non-diazotrophic ones.

Across the Mediterranean Sea, filamentous cyanobacteria were likely inhibited in surface waters by iron and inorganic phosphate, which 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 is considered as the first indicator of phosphate availability in oligotrophic marine ocean, is 5 times lower in surface Mediterranean waters than the one defined to prevent *Trichodesmium* spp. growth (Moutin et al., 2005, 2008; Mauriac et al., 2010). Conversely, UCYN have been shown to grow on deprived iron waters and to reach 10^2 to 10^3 cell ml^{-1} in marine areas with similar or lower inorganic phosphate concentrations (Berman-Frank et al., 2007; see review in Le Moal and Biegala, 2009). Moreover, phosphate enrichment experiment performed at station A, where UCYN-A were detected did not induce any growth enhancement (Ridame et al., 2010). These differential cell types requirement towards phosphate are not reflected by their genomic potential (Dyhrman and Haley, 2006; Orchard et al., 2009). Both *Trichodesmium* and UCYN-B hold a broad spectrum of genes coding (i) for high affinity system to acquire weakly concentrated inorganic phosphate and (ii) for the scavenge of phosphomonoester, the dominant form of organic phosphate in the marine environment (75%, Kolowith et al., 2001). In addition, we provide in this study (Table 3) the analysis of UCYN-A genome, which despite its reduce size, has conserved similar genetic equipment, in accordance with that of other picoplanktonic cyanobacteria (Scanlan et al., 2009; Tripp et al., 2010). Interestingly, *Trichodesmium* seems to be the only marine cyanobacteria that have 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), hence the low surface/volume ratio of filamentous cyanobacteria seems an obvious disadvantage compare to UCYN to efficiently grow in oligotrophic environment.

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When iron and phosphate seems to control the development of *Trichodesmium* and *Richelia*, the temperature has been recently suspected to limit the one of UCYN (Moisander et al., 2010). At stations where species richness was characterised, temperature was 25 °C (Moutin et al., 2010), being slightly under or above the optimal

5 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). The absence of UCYN-B and C in this study and the one of UCYN-A in the central and eastern part of this transect indicate that other factors must co-limit or prevent their development. Still, UCYN-A limiting factors seems slightly relaxed in the less oligotrophic western basin and at coastal

10 station off Israel, where they were recovered (Man-Aharanovich et al., 2007). We suspects 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 dependant from an unknown organic source of carbon, a unique feature among planktonic diazotrophic cyanobacteria. The origin of

15 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,

20 2009). Although it has not been proven that those labelled cells belong to UCYN-A, the conspicuous similitude between UCYN-A and plastid's genome and metabolism suggests these association highly probable (Neuhaus and Emes, 2000; Tripp et al., 2010). This information on UCYN-A further sustain the narrow link between UCYN lineage and plastids.

25 In this first Mediterranean basin wide study the photosynthetic diazotrophs *Trichodesmium* sp., UCYN-B and C were not detected, although these free living organisms show significant degree of nutritional independence towards carbon and nitrogen. While low phosphate and iron concentrations could explain the absence of *Trichodesmium* sp. the limiting factors for the development of UCYN-B and C remain

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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) similarly as their terrestrial counterparts, rhizobia may form punctual symbiosis with marine higher plants such as seagrass; (ii) when Bradyrhizobia, the most widely distributed diazotrophs in this Mediterranean study, could also acquire carbon through photosynthesis; (iii) UCYN-A, which by many ways resemble plastids with their genome size and metabolic pathways, probably developed associations and/or symbiosis with inert particles or eukaryotes to acquire organic carbon necessary for their development. This close relationship between UCYN and plastids sustains other recent biochemical and phylogenetic studies which suggested that UCYN could be plastid ancestor. All these lines of evidence are in accordance with the recovery of many plastid sequences in this study using a specific UCYN oligonucleotide.

To answer all those hypothesis, further works will be necessary to characterize the different strategies developed by picoplanktonic diazotrophs to acquire carbon in oligotrophic environment. Among UCYN, this area of research may help us to better understand their suggested role in plastid evolution.

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

Primer	Target	Sequence (5′ to 3′)	Reference
27F	16S rDNA of prokaryotes	AGA GTT TGA TCM TGG CTC AG	Gordon and Giovannoni, 1996
1518R	16S rDNA of prokaryotes	AAG GAG GTG ATC CAN CCR CA	Gordon and Giovannoni, 1996
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
nifH3	nifH of prokaryotes	ATR TTR TTN GCN GCR TA	Zani et al., 2000
nifH4	nifH of prokaryotes	TTY TAY GGN AAR GGN GG	Zani et al., 2000
nifH1	nifH of prokaryotes	TGY GAY CCN AAR GCN GA	Zehr and McReynolds, 1989
nifH2	nifH of prokaryotes	ADN GCC ATC ATY TCN CC	Zehr and McReynolds, 1989

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

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
	Group 2	5	BOUM_C5	Uncultured bacterium clone S25_1306 (EF574962)	99
				Chrysosphaera sp. CCMP296 plastid (AY702107)	91
	Group 3	1	BOUM_B2	Uncultured cyanobacterium clone SHAB462 (GQ348575)	97
				Rhizochromulina sp. CCMP1253 plastid (AY702125)	95
	Group 4	1	BOUM_B6	Unidentified eukaryote OM270 (U70723)	91
				Chroomonas sp. SAG 980-1 plastid (AF545625)	91
	Group 5	1	BOUM_C9	Unidentified eukaryote OM270 (U70723)	96
				Chroomonas sp. SAG 980-1 plastid (AF545625)	92
	Group 6	3	BOUM_B1	*Crustomastix stigmatica plastid (FN563093)*	88
	Group 7	3	BOUM_B7	*Crustomastix stigmatica plastid (FN563093)*	89
	Group 8	2	BOUM_C6	*Dolichomastix tenuilepis plastid (FN563094)*	93
	Group 9	1	BOUM_B8	Uncultured bacterium (AB307974)	99
				Euptilota articulata (DQ026695)	86
nifH	Group 1′	3	BOUM_A4	Uncultured marine bacterium clone HT70A1_T7 (DQ118201)	100
				Cyanobacterium 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	Unculture microorganism clone H05_DNA_E10 (EF568515)	98
				Denitrovibrio acetiphilus DSM 12809 (CP001968)	77

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

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 component	ATPase and permease	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.
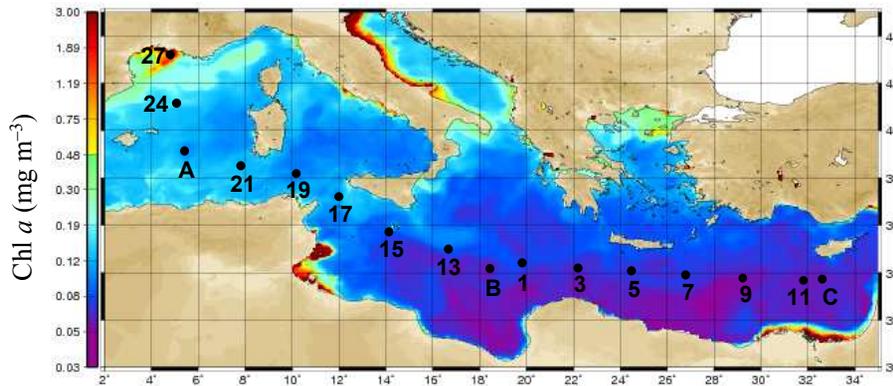
Fig. 1. Position of sampled stations during the BOUM transect across the Mediterranean Sea. Numbers (1–27) represent stations where surface TSA-FISH analyses were done and letters (A, B, and C) represent stations where TSA-FISH analyses were done at 12.5 m and DCM$^+$ as well as 16S and nifH phylogenies at 12.5 m.
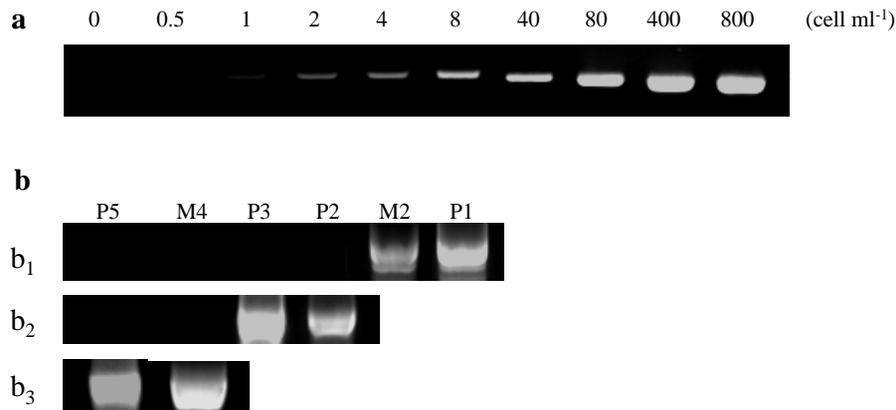
Fig. 2. Different PCR amplification tests for the detection of scarce microorganisms. **(a)** Definition of PCR detection limit of UCYN using a range of concentrations of *C. watsonni* with Nitro821/Cya359 16S primers. **(b)** Test of stability of PCR efficiency with time on different type of DNA templates collected in the Pacific Ocean (P) or in the Mediterranean Sea (M) and stored at −80 °C for 1 to 5 years. 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.

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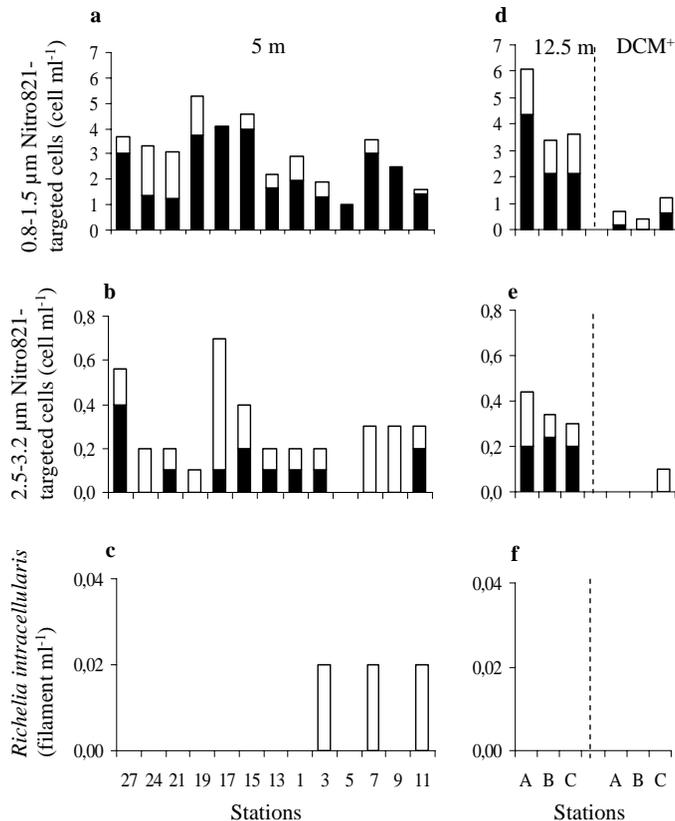
Fig. 3. Epifluorescent microscopic counts of Nitro821-targeted UCYN and filamentous diazotrophic cyanobacteria across the Mediterranean Sea within the 0.2–3 µm (black bars) and the 3–10 µm combined with >10 µm (white bars) size fractions. Counts were done at 5 m depth **(a, b, c)** and at two nutrient 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).

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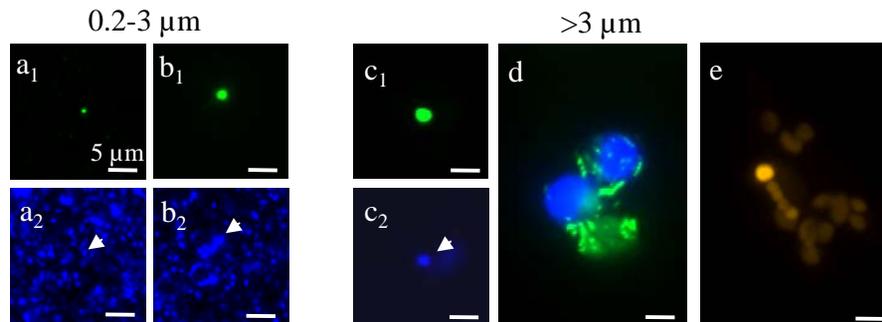
Fig. 4. Epifluorescent microphotographs from Nitro821-targeted UCYN and filamentous diazotrophic cyanobacteria detected in the Mediterranean Sea. Nitro821-targeted UCYN were labelled with FITC (green fluorescence, a_1, b_1, c_1, d) using TSA-FISH technique. a_2, b_2, c_2 and d showed corresponding microphotograph 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.

90 ┌ BOUM_B4
92 ├ BOUM_C10
90 ├ BOUM_C3   **Group 2**
92 ├ BOUM_C4
├ BOUM_C5
90 └ EF574962
85
91   Chrysosphaera sp. CCMP296
92 ┌ Skeletonema costatum
├ Odontella sinensis   **Group 3**
90 ├ BOUM_B2
├ GQ348575
└ Rhizochromulina sp. CCMP1253

67 ┌ Chrysochromulina sp. MBIC10513
├ Imantonia rotunda
98 ┌ Emiliania huxleyi
└ Ochrosphaera neapolitana
90 ┌ BOUM_B6   **Group 4**
├ BOUM_C9   **Group 5**
└ U70723

Heterokon-
tophyta

Haptophyta

92 ┌ Aristoptilon mooreanum
66 ├ Gracilaria tenuistipitata var. liui
└ Palmaria palmata

Rhodophyta

87
86
90 ┌ Pyrenomonas helgolandii
├ Storeatula sp. CCMP1868
└ Cryptomonas ovata

Cryptophyta

90 ┌ BOUM_B1
92 ├ BOUM_B5
├ BOUM_B9   **Group 6**
90 └ Crustomastix stigmatica
90
90 ┌ BOUM_B10
├ BOUM_A2   **Group 7**
├ BOUM_B7
74 └ Dolichomastix tenuilepis
┌ BOUM_B3   **Group 8**
74 └ BOUM_C6
└ Mantoniella squamata
74 ┌ BOUM_B8   **Group 9**
70 └ Chlorella vulgaris

Chlorophyta

73
84
88   Chlorarachnion reptans
Chlamydomonas reinhardtii
┌ Vitis vinifera
└ Mesostigma viride

Land plants

93
89   Cyanophora paradoxa
cyanobacterium UCYN-A

Glaucophyta

90 ┌ BOUM_A5
90 ├ BOUM_A7
90 ├ BOUM_A1   UCYN-A
├ BOUM_A9
90 ┌ BOUM_A4   **Group 1**
└ BOUM_A8
92 ┌ BOUM_A6
90 └ BOUM_A10
└ BOUM_A3

72 ┌ Cyanothece sp. ATCC 51142   UCYN-C
85 ├ symbiont of Climacodium frauenfeldianum
├ Crocosphaera watsonii WH 8501   UCYN-B
52 ├ Rhopalodia gibba   UCYN-C
├ Cyanothece sp. PCC 8801
61 ├ Trichodesmium erythraeum IMS101
61 ├ Oscillatoria spongeliae
95 94 ┌ Nodularia baltica
80 └ Nostoc sp. PCC 7120
90 ├ Calothrix sp. PCC 7507
61 ┌ Synechococcus sp. Minos02-C2C2
67 ├ Prochlorococcus marinus str. MIT 9303
99 ├ Leptolyngbya sp. PCC 7375
└ Gloeobacter violaceus PCC 7421

Cyanobacteria

Plastids

Methanosarcina barkeri   Archaeabacteria

0.10

Fig. 5. Phylogenetic tree of 16S rDNA sequences from Cyanobacteria and picoeukaryotes or land plants plastids. 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.
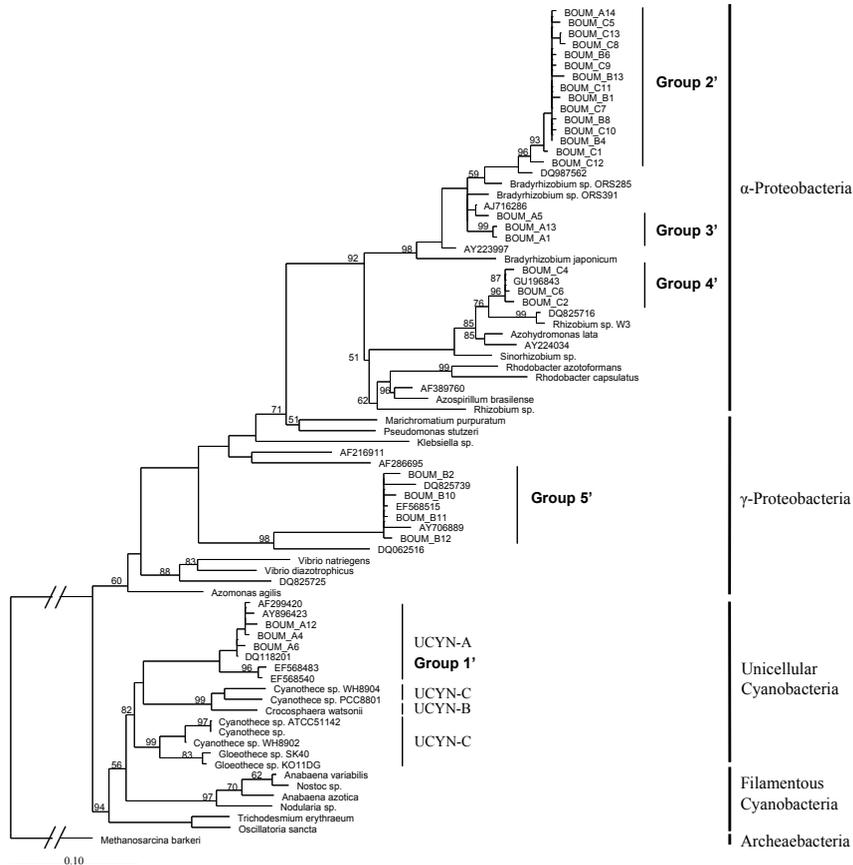
Fig. 6. Phylogenetic tree of nifH sequences. Sequences obtained in this study are referred to (i) by the oceanographic transect BOUM, (ii) the station at which they were sampled (A, B, or C), and (iii) their clone number Bootstrap values >50% are indicated at the nodes. Scale bar = 0.1 substitution per nucleotides.