Diazotrophic unicellular cyanobacteria in the northwestern Mediterranean Sea: A seasonal cycle

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Abstract

Unicellular diazotrophic cyanobacteria ($UCYN_2$-Fix lineage) were detected using whole-cell hybridization of specific Nitro821 oligonucleotide probe at the coastal and oligotrophic station SOMLIT off Marseilles (France). This station was sampled monthly, for a year and a half (June 2006–November 2007). The $UCYN_2$-Fix community was dominated at 99.9% by picoplankters (0.7–1.5 μm) mainly as free living in the 0.2–3-μm size fraction. They were present all the year long with a mean density of 4.6 cell mL^{-1}, except in summer 2006, when concentrations reached 1.9×10^3 cell mL^{-1} and 5.3×10^3 cell mL^{-1} in June and July, respectively. During this bloom, picoplanktonic $UCYN_2$-Fix represented 10.7% of the picocyanobacterial community. The larger size fractions (3–10 μm and >10 μm) were also dominated (98.9%) by picoplanktonic $UCYN_2$-Fix cells, associated with inert particles, or dinoflagellates. However, hardly any nanoplanktonic $UCYN_2$-Fix were detected (0–0.12 cell mL^{-1}), and *Trichodesmium* sp. was observed only once in summer at low concentration (0.03 trichome mL^{-1}). We hypothesize that a combination of abiotic parameters, such as elevated temperature, absence of nitrate, presence of phosphate, and an exceptionally high urban pollution event, explain the large bloom of potentially diazotrophic picocyanobacteria. Further studies are needed to confirm the identity of these small cells and their role in nitrogen cycle and marine productivity, especially since some effects of climate change (e.g., increased surface warming and upper-water-column stratification) may increase their importance in a near future.

The Mediterranean Sea has been long known to be very oligotrophic with phytoplankton and bacterial communities being limited by very low concentrations of nutrients in surface water and, in particular, phosphate (Thingstad et al. 1998). Despite this oligotrophy through the central basin, littoral, and coastal waters are enriched with phosphate and nitrate because of river discharges (Béthoux et al. 1992). In addition, Saharan and anthropogenic particles provide a source of phosphate, nitrate, and iron (Bonnet and Guieu 2006; Sandroni et al. 2007). Furthermore, it has been shown that a significant source of new nitrogen in the Mediterranean Sea could be of biological origin through the fixation of dissolved atmospheric N_2 by diazotrophic prokaryotes. Unlike other water bodies which have the classic Redfield N : P ratio of 16 : 1, Mediterranean deep waters show unusually high N : P ratios, respectively, 22 and 24 for the occidental and oriental basins (Béthoux and Copin-Montégut 1986). In addition, based on isotope studies, Pantoja et al. (2002) estimated that nitrogen fixation could explain 20% and 90% of the isotopic excess of deep nitrogen in the occidental and oriental Mediterranean basins, respectively. Finally, significant N_2 fixing activity has been recently measured both in the north of the Mediterranean Sea at the offshore DYFAMED (DYnamique des Flux Atmosphériques en MEDiterranée) station (Garcia et al. 2006) and south of Cyprus in the most oligotrophic site of the oriental basin (Rees et al. 2006). At the DYFAMED site, diazotrophy sustains up to 7% of new primary production over a full annual cycle and could reach up to 55% in summer (Garcia et al. 2006).

Although diazotrophy could significantly contribute to the Mediterranean biogeochemical cycles, very few studies have yet directly examined the organisms responsible for this activity. According to Béthoux and Copin-Montégut (1986), two-thirds of the diazotrophy may be attributable to planktonic activity. Among the planktonic diazotrophs, *Trichodesmium* sp. is a well-known filamentous cyanobacterium that can produce massive surface accumulations visible from space (Dupouy et al. 2000). In the Mediterranean Sea such accumulations have never been reported. However, Trégouboff (1957) described *Trichodesmium thiebautii* and *Richelia intracellularis* as Mediterranean planktonic species. Trichomes have been detected in the northwestern basin but in such low concentration that it could explain only 0.6% of the planktonic nitrogen fixation (Béthoux and Copin-Montégut 1986). Consequently, a large majority of the remaining planktonic activity (99.4%) may be attributable to unknown microorganisms.

Recently, active diazotrophic Archaea, heterotrophic Bacteria, and Cyanobacteria were detected at different seasons at coastal and offshore stations near Israel (Man-Aharonovich et al. 2007). Among diazotrophic cyanobacteria, only unicellular cells affiliated to group A were detected. Yet three groups (A, B, and C) of unicellular diazotrophic cyanobacteria have been regularly sequenced in both the Pacific and the Atlantic Oceans (Zehr et al. 2001; Falcón et al. 2004; Foster et al. 2007). While groups B and C are closely affiliated to the nanoplanktonic species *Crocosphaera* sp. and *Cyanothece* sp., respectively (Church et al. 2005; Foster et al. 2007), group A is distantly related to group C and has never been observed or cultivated so far (Foster et al. 2007; Zehr et al. 2007).

Unicellular diazotrophs diversity has been investigated using the polymerase chain reaction (PCR) targeting *nifH* genes and transcripts that code for a structural component of nitrogenase, the enzyme complex responsible for

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diazotrophic activity (Zehr et al. 2001). 16S rDNA has also been used as a target gene to detect unicellular diazotrophic cyanobacteria ($UCYN_2$-Fix lineage) using the specific PCR primer Nitro821 (Mazard et al. 2004). This was possible as both 16S rDNA and *nifH* genes have coevolved and their phylogeny show strong similarity (Rosado et al. 1998; Zehr et al. 2003). Recently, 16S rRNA-specific Nitro821 probe was used in association with the tyramide signal amplification fluorescent in situ hybridization (TSA-FISH) technique in several studies to describe size class diversity within the $UCYN_2$-Fix lineage (Biegala and Raimbault 2008; I. C. Biegala unpubl.). These studies revealed for the first time significant concentrations of coastal and oceanic picocyanobacteria over a large range of nutrient concentrations. These organisms were either free living or associated with particles and eukaryotes.

Our views on the distribution of photosynthetic diazotrophs have recently evolved, especially concerning unicellular phylogenetic groups. Their distribution is much wider than previously thought and includes temperate and coastal waters (Needoba et al. 2007; Biegala and Raimbault 2008) as well as nutrient-rich environments (Foster et al. 2007; Short and Zehr 2007). Although these spatially extensive studies have provided highly valuable information by revealing the global importance of this functional group of organisms, these snapshot studies would benefit from time-series investigations to improve our understanding of diazotroph seasonal dynamics and bloom formation. So far, very few studies have examined the temporal succession of photosynthetic diazotroph abundance and/or activity (Orcutt et al. 2001; Garcia et al. 2006), and to our knowledge none have previously involved unicellular diazotrophic cyanobacteria.

The aims of this study were (1) to find out whether unicellular diazotrophic cyanobacteria were present in the northwestern Mediterranean Sea, (2) to assess their changes in size class diversity over a year-and-a-half seasonal cycle, and (3) to find out whether the cells were free living or attached to inert particles or organisms. The coastal SOMLIT station off Marseilles (France, 830,000 habitants) was chosen, as it is mainly oligotrophic, although it receives high concentrations of nutrients from the Rhône River flood each spring. Cells from $UCYN_2$-Fix lineage were detected within three size fractions (0.2–3 μm, 3–10 μm, and >10 μm), using the specific Nitro821 probe associated with TSA-FISH technique.

Methods

Study site, sampling and size fractionation—Environmental samples were collected from June 2006 to November 2007 while on board the RV *Antédon II* at the SOMLIT (Service d'Observation en Milieu Littoral) station (5°17′30″E and 43°14′30″N, 5 km off Marseilles; Fig. 1). This station belongs to the national SOMLIT network of coastal stations regularly sampled for 13 years. Once a month, 10 liters of subsurface (1-m depth) seawater were collected with a Niskin bottle for TSA-FISH assays. An additional 10 other liters were collected for nutrients and chlorophyll *a* (Chl *a*) concentrations, and temperature and salinity were measured
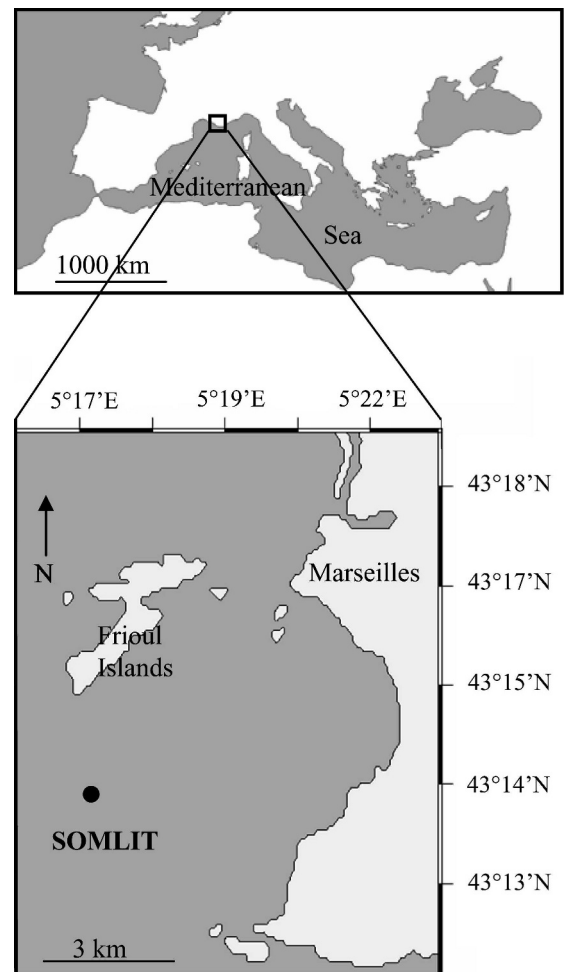

Fig. 1.   Localization of the SOMLIT-Marseilles station in the bay of Marseilles (France).

in situ with a conductivity temperature depth profiler (SBE 19+, Seabird).

Plankton from seawater samples were collected from three size fractions (0.2–3 μm, 3–10 μm, and >10 μm). Depending on the degree of oligotrophy, 1.1–8.8 liters were filtered by gravity through 10-μm ISOPORE™ (Millipore) 47-mm filters; 650 mL–2.5 liters of the remaining filtrate were then collected by gravity on 3-μm ISOPORE 47-mm filters. Finally, 200 mL of the <3-μm filtrate were collected under 200-mmHg vacuum on 0.2-μm ISOPORE 47-mm filters. Cells were then fixed with buffered paraformaldehyde, dehydrated with ethanol and, stored at −80°C according to Biegala and Raimbault (2008). For the larger size fractions, it is important to use gravity filtration to avoid the filters clogging and trapping free-living picoplankton within >3-μm size fractions.

TSA-FISH—Before hybridization, filters were covered with 0.1% warm agarose to avoid cell loss, according to the protocol of Biegala and Raimbault (2008). However, 0.1% of agarose treatment was not sufficient to avoid cell loss for larger organisms and particles and was significantly improved by using 0.4% on 10-μm filters (data not shown).
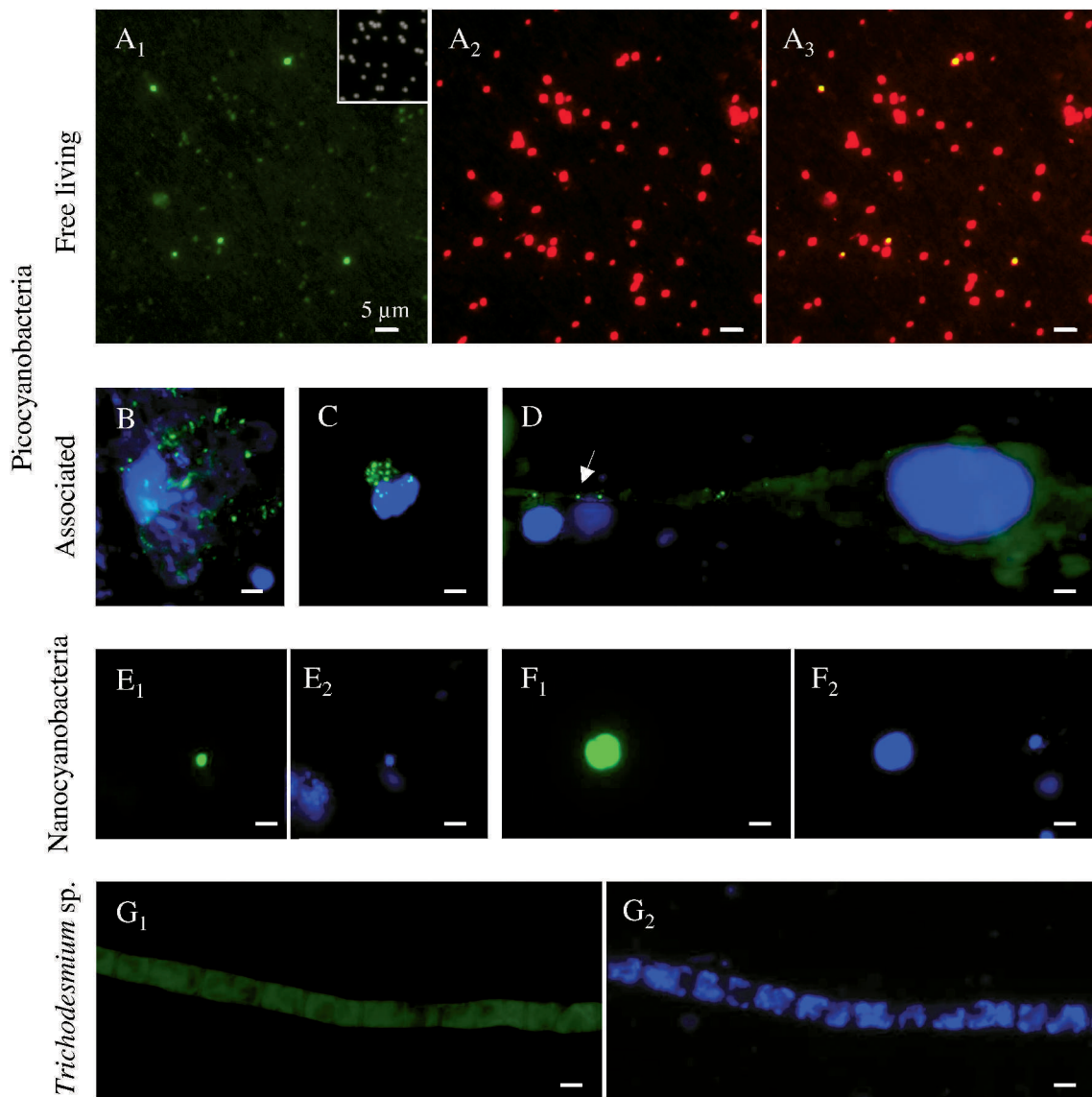
Fig. 2. Microphotographs from unicellular diazotrophic cyanobacteria hybridized with the specific probe Nitro821, labeled with FITC (green fluorescence, A_1, B–D, E_1, F_1), while the filamentous diazotrophic cyanobacteria *Trichodesmium* sp. was not hybridized by Nitro821 and showed only green autofluorescence (G_1). (A_2) Corresponding microphotograph to A_1 revealed cells hybridized with the general cyanobacterial probe Cya664 colored with CY3 (red fluorescence). (A_3) Merging microphotograph of A_1 and A_2 showing cells double hybridized (in yellow). B–D, E_2, F_2, and G_2 showed DAPI-stained DNA (blue fluorescence). Diazotrophic picocyanobacteria were either (A) free living in the water column or associated (B) with inert particles or (D) with dinoflagellates. They were either detected (C) close to the nucleus of nonthecate dinoflagellate or (D) at the end of a *Ceratium* sp. theca. E and F: free-living diazotrophic nanocyanobacteria. Insert microphotograph in A_1 shows 0.97-μm beads. Arrow points to the smallest Nitro821-hybridized picocyanobacteria detected (0.7 μm). Scale bar = 5 μm.

Hybridizations were done according to Biegala and Raimbault (2008) (Figs. 2, 3). In brief, each sample from the different-size fractions were hybridized with the specific horseradish peroxidase (HRP)-labeled 16S rDNA Nitro821 probe (Thermo, CAA GCC ACA CCT AGT TTC; Mazard et al. 2004) and subsequently stained with FITC (Fluorescein IsoThioCyanate, TSA-Kit, Perkin Elmer). In order to measure the proportion of Nitro821-targeted cells within the overall picocyanobacterial community, double hybridizations were done with the general cyanobacterial HRP-labeled 16S rDNA Cya664 probe (GGA ATT CCC TCT GCC CC; Schönhuber et al. 1999) and subsequently stained with Cyanine 3 (CY3; TSA-Kit, Perkin Elmer). These double hybridizations were done in July 2006 and November 2006 at contrasting concentrations of Nitro821-targeted cell (Figs. $2A_1$–A_3, 3). All cells were counterstained with DAPI (Sigma-Aldrich), a specific dye for prokaryotic and eukaryotic DNA.

Microscopy—Microphotographs (Fig. 2) were acquired with an epifluorescence BX61 microscope (Olympus Optical) equipped with a 40× (NA 0.75 N Plan Fluor
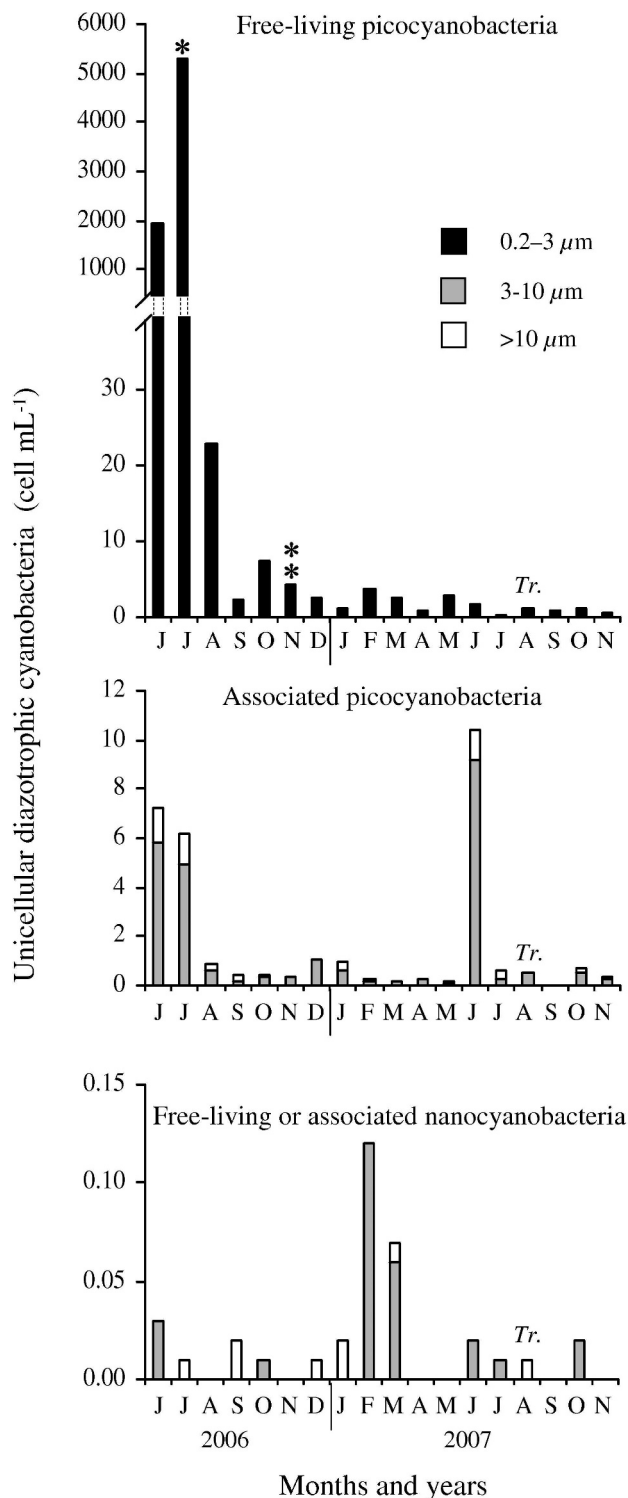
Fig. 3. Concentrations of unicellular diazotrophic pico and nanocyanobacteria at the SOMLIT-Marseilles station over a year and a half. Counts of Nitro821-labeled cells were recovered from three size fractions (0.2–3 μm, 3–10 μm, and >10 μm). Diazotrophic picocyanobacteria were recovered either as free living in the 0.2–3-μm size fraction (black bars) or attached to inert particules or dinoflagellates in the 3–10-μm and >10-μm size fractions (gray and white bars, respectively). Most of the nanocyanobacteria were recovered free living in the 3–10-μm size

PH2 DT: 0.51 mm, Olympus) objective and a mercury lamp (HBO 100W/2, Osram), a camera (Retiga-SRV 1394, QImaging), and the Image-Pro+ software (Media Cybernetics). Different excitation (ex.) and emission (em.) dichroic filters were used according to the different dyes: (360 ± 20 ex., 410 ± 5 em. for DAPI [blue fluorescence]; 480 ± 15 ex., 535 ± 20 or 510 long-pass em. for FITC [green fluorescence]; 540 ± 12 ex., 605 ± 22 em. for CY3 [red fluorescence]).

Cells counts and cells size—Entire surfaces of each filter portion (~0.6 cm²) were counted (Fig. 3). According to oligotrophic status, surfaces corresponded to 75–320 mL of water for the >10-μm fraction, 40–160 mL for the 3–10-μm fraction, and 13 mL for the 0.2–3-μm fraction. Entire surfaces were counted for most of the samples, but when cell densities were over 10 cells per microscopic field, only 10 randomly chosen fields were counted. The sizes of the unicellular cells were determined according to 0.97-μm calibration beads (Fig. 2A$_1$).

Environmental parameters—Environmental parameters were measured according to standard protocols recorded in Aminot and Chaussepied (1983). Seawater samples for nitrate (NO$_3^-$), nitrite (NO$_2^-$), and phosphate (PO$_4^{3-}$) determinations were collected with 100-mL polyethylene flasks and analyzed with an automated Technicon analyzer (Technicon III, Brian and Luebbe, Axflow). Detection limit for these nutrients were 0.05 μmol L^{-1} (±0.05), 0.02 (±0.02) μmol L^{-1}, and 0.02 μmol L^{-1} (±0.02), respectively. Samples for ammonium (NH$_4^+$, 0.05 μmol L^{-1} ± 0.05) were collected in 100-mL glass flasks, and concentrations were measured with a spectrometer (UV 160A, Shimadzu). Total Chl a concentrations were measured from 1 liter of water collected on GF/F filters and analyzed with a fluorometer (10.005R, Turner Designs).

Results

Size class diversity—Nitro821-labeled cells were detected all along the year at the coastal station off Marseilles (Fig. 1). These cells were picoplankters and nanoplankters ranging from 0.7 μm to 1.5 μm and 3 μm to 10 μm in diameter, respectively (Fig. 2A–F). A large majority of the Nitro821-targeted picocyanobacteria were dually labeled by the general cyanobacterial probe Cya664 (85%) in July and November 2006. The larger filamentous diazotrophic cyanobacteria *Trichodesmium* sp. was also detected at the SOMLIT-Marseilles station because of its remaining autofluorescence, as it was not targeted by the Nitro821 probe (Fig. 2G). Diazotrophic picocyanobacteria were

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fractions, while they were either free living or attached to inert particles in the largest size fraction. Single and double asterisks mean the percentage of Nitro821-targeted cells within the Cya664-targeted community, which represents 10.7% in July and 0.01% in November, respectively. *Tr.* means that *Trichodesmium* sp. was detected at 0.03 trichome mL^{-1}.

either free living (Fig. 2A$_1$) or associated with inert particles (Fig. 2B) or dinoflagellates (Fig. 2C,D), which were easily identified by their condensed chromosomes (data not shown). Within dinoflagellates, labeled cells were often observed close to the nucleus of small (<10 μm) nonthecate species (Fig. 2C) or associated with *Ceratium* sp. theca (Fig. 2D). Similarly, as for picocyanobacteria, Nitro821-labeled nanocyanobacteria were either free living (Fig. 2E,F) or associated with inorganic particles (data not shown). However, associations with eukaryotes have never been observed for these larger unicellular diazotrophs.

Abundance—The Nitro821-labeled cells were predominantly picoplankters (99.996%); very few nanoplankters were detected (0.004%) with this probe. The concentration of these picoplankters was extremely high over at least a 1-month period, reaching 1.9×10^3 cell mL^{-1} and 5.3×10^3 cell mL^{-1} in June and July 2006, respectively (Fig. 3). The minimum of Nitro821-labeled picoplanktonic cells was detected in July 2007 with 1 cell mL^{-1}, when they averaged 4.6 cell mL^{-1} for the remaining months. Among these picoplanktonic cells, 99.6% were free-living organisms, recovered in the 0.2–3-μm fractions. The remaining 0.4% were associated with inert particles and often dinoflagellates within the larger size fractions (3–10 μm and >10 μm; Fig. 3). The blooms of diazotrophic picoplanktonic cells associated with dinoflagellates appeared mainly in early summer, when picocyanobacteria associated with particles were recovered all year long at low abundance (<1 cell mL^{-1}; data not shown). Maximum labeled nanocyanobacteria were detected in late winter, and *Trichodesmium* sp. was observed only once, in August 2007, at low concentration (0.03 trichome mL^{-1}). Among the total picocyanobacteria community, diazotrophs accounted for 10.7% in July 2006 and only 0.01% in November 2006, when the community concentrations averaged $5.0 \pm 2.7 \times 10^4$ and $3.0 \pm 2.3 \times 10^4$ cell mL^{-1}, respectively.

Environmental parameters—All the environmental parameters were compared to the average over the past 10 years. For the full studied period, temperature (mean = $18.5 \pm 3.5°C$), salinity (mean = 38.2 ± 0.2), and Chl *a* (mean = 0.49 ± 0.37 μg L^{-1}) concentrations were, respectively, $0.7°C$, 0.1, and 0.21 μmol L^{-1} over the past 10 years' means; nutrient concentrations were three times below the 10-year means for NH$_4^+$ (mean = 0.32 ± 0.23 μmol L^{-1}) and PO$_4^{3-}$ (mean = 0.1 ± 0 μmol L^{-1}) and seven times for NO$_3^- +$ NO$_2^-$ (mean = 0.27 ± 0.39 μmol L^{-1}; Fig. 4). Sea surface temperature doubled between the minimum and the maximum values, respectively: $13.6°C$ in March 2007 and $27.2°C$ in July 2006. This exceptionally high temperature was $6.4°C$ above the mean of previous July months and has been reached only once before in July 2005 over the past 10 years. In contrast, July 2007 was $5.2°C$ below the mean of July months. Changes of nutrients concentrations revealed different pattern of variability. Phosphate and ammonium showed homogeneous trends over the studied period, whereas nitrate and nitrite concentrations clearly increased in winter and spring and showed very low concentrations all through summer and fall.

Discussion

Size class diversity—In the Mediterranean north occidental basin, three types of diazotrophic cyanobacteria were detected: picoplanktonic, nanoplanktonic, and filamentous *Trichodesmium* sp. Among these three groups, only *Trichodesmium* sp. has been previously observed in the Mediterranean Sea (Trégouboff 1957; Béthoux and Copin-Montégut 1986). Although the report of diazotrophic picocyanobacteria is new for the Mediterranean Sea (Figs. 2A$_1$,A$_2$,B–D), similar cell types (0.7–1.5 μm) have been recently discovered in coastal and oceanic Pacific waters using the same technical approach (Biegala and Raimbault 2008; I. C. Biegala unpubl.). In this study we confirmed the phylogenetic affiliation of most of these picoplanktonic cells to Cyanobacteria phylum, as 85% of Nitro821-targeted cells were double hybridized by the general cyanobacterial probe Cya664 (Fig. 2A$_1$–A$_3$). However, 15% of them were not targeted by Cya664. This may be due to the fact that this general probe is not fully comprehensive for targeting all unicellular cyanobacteria, as shown by Schönhuber et al. (1999). Another hypothesis is that oligonucleotide probes or primers are molecular tools that may target organisms for which they were not primarily designed and for which sequences may have not yet been downloaded on genetic databases. The identity of the dually labeled diazotrophic picocyanobacteria is unknown, although Biegala and Raimbault (2008) hypothesized that they could belong to group A, and Zehr et al. (2007) recently suggested that group A could be of picoplanktonic size. Furthermore, among unicellular diazotrophic cyanobacteria, Man-Aharanovich et al. (2007) detected only the group A cell type in March and July 2006 at a coastal station in the Mediterranean oriental basin. In addition to these picoplanktonic cells, this study provides the first report of potentially diazotrophic nanoplanktonic cyanobacteria in the Mediterranean Sea. Nanoplanktonic diazotrophic organisms have been essentially affiliated to groups B and C of diazotrophic cyanobacteria with known genus such as *Crocosphaera* sp. and *Cyanothece* sp. (Zehr et al. 2001; Falcón et al. 2004).

The Nitro821-targeted picocyanobacteria detected in this study were recovered in all size fractions and were either free living or associated with inert particles or dinoflagellates (Fig. 2A–D). Similar associations with nonthecate dinoflagellates and other eukaryotes have been mentioned in coastal and oceanic southwest Pacific waters and were hypothesized to be of symbiotic origin (Biegala and Raimbault 2008; I. C. Biegala unpubl.). We show in this study that such intimate relationship may be extended to the thecate dinoflagellates *Ceratium* sp. (Fig. 2D). Contrary to potentially diazotrophic picocyanobacteria, Nitro821-targeted nanocyanobacteria from the >10-μm size fraction were not associated with eukaryotes or with particles but were associated in unstained mucilage as previously described (Biegala and Raimbault 2008; I. C. Biegala unpubl.). Still, the presence of nanoplanktonic
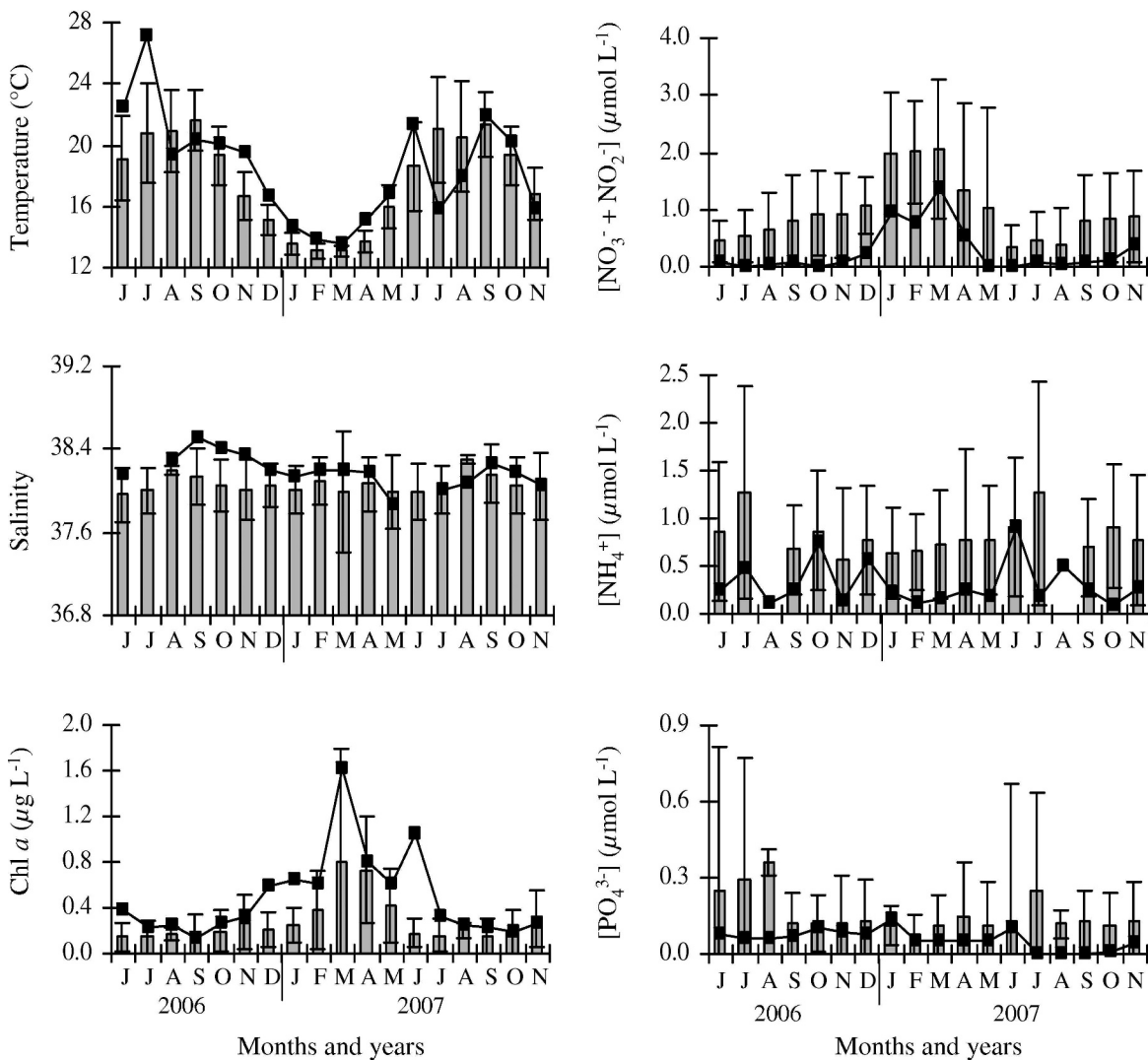
Fig. 4.   Comparison of environmental parameters measured at the SOMLIT-Marseilles station over a year and a half (black square, June 2006–November 2007) with the averages over the past 10 yr (gray bars).

diazotrophic cyanobacteria symbionts of nonthecate *Histoneis* sp. dinoflagellates has previously been mentioned (Foster et al. 2006).

Seasonal changes of diazotrophic cyanobacteria—The diazotrophic cyanobacterial community was essentially dominated by picoplankters. These small UCYN$_2$-Fix–targeted cells (Mazard et al. 2004) were present all year long within a very large range of temperature (13.6–27.2°C) and nutrient concentrations ($<$0.05–1.38 μg L^{-1}, 0.11–0.92 μg L^{-1}, and $<$0.02–0.14 μg L^{-1} for NO$_3^-$ + NO$_2^-$, NH$_4^+$, and PO$_4^{3-}$, respectively) (Figs. 3, 4). These results are in accordance with recent literature where group A has been detected in water temperature as low as 12°C in the Chesapeake Bay and 14–19°C in the northern Pacific Ocean (Needoba et al. 2007; Short and Zehr 2007). In the northwestern Mediterranean Sea, nitrogen fixation has been detected all year long at the offshore DYFAMED station, which shows the same range of oligotrophic and mesotrophic conditions as the SOMLIT-Marseilles station

(Garcia et al. 2006; Fig. 4). In addition, similar UCYN$_2$-Fix picocyanobacteria as the one mentioned in this study were detected in nutrient-rich coastal and oceanic Pacific waters (Biegala and Raimbault 2008; I. C. Biegala unpubl.). All these results support the fact that a part of the UCYN$_2$-Fix community tolerates very large range of environmental conditions.

In addition of having a ubiquitous distribution, UCYN$_2$-Fix cells reached the highest concentrations ever recorded in the literature in the summer of 2006 (Table 1). One reason for such elevated concentrations could be the use of different techniques to estimate unicellular diazotrophic concentrations. Ten studies out of the 16 cited in Table 1 used direct fluorescent microscopy counts based on natural or artificial cell fluorescence. One technique estimated UCYN$_2$-Fix cell concentration by 16S rDNA PCR amplification with a calibration over known cell concentrations (Mazard et al. 2004). The five other studies used a proxy for cell concentrations (i.e., nifH gene copy numbers) quantified by the quantitative polymerase chain reaction

Table 1. Literature review of maximum concentrations recorded for unicellular diazotrophic cyanobacteria (n, concentrations of $NO_3^- + NO_2^-$; —, not mentioned by authors).

Concentrations (mL⁻¹)	Cell information		T (°C)	NO_3^- (μmol L⁻¹)	PO_4^{3-} (μmol L⁻¹)	Chl a (μg L⁻¹)	Date	Location	References
	Size (μm)	Phenotype or phylotype						Ocean or sea, station or area, (lat.; long.)	
>1000*	1.8–3.9	Orange Unicell. Cyano.	28	<1ⁿ	—	<0.1	Summer 1990	Pac. (18–20°N; 175°E)	Ishizaka et al. (1994)
928*	2–8	Orange Unicell. Cyano.	—	—	—	—	Sep 1993	Pac., Sta. ALOHA (22°N; 158°W)	Campbell et al. (1997)
500*	2–3	Orange Unicell. Cyano.	26–29	<0.1ⁿ	0.12	—	Nov 1994	Pac., Sta. 21 (16°S; 150°W)	Neveux et al. (1999)
52*	3–10	Orange Unicell. Cyano.	—	0.0096	—	—	Jul 2000	Pac., Sta. ALOHA (22°N; 158°W)	Zehr et al. (2001)
520*	2.5	Orange Unicell. Cyano.	—	—	—	—	Summer 2001 and spring 2002	Atl. (7–12°N; 45–55°W)	Falcón et al. (2004)
30*	3–7	Orange Unicell. Cyano.	—	—	—	—	Fall 2002	Pac., Sta. ALOHA (22°N; 158°W)	Falcón et al. (2004)
200*	3–7	"Crocosphaera-like"	—	—	—	—	Aug 2001	Pac., Sta. ALOHA (22°N; 158°W)	Montoya et al. (2004)
~380†	>3	B	>29	<0.01–0.15	0.02–2.5	—	Sep 2001	Arabian Sea, Sta. 2 (0°N; 67°E)	Mazard et al. (2004)
1600*	2–3	"Crocosphaera-like"	28	0.16ⁿ	0.11	—	Mar, Apr 1998	Pac., Sta. 22–25 (18°S; 170–175°W)	Campbell et al. (2005)
200‡	—	A	25	0.0025ⁿ	—	0.1	Dec 2002	Pac., Sta. ALOHA (22°N; 158°W)	Church et al. (2005)
2‡	—	B	25	0.0025ⁿ	—	0.1	Dec 2002	Pac., Sta. ALOHA (22°N; 158°W)	Church et al. (2005)
~1000‡	—	B	20–25	—	—	0.16	Aug 2001	Pac., Sta. ALOHA (22°N; 158°W)	Zehr et al. (2007)
~70‡	—	A	20–25	—	—	0.48	Feb 2002	Pac., Kane'ohe Bay (21°N, 157°W)	Zehr et al. (2007)
1300‡	—	B	27	<0.01	<0.02	—	May 2003	Atl., Amazon plume, Sta.37 (10°N; 48°W)	Foster et al. (2007)
260‡	—	A	26	<0.01	<0.02	—	May 2003	Atl., Amazon plume, Sta.37 (10°N; 48°W)	Foster et al. (2007)
140‡	—	C	27	<0.01	<0.02	—	May 2003	Atl., Amazon plume, Sta.37 (10°N; 48°W)	Foster et al. (2007)
~500‡	—	A	24	<0.01	0.1	0.1	Apr 2003	Pac. (23°N; 157°W)	Church et al. (2008)
~100‡	—	B	26	<0.01	0.01	0.1	Oct 2003	Pac. (29°N; 156°W)	Church et al. (2008)
1000‡	—	A	20–23	0.01	0–1.5	—	Mar 2002	Atl. (17–25°N; 27–30°W)	Langlois et al. (2008)
30‡	—	B	26.7	—	0–1.5	—	Nov 2000	Atl. (6°N; 33°W)	Langlois et al. (2008)
10‡	—	C	26.7	—	0–1.5	—	Nov 2000	Atl. (16°N; 53°W)	Langlois et al. (2008)
150§	1–1.5	UCYN₂-Fix lineage	23–25	0.2ⁿ	—	0.25	Oct 2005	Atl., Noumea's lagoon, Sta. D (22°S; 166°E)	Biegala and Raimbault (2008)
3§	3–13	UCYN₂-Fix lineage	23–25	0.2ⁿ	—	—	Oct 2005	Pac., Noumea's lagoon, Sta. C (22°S; 166°E)	Biegala and Raimbault (2008)
17§	0.7–1.5	UCYN₂-Fix lineage	30	0.1	<0.02	0.05	Sep 2006	Pac., Sta.18 (0°N; 165°E)	I. C. Biegala (unpubl.)
0.54§	3–10	UCYN₂-Fix lineage	29	<0.03	0.06	0.25	Sep 2006	Pac., Sta.27 (0°N; 145°E)	I. C. Biegala (unpubl.)
5289§	0.7–1.5	UCYN₂-Fix lineage	27.2	<0.05ⁿ	0.06	0.23	Jul 2006	Med. Sea, Sta. SOMLIT (43°N; 5°E)	This study
0.12§	3–10	UCYN₂-Fix lineage	13.8	0.78ⁿ	0.05	0.61	Feb 2007	Med. Sea, Sta. SOMLIT (43°N; 5°E)	This study

* Phycoerythrin-containing cells counted by epifluorescence microscopy according to their orange fluorescence (under 450–480 nm ex., >515 nm em.).
† Cells concentration from UCYN₂-Fix lineage estimated by 16S rDNA PCR amplification.
‡ nifH gene copies quantify by QPCR.
§ Cells detected by whole-cell TSA-FISH technique using a 16S rDNA probe (Nitro821) specific for the UCYN₂-Fix lineage.

(QPCR) technique. This latter technique may provide an overestimation of specific species concentrations, as the number of nifH gene copies per cell has been shown to vary among diazotrophs (Rosado et al. 1998; Langlois et al. 2008). Nonetheless, Zehr et al. (2007) mentioned a good agreement between group B gene copy numbers and nanoplanktonic orange cell counts. We suspect however, that group A concentrations have been more often likely underestimated using the QPCR approach. Recent studies have mentioned difficulties in amplifying the nifH gene from group A using either degenerate or nondegenerate PCR primers when reverse-transcription QPCR or 16S rDNA amplification demonstrated their presence in each sample investigated (Mazard et al. 2004; Man-Aharanovich et al. 2007). Underestimation of cell concentration could also happen using the TSA-FISH technique, as targeted organisms may harbor an unusually thick cell wall or low ribosome content. One requirement for accurate quantification is for cell wall perforation to allow the large HRP-labeled probes to reach their 16S rRNA target. In this study we applied a perforation protocol developed for prokaryotes with a thick peptidoglycan layer, using both lysozyme and acromopeptidase. Although low ribosome content has been suspected to underestimate TSA-FISH counts, a clear quantification of photosynthetic picoplanktonic cells, as small as 0.8 μm and in a stationary phase of growth, has been demonstrated earlier (Biegala et al. 2003). Despite the fact that each quantification technique mentioned has its limits and holds a certain range of error, the results they provide fall into the same range of maxima, except for the one of this study (Table 1). The use of different methods could thus partially explain the highest concentrations of picoplanktonic UCYN$_2$-Fix at the SOMLIT-Marseilles station, but specific environmental conditions are also suspected to play a role in such bloom formation.

Elevated temperature and oligotrophic conditions have been regularly mentioned to explain high concentrations of unicellular diazotrophic cyanobacteria (Mazard et al. 2004; Langlois et al. 2008). During this seasonal study, we went through two exceptional July months, a very hot one in 2006 and an unusually cold one in 2007. These two events were associated with the highest and lowest concentrations of free-living UCYN$_2$-Fix picocyanobacteria, respectively. However, similar surface water temperatures were measured in June 2006 and 2007, and cell concentrations were three orders of magnitude different among those years, suggesting that parameters other than temperature may contribute to the growth of UCYN$_2$-Fix picocyanobacteria. Recent results over the Pacific support this finding by showing no clear relationship between unicellular diazotrophic cyanobacteria concentrations and temperature (Church et al. 2008). Among recorded nutrients at the SOMLIT-Marseilles station (Fig. 4), only phosphate concentrations show significant differences between the summers of 2006 and 2007, with 0.07 ± 0.01 μg L^{-1} in 2006 and values below the detection limit during most of 2007. Phosphate has been shown to control oceanic nitrogen fixation of *Trichodesmium* sp. in the North Atlantic (Sañudo-Wilhelmy et al. 2001). However, in June 2007, phosphate concentration was the second highest from the

seasonal cycle (0.08 μg L^{-1}) and did not enhance a bloom of Nitro821-targeted cells. This result is in accordance with recent experiments of phosphate enrichments of natural Pacific waters, which did not show any increase of growth rates for group A or B unicellular diazotrophs (Zehr et al. 2007).

Together with the long period of elevated temperature recorded in June and July 2006, the city of Marseilles went through the highest atmospheric urban pollution event ever recorded for the past 10 yr (http://www.atmopaca.org/files/ba/Plaquette_O3_2006.pdf). Urban pollution is rich in greenhouse gas, such as CO_2 (George et al. 2007), and anthropogenic particles (http://www.atmopaca.org/html/polluants_seuils_effets_sur_la_sante.php). On the one hand, the greenhouse gas CO_2 has recently been shown to enhance *Trichodesmium* sp. growth rate and nitrogen fixation as well as the growth rate of the nondiazotrophic picocyanobacteria *Synechoccocus* (Fu et al. 2007; Hutchins et al. 2007). On the other hand, a summer peak of nitrogen fixation in the northwestern Mediterranean Sea (DYFAMED station) was shown to co-occur with significant concentrations of anthropogenic particles (Bonnet and Guieu 2006; Sandroni et al. 2007). Anthropogenic particles, together with Saharan dust, were shown to contain iron, phosphate, and nitrate in various concentrations and solubility and to increase significantly the biomass of picocyanobacteria from the DYFAMED site (Bonnet et al. 2005). Bonnet et al. (2005) suspected these blooming picocyanobacteria to be diazotrophs, as shortages of iron and phosphate or Saharan dust have been shown to colimit nitrogen fixation (Mills et al. 2004). It is thus probable that the environmental conditions encountered in June and July 2006 at the coastal SOMLIT-Marseilles station represented a confluence of the parameters that can stimulate the growth and activity of cyanobacterial nitrogen fixers (i.e., dissolved inorganic nitrogen concentrations under detection limit, elevated temperature, detectable concentrations of phosphate, highly soluble iron rich urban aerosol, and significant concentrations of urban greenhouse gas). Although the latter hypothesis is tempting, one must be cautious, as urban pollution is also known to contain high concentrations of oxygen-rich pollutants, such as NO_x, HNO_3, SO_2, H_2SO_4, and O_3 and volatile organic compounds that may harm photosynthetic organisms (Gregg et al. 2003) or oxygen-sensitive physiological activities, such as nitrogen fixation (Pienkos et al. 1983). Furthermore, one must not forget that unknown biotic factors may also enhance the growth rate and biomass of specific species or contribute to a rapid decline of a population. As such, the sudden drop in UCYN$_2$-Fix picocyanobacteria population in August 2006 may be explained by a rapid decrease of sea surface temperature and/or virus or predation attacks.

Free-living UCYN$_2$-Fix accounted for 10.7% of the picocyanobacteria community in July 2006 and only 0.01% in November 2006. In July and November 2006, the picocyanobacteria community concentrations were $5.0 \pm 2.7 \times 10^4$ cell mL^{-1}and $3.0 \pm 2.3 \times 10^4$ cell mL^{-1}, respectively, thus in the range of those described by Grégori et al. (2001) over a 2-yr seasonal cycle at the same station.

As previously shown by Campbell et al. (2005), unicellular diazotrophic cyanobacteria were one to four orders of magnitude less abundant than other nondiazotrophic marine cyanobacteria. Despite these low concentrations, unicellular diazotrophs have been shown to contribute 50% or more (83%) to the global N_2 fixation in Pacific waters (Montoya et al. 2004; S. Bonnet unpubl.) and up to 55% in the Mediterranean Sea (Garcia et al. 2006). In addition to their contribution to global biogeochemical cycles, these organisms may be of great use as symbionts for other eukaryotes by providing additional nutrients. Here we show that picodiazotrophs associated within small non-thecate dinoflagellates were 16 times more abundant in early summer than at other time of the year. These blooms were probably related to one of their host *Gyrodinium* or *Gymnodinium* genera (8–15 μm), which are known to appear in the northwestern Mediterranean Sea at that time of the year (Gómez and Gorsky 2003; B. Becker pers. comm.). This kind of association probably explains the reason why the nifH gene from unicellular diazotrophic cyanobacteria has been regularly recovered in the larger size fraction (>10 μm; Zehr et al. 2007). However, care must be taken when filtering seawater, as mesh or filters can rapidly clog, and the smaller free-living diazotrophs may be trapped on larger size fractions.

Unlike diazotrophic picocyanobacteria, low abundances of nanoplanktonic cyanobacteria were measured over the seasonal cycle. This observation is consistent with the fact that a significant population of nanoplanktonic cyanobacteria had never been observed over previous seasonal studies at the SOMLIT-Marseilles station or surrounding waters (Grégori et al. 2001). Despite their low concentrations, their maximal abundance was observed in February and March 2007 under low temperature, high nutrients, and high Chl *a* concentrations (Fig. 4). Although the maximal abundances of diazotrophic nano-planktonic cyanobacteria from groups B and C have generally been noted in warm waters (20°C to >29°C) exhibiting low nitrate concentrations (Table 1), many recent studies mentioned that these organisms are able to grow in a wide range of environmental conditions. Langlois et al. (2005) pointed out their presence under a large temperature range (15–30°C) and nutrient-enriched oceanic waters (21.9 μg mL^{-1}). These microorganisms were also detected in coastal waters under anthropogenic influences (Biegala and Raimbault 2008) or close to the Amazon River plume (Foster et al. 2007). Finally, the highest annual nitrogen fixation activity (17 nmol N L^{-1} d^{-1}) measured in the northwestern Mediterranean Sea was in March 2003, under similar environmental conditions as the ones measured at the SOMLIT-Marseilles station in February–March 2007 (Garcia et al. 2006). This finding suggests that some diazotrophs can sustain much wider environmental conditions than the warm and oligotrophic conditions where the larger bulk of recent observations have taken place. Among filamentous diazotrophs, only *Trichodesmium* sp. was detected and just once at low concentration (0.03 trichome mL^{-1}) over the studied period. This observation is in accordance with those of Béthoux and Copin-Montégut (1986) and Trégouboff (1957) and contrasts with the high

accumulations of this genus in other oceans (Dupouy et al. 2000).

In conclusion, unicellular diazotrophic cyanobacteria were described and quantified over a complete seasonal cycle for the first time in the Mediterranean Sea. This community was present all year long and was dominated largely by free-living picocyanobacteria, reaching in July 2006 the highest concentration of UCYN$_2$-Fix ever recorded. This bloom was related to a singularly hot and oligotrophic summer and may have been promoted by significant urban air pollution. We thus presume that expected anthropogenically forced global changes, including elevated temperatures, increased stratification, and deposition of certain nutrients, may together favor the development of potentially diazotrophic picocyanobacteria, at least in oligotrophic coastal environments. Because this new type of diazotrophic cyanobacteria have been recently discovered in coastal and oceanic Pacific waters, we expect their distribution to be worldwide. Further studies will be necessary to demonstrate their identity and their contribution to global nitrogen fixation. Another important result from this study confirms the large range of environmental conditions that are tolerated by pico, nano, or filamentous diazotrophic cyanobacteria, although high temperature and oligotrophic conditions seem to correspond to their optimal environment.

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