

Dominance of unicellular cyanobacteria in the diazotrophic community in the Atlantic Ocean

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Abstract

The horizontal and vertical distribution of representatives of diazotrophic unicellular cyanobacteria was investigated in the subtropical northeast Atlantic Ocean (28.87 to 42.00°N; 9.01 to 20.02°W). Samples from stations encompassing different water conditions (from oceanic oligotrophic waters to upwelling areas and a temperature range of 13.1°C to 24.2°C) were size fractionated and analyzed for *nifH* by a nested polymerase chain reaction (PCR) and by tyramide signal amplification–fluorescence in situ hybridization (TSA-FISH) using probe Nitro821. In samples from the surface, mixed-layer depth, and deep chlorophyll maximum waters, most (> 50%) of the *nifH* recovered was from the 0.2–3 μm fraction and was consistent with TSA-FISH counts. The < 3 μm Nitro821-positive cells were more abundant than the larger cells, and the proportion of single cells was larger than that associated with particulate matter or with larger cells. Phylogenetic analysis of representative samples revealed that most of the sequences belong to diazotrophic unicellular cyanobacteria Group A (UCYN-A or *Candidatus* Atelocyanobacterium thalassa). N₂ fixation in the 0.2–3 μm fraction, putatively representing the activity of UCYN-A, contributed more than 50% of the total N₂ fixation. There was a positive relationship of this putative UCYN-A abundance and activity with temperature, and a negative relationship with dissolved O₂. The dominance of these putative UCYN-A organisms in nitrate-rich upwelling filament regions suggests that the activity of this group of organisms may not be strongly controlled by the availability of fixed N.

For a long time, *Trichodesmium* was thought to be the only abundant and important N₂-fixing microorganism in the oceans (Capone et al. 1997). The discovery of diazotrophic unicellular cyanobacteria (< 10 μm) was made possible by the use of molecular genetic techniques that showed the presence of fragments of the unicellular cyanobacterial *nifH* (a gene encoding the nitrogenase Fe-protein component [dinitrogenase reductase]; Zehr et al. 1998). The tyramide signal amplification–fluorescence in situ hybridization (TSA-FISH), using the primer Nitro821R targeting the 16S ribosomal ribonucleic acid (rRNA) gene of diazotrophic unicellular cyanobacteria (Mazard et al. 2004), has been employed in order to estimate the in situ abundance of diazotrophic unicellular cyanobacteria of different sizes (Biegala and Raimbault 2008). During the past decade, it has been suggested that the contribution of diazotrophic unicellular cyanobacteria to oceanic N₂ fixation is similar to or exceeds that of the larger (> 100 μm) filamentous cyanobacteria *Trichodesmium* spp. (Montoya et al. 2004; Moisander et al. 2010; Benavides et al. 2011).

To date, three phylogenetically different groups of diazotrophic unicellular cyanobacteria (UCYN) are recognized: Group A (UCYN-A or *Candidatus* Atelocyanobacterium thalassa [$\leq 1 \mu\text{m}$], an uncultured symbiotic group of organisms [Thompson et al. 2012]), Group B (UCYN-B [3–8 μm], *Crocospaera watsonii* and related organisms), and

Group C (UCYN-C [2.5–6 μm], comprising organisms that are related to *Cyanothece* sp. [Needoba et al. 2007]). Since their discovery, Group A (UCYN-A) members have received much attention because of their peculiar properties as deduced from their high degree of genome streamlining (1.44 megabase pairs; Zehr et al. 2008; Tripp et al. 2010; Thompson et al. 2012). For instance, these organisms lack the following cellular components: (1) the oxygen-evolving photosystem II complex (Zehr et al. 2008), (2) the CO₂-fixing enzyme (ribulose-1,5-bisphosphate carboxylase-oxygenase), and (3) the major elements of the tricarboxylic acid cycle (TCA; Tripp et al. 2010). Although considerable advances in our knowledge of the molecular and metabolic features of diazotrophic unicellular cyanobacteria have been made (Zehr et al. 2008; Tripp et al. 2010; Thompson et al. 2012), studies on their environmental growth requirements and ecology are scarce. Unlike the larger N₂ fixer, *Trichodesmium*, little is known about the spatial distribution of diazotrophic unicellular cyanobacteria. There are indications that unicellular diazotrophic cyanobacteria have spatial distributions that are distinctly different from those of *Trichodesmium* (Langlois et al. 2005; Goebel et al. 2010; Moisander et al. 2010). Most of the reports on the distribution of diazotrophic unicellular cyanobacteria (particularly UCYN-A) were from studies carried out in the Pacific Ocean (Zehr et al. 2008; Moisander et al. 2010), whereas only a few surveys were done in the Atlantic Ocean. In the Atlantic Ocean, previous studies were focused on the tropical and subtropical northern regions

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(< 30°N; Langlois et al. 2005, 2008; Goebel et al. 2010). In areas further north (> 30°N), few studies reported the presence of diazotrophic unicellular cyanobacteria (Langlois et al. 2008; Rees et al. 2009). Moreover, studies focusing on the upwelling regions in the northern latitudes (> 30°N) are lacking (Luo et al. 2012). Hence, more knowledge is required on the spatial distribution of diazotrophic unicellular cyanobacteria in the open ocean in order to be able to assess their contribution to global N₂ fixation.

Here, we investigated the horizontal and vertical distribution of diazotrophic unicellular cyanobacteria in the subtropical northeast Atlantic Ocean (28.87 to 42.00°N, 9.01 to 20.02°W) by amplifying *nifH* in size-fractionated samples (> 10 μm, 3–10 μm, and 0.2–3 μm), by using a nested polymerase chain reaction (PCR; Langlois et al. 2005), and by counting the cells after having visualized them by TSA-FISH using probe Nitro821 (Biegala and Raimbault 2008). TSA-FISH allows visual inspection of unicellular diazotrophic cyanobacteria, to observe whether they are single cells, are associated with other organisms, or are attached to nonliving particulate organic matter. In addition, we investigated the correlations of size-fractionated *nifH* gene analysis and TSA-FISH counts with the raw data used in Benavides et al. (2011) of N₂ fixation rates in the same samples. The N₂-fixation rates were measured using acetylene reduction assay (ARA), which measures gross N₂ fixation (Stal 1988; Capone 1993; Gallon et al. 2002), and the ¹⁵N₂ stable isotope technique, which measures net incorporated N₂ fixation (Montoya et al. 1996). Using both methods enhanced the robustness of the patterns of N₂ fixation in the different size classes. In Benavides et al. (2011), the N₂ fixation rates were reported for the > 10 μm and < 10 μm fractions. In this paper, we present N₂ fixation in the < 10 μm fractions in a higher resolution of size (i.e., in the < 3 μm and in the 3–10 μm fractions). Moreover, we report N₂ fixation, expressing the ARA as hourly rates, and distinguish light and dark rates separately. Benavides et al. (2011) presented these data as total daily rates. In this manner, the N₂ fixation data allow a more robust comparison with the size-fractionated *nifH* gene analysis and the TSA-FISH counts. This study was carried out in oceanic oligotrophic waters and in upwelling areas at different depths, covering a temperature range of 13.1°C to 24.2°C. The stations surveyed encompassed a wide range of water conditions that allowed further analysis of how these environmental parameters affected the distribution and activity of diazotrophic unicellular cyanobacteria.

Methods

Study sites, hydrography, primary production, and nutrient analyses—This study was carried out on board the R/V *Sarmiento de Gamboa* during three legs of the cruise of the project Shelf–Ocean Exchanges in the Canaries–Iberian Large Marine Ecosystem (CAIBEX) during the summer of 2009. The CAIBEX project was planned to carry out interdisciplinary studies of two active upwelling filament systems that export water from the coastal zone into the oligotrophic subtropical gyre of the

North Atlantic Ocean. The two upwelling filament systems studied during small-scale surveys were Cape Silleiro, northwest Iberia, from 06 to 24 July (leg 1) and Cape Ghir, northwest Africa, from 16 August to 05 September (leg 3; Fig. 1). A large-scale survey was also conducted from 25 July to 14 August in a box between 20°W and the Canary Islands, encompassing the two upwelling filament systems (leg 2, CAIBOX, grid box). The Cape Silleiro filament system appears intermittently but recurrently every summer, whereas the Cape Ghir filament system is a strong and quasi-permanent feature (Aristegui et al. 2009). During the time of the cruise, the Cape Silleiro filament did not appear, and the stations sampled were coastal upwelling (Sta. S05 to S08) and open-ocean stations (Sta. S01 to S03) during leg 1 (Fig. 1). During leg 2, 17 stations were sampled during the large-scale survey; and, finally, during leg 3, stations sampled were those along the path of the Cape Ghir filament (G12, G17, G22, G40, G44, and G48) and those outside the filament (GM2 and GT2 located inside and outside the upwelling area, respectively; Fig. 1).

At each station during the three legs of the cruise, temperature, salinity, and chlorophyll fluorescence were recorded by means of a conductivity–temperature–depth (CTD) SeaBird 911 Plus system, mounted on a General Oceanics 24-bottle rosette sampler equipped with 12 liter Niskin bottles. Dissolved O₂ and chlorophyll fluorescence were also measured with auxiliary sensors attached to the CTD system. Samples from cruise leg 1 (Cape Silleiro) and leg 2 (grid box) for the determination of the dissolved inorganic PO₄³⁻ and the NO₃⁻ + NO₂⁻ concentrations were immediately analyzed on board using a Prescop Alphen autoanalyzer with detection limits of 0.1 μmol L⁻¹ for NO₃⁻ and 0.02 μmol L⁻¹ for NO₂⁻ and PO₄³⁻. Samples from leg 3 (Cape Ghir) were kept frozen until analysis, following standard spectrophotometric methods (Hansen and Koroleff 1999) in a Bran + Luebbe AA3 autoanalyzer with detection limits of 0.01, 0.003, and 0.024 μmol L⁻¹ for NO₃⁻, NO₂⁻, and PO₄³⁻, respectively. Primary production of samples from surface water was measured using the ¹³C isotope technique as described in Hama et al. (1983).

Size-fractionated N₂ fixation measurement—N₂ fixation was measured in whole water and in size-fractionated (< 10 and < 3 μm) surface-water samples (~ 5 m depth) collected with 12 liter Niskin bottles, using ARA (Stal 1988; Capone 1993) and the ¹⁵N₂ stable isotope technique (Montoya et al. 1996) during leg 2 (CAIBOX, grid box, Fig. 1). Due to logistical constraints, only N₂ fixation measurements were made in whole and < 10 μm fractions during leg 1 (Cape Silleiro) and leg 3 (Cape Ghir). The size-fractionated samples were obtained by filtering through Osmonics™ polycarbonate (PC) membrane filters (diameter, 47 mm; pore size, 3 or 10 μm).

For the ARA technique, six replicate 2 liter samples (of whole unfractionated and size-fractionated samples) were filter-concentrated on Whatman GF/F filters (< 100 mm Hg) using the approach and recommendations of Staal et al. (2001) to circumvent problems of gas diffusion. The concentration of samples on a filter was necessary to

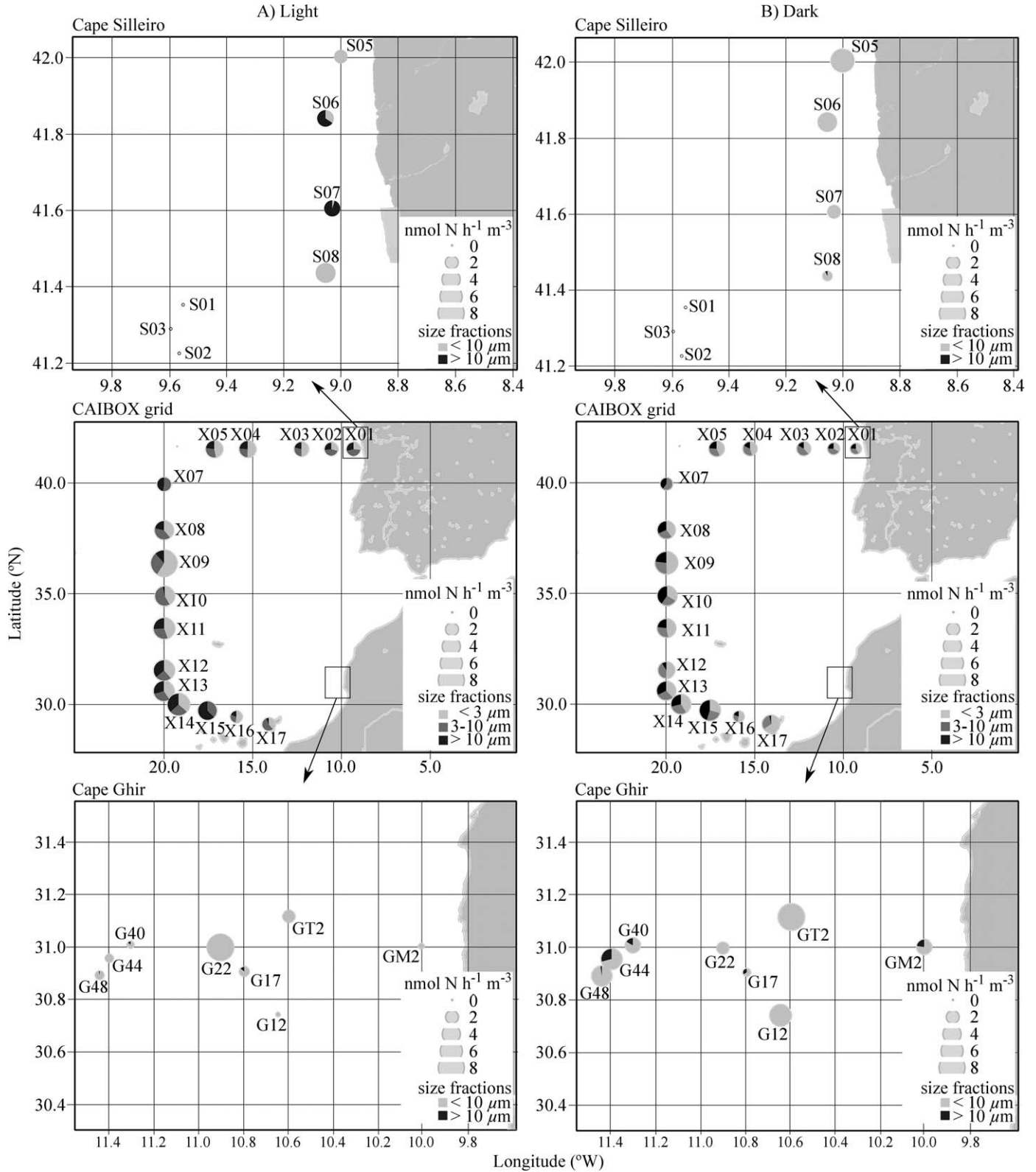


Fig. 1. Relative contribution of the different size fractions to overall N_2 fixation rates ($\text{nmol N h}^{-1} \text{m}^{-3}$) based on acetylene reduction assays in surface waters in the three legs of the cruise (Cape Silleiro, CAIBOX grid, and Cape Ghir) measured in (A) light and (B) dark incubations. The sizes of the circles are proportional to total N_2 fixation rate, as indicated in the legend.

obtain sufficiently high ARA rates to measure without extending incubation times too much, as well as to prevent artifacts caused by gas diffusion (i.e., exchange of acetylene and ethylene, and CO₂ depletion and accumulation of O₂; Staal et al. 2001). The filters were placed in 10 mL crimp-top vials, humidified with 0.5 mL GF/F filtered seawater, and sealed with a rubber stopper and an aluminum cap. After sealing, 2 mL of acetylene was injected using a gas-tight Hamilton syringe. Acetylene was generated from calcium carbide (CaC₂; Sigma Aldrich) by adding Milli-Q water in a reaction flask (Stal 1988). The gas was recovered in 1 liter Tedlar gas bags with polypropylene valves (SKC Incorporated). Triplicate vials were subsequently incubated for 3 h in on-deck aquaria with running circulating seawater to maintain ambient temperature. The 3 h incubations were sufficient to determine nitrogenase activity while minimizing bottles effects. One set was incubated in the dark, and another set of vials was incubated in ambient light that was attenuated to the light intensity at 5 m depth. At 5 m depth, light intensity was 85% of surface irradiance, and this light intensity was achieved by covering the on-deck aquaria with one layer of neutral density screen (Lee filter sheet 210, 0.6 neutral density). After incubation, 10 mL of headspace was taken using a gas-tight Hamilton syringe and was transferred to Hungate tubes that had been evacuated using a vacuum pump. The puncture holes were sealed with hot melt adhesive glue (SALKI, ref. 0430308). Ethylene and acetylene were determined in the laboratory back on land using a gas chromatograph (Agilent Technologies, model HP-5890) equipped with a flame ionization detector (FID). The column was a Varian wide-bore column (ref. CP7584) packed with Cole Parmer-Pora Porous Layer Open Tubular (CP-Poraplot U; 27.5 m length, 0.53 mm inside diameter, 0.70 mm outside diameter, 20 μm film thickness). Helium was used as the carrier gas at a flow rate of 30 mL min⁻¹. Hydrogen- and air-flow rates were set at 30 mL min⁻¹ and 365 mL min⁻¹, respectively. The split flow was used so that the carrier gas flow through the column was 4 mL min⁻¹ at a pressure of 34 kPa. Oven, injection, and detector temperatures were set at 52°C, 120°C, and 170°C, respectively. The acetylene reduction rates were converted to N₂ fixation using a factor of 4:1 (C₂H₄:N₂ reduced) following the theoretical ratio derived from the equations: N₂ + 8[H⁺] → 2NH₃ + H₂ and C₂H₂ + 2[H⁺] → C₂H₄, which ratio was experimentally proven (Jensen and Cox 1983; Gallon et al. 2002); however, this ratio may be as low as 3:1, depending on the physiological condition of the organism (Montoya et al. 1996). The acetylene reduction rates were calculated according to the equations in Stal (1988) using acetylene as an internal standard, which circumvents inaccuracies due to gas losses during experimental handling, storage, and transport.

For the ¹⁵N₂ technique, whole surface seawater and size-fractionated samples (< 10 and < 3 μm) were transferred to 1.24 liter transparent polycarbonate bottles with septum screw caps. The bottles were filled to the top, using silicone tubing and avoiding air bubbles. Subsequently, the bottles were sealed with septum screw caps before trace additions of ¹⁵N₂ (2 mL, at 99% ¹⁵N₂, Cambridge Isotope

Laboratories) were injected through the septum using a gas-tight Hamilton syringe. Enrichments with ¹⁵N varied from 9.8% to 11.2%. The bottles were placed in on-deck aquaria (described above) for 24 h. Incubations started between 08:00 h and 12:00 h Coordinated Universal Time. After incubation, samples were filtered through precombusted (4 h, 450°C) 25 mm GF/F filters, wrapped in precombusted (4 h, 450°C) aluminum foil, and stored at -80°C until analysis. The particulate organic nitrogen (PON) content and isotopic ratio of samples were measured with a Thermo Flash EA 1112 elemental analyzer connected to a Thermo Delta Advantage isotope ratio mass spectrometer. N₂ fixation estimated from the incorporation of ¹⁵N was calculated according to Montoya et al. (1996).

Size-fractionated nifH gene nested PCR analysis, cloning, and sequencing—Five liters of seawater from three depths (surface, the bottom of the mixed-layer depth [MLD], and deep chlorophyll maximum [DCM]) were collected during the three legs of the cruise. The samples were filtered (vacuum < 100 mm Hg) through a series of three connected filter holders containing filters of different pore sizes: (1) 10 μm (Fluoropore, 47 mm), (2) 3 μm (MF-Millipore, 47 mm), and (3) 0.22 μm (Durapore, 47 mm). The in-line filtering system allowed the same sample volume for the different size fractions for estimations of relative *nifH* content. The filters were frozen and stored at -80°C until deoxyribonucleic acid (DNA) extraction (Nogales et al. 2007). The DNA was obtained from the size fractions >10 μm, 3–10 μm, and 0.2–3 μm. The volume of DNA extracted from each filter was 50 μL.

Before *nifH* gene amplification, we checked all our samples for the absence of PCR inhibitors, using amplification controls employing DNA primers (universal primer 16S [F27/R1492] and cyanobacterial primer 16S [OXY107F/OXY13013R]; Lane 1991). The results indicated the absence of inhibitors. *NifH* was subsequently amplified using a nested PCR (Langlois et al. 2005) with modifications. The first round of PCR used 1 μL of the original extracted DNA added to 0.5 μL Taq DNA Polymerase (GE Healthcare) and 11.5 μL mixture of buffers and primers (10× buffer II, MgCl₂ [final concentration, 4 mmol L⁻¹], 10 mmol L⁻¹ deoxynucleoside triphosphates, 80 pmol of each primer: *nifH4* [*A. vinelandii* positions 546 to 562; 5'-TTY TAY GGN AAR GGN GG-3'] and *nifH3* [*A. vinelandii* positions 1018 to 1002; 5'-ATR TTR TTN GCN GCR TA-3']; Zehr and McReynolds 1989; Zani et al. 2000). The thermocycler program was 10 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 45°C, and 1 min at 72°C, with a final elongation for 10 min at 72°C. The second round of PCR consisted of two separate PCR setups: addition of 80 pmol of the *Trichodesmium* primer (see below) and addition of 80 pmol mixture of primers specific for groups A, B, and C unicellular diazotrophs (see below), to 1 μL of the first PCR product, 0.5 μL Taq DNA Polymerase (GE Healthcare) and 11.5 μL mixture of 10× buffer II, MgCl₂ (final concentration, 6 mmol L⁻¹), 10 mmol L⁻¹ deoxynucleoside triphosphates. The thermocycler program was the same as described above but using 28 cycles and with an annealing

temperature of 54°C. The four primers amplifying *nifH* fragments for the unicellular N₂-fixing cyanobacteria groups A, B, and C (Needoba et al. 2007) and *Trichodesmium* used in the second nested PCR were designed by selecting 52 sequences of cyanobacterial *nifH* genes (Group A: forward primer: 5'-CGTYTAATGTTGCAYTGTAAAGC-3', reverse primer: 5'-CCTAATACATCATAGATACG-3'; Group B: forward primer: 5'-CGTTTAATCCTCAACTGTAAAGC-3', reverse primer: 5'-CCTAATACGTCATAGGATACG-3'; Group C: forward primer: 5'-CGT TTG ATG CTA CAC ACT AAA GC-3', reverse primer: 5'-CCTAATACGTCATAGGATACG-3'; *Trichodesmium*: forward primer: 5'-CGTTTAATCTTAAACGCTAAAGC-3', reverse primer: 5'-CCTAGTACGTCATAGCTTACG-3'). We included negative controls in our PCR reactions, using nondiazotrophic cultures of PCC 73106 and PCC 6803. We validated the primers and the nested PCR design (see Web Appendix, www.aslo.org/lo/toc/vol_59/issue_2/0623a.pdf). The relative quantity of *nifH* amplicons (ng) of the nested PCR among the samples was estimated from the intensity of the bands on the agarose gels relative to the bands of the 100 Base-Pair Ladder DNA standard (GE-Healthcare) using Image Lab Software (Bio-Rad). We also checked the estimation of the relative quantity of *nifH* using a dilution series of a mixture of positive and negative *nifH* environmental samples (see Web Appendix). Amplicons of ~ 200 base pairs (bp) size, as revealed by agarose gel electrophoresis in 15 representative samples that contained a sufficient quantity of DNA, were cloned using the TOPO[®]-TA cloning kit (Life Technologies) according to the manufacturer's instructions. Ten clones from each library were picked randomly and were sequenced in the forward direction using the M13F primer with BigDye vs. 3.1 in the ABI PRISM 377 DNA Sequencer (Perkin-Elmer Biosystems). The phylogenetic analysis of the ~ 150 *nifH* sequences obtained was done using BLASTn (<http://www.ncbi.nlm.nih.gov>). The closest related *nifH* sequences were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/genbank>) and were aligned to other sequences obtained during this study using CLC Sequence Viewer 6. Alignments based on a 199-bp DNA sequence were used to construct a neighbor-joining phylogenetic tree rooted to the *Methylobacterium rubra* clone 23-4 nitrogenase iron protein (*nifH*) gene (AF484673), and relationships were bootstrapped 1000 times using CLC Sequence Viewer 6.

Size-fractionated TSA-FISH using probe Nitro821—In situ whole-cell detection of probe Nitro821-targeted unicellular N₂-fixing cyanobacteria was done following the methods in Zwirgmaier (2005) and Biegala and Raimbault (2008). Depending on the degree of “clogging” of filters by the retained planktonic cells and particles, volumes between 250 mL and 4 liters of seawater from the three depths (surface, MLD, and DCM) were filtered (vacuum < 100 mm Hg) on 47 mm Isopore[™] PC membrane filters of three different pore sizes (10 μm, 3 μm, 0.22 μm) on individual filter manifolds. Subsequently, the cells were fixed with 1% paraformaldehyde (pH 8.2), buffered with phosphate buffer solution (PBS) for 15 min at room temperature, and washed with PBS. The filters

were air-dried for 5 min; subsequently, the cells were dehydrated with molecular grade ethanol series (50%, 80%, and 100%), each for 10 min at room temperature, and then stored at -20°C until further analysis. Samples that gave *nifH* positive results by PCR were also analyzed by TSA-FISH. A subsample of the filter (1/8) was used for TSA-FISH. Hybridization with horseradish peroxidase (HRP)-labeled (Thermo Fisher Scientific GmbH) 16S rRNA Nitro821 probe was done after the cells were made permeable with lysozyme (5 mg mL⁻¹ in buffer, Roche) for 30 min at 37°C; then the cells were rinsed three times with sterile Milli-Q water and were subjected to molecular grade ethanol series (50%, 80%, and 100%). The cells were subsequently processed for hybridization and TSA reactions using 50% formamide in the hybridization buffer (Biegala and Raimbault 2008). Hybridization was done at 37°C for 2 h. Finally, the filters were mounted on a microscope slide using mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; product H-1200, Vectashield[®]). The unicellular diazotrophic cyanobacterium *Cyanothece* sp. PCC 8801 was used as the positive control, and the nano- and picoplanktonic, nondiazotrophic cyanobacteria *Gloeocapsa* sp. PCC 73106 and *Synechocystis* sp. PCC 6803 were used as negative controls for TSA-FISH.

The images of the prepared TSA-FISH slides were captured using an epifluorescence microscope (Leica DM 2500) equipped with the following filters: (1) 360 ± 20 excitation (ex.), 410 ± 5 emission (em.) for the DAPI (blue fluorescence); (2) 480 ± 40 (ex.), 527 ± 30 (em.) for the fluorescein isothiocyanate (FITC) associated with Nitro821 probe (green fluorescence); and (3) 450–490 (ex.), 515 long pass (em.) for chlorophyll fluorescence (orange fluorescence). The digital camera (Leica DFC420C) with software for control of time of exposure, zoom, and color allowed adjustment of the exposure time for improved visualization of FITC-labeled cells (to minimize the interference of the chlorophyll fluorescence). Nitro821-positive cells were counted using the objective 100×. The 1/8 filter slices were scanned, and all cells were counted. Ranges of Nitro821-positive cell counts per filter slice were from 6 to 1018, 0 to 401, and 0 to 359 cells for samples in Cape Ghir, CAIBOX, and Cape Silleiro, respectively. Cells were recorded as (1) single cells, (2) symbionts of larger organisms, or (3) associated with organic matter, and as small (< 1 μm), medium (1–3 μm), and large (> 3 μm).

Data and statistical analyses—Size-fractionated N₂ fixation rates for >10 μm and 3–10 μm fractions were calculated as the difference between N₂ fixation rate of whole water sample and < 10 μm fraction and as the difference between N₂ fixation rate of < 10 μm and < 3 μm fractions, respectively. Normal distribution of data was checked with the Shapiro–Wilk W test, and, if necessary, data were logarithmically transformed prior to analyses. Pearson correlation and regression analyses were used to determine the relationships between N₂ fixation rates, size-fractionated *nifH* gene nested PCR analysis, TSA-FISH counts, and a variety of physicochemical factors (temperature, salinity, chlorophyll fluorescence, dissolved O₂, total

Table 1. Average \pm standard deviation (SD) of surface values (~ 5 m depth) of selected physicochemical parameters, hydrography, and primary production in the different stations in each leg of the CAIBEX cruise.

Leg	<i>n</i>	MLD (m)	DCM (m)	Temperature (°C)	Salinity	Fluorescence (arbitrary units)	Dissolved O ₂ (mg L ⁻¹)	NO ₃ ⁻ + NO ₂ ⁻ (μmol L ⁻¹)	PO ₄ ³⁻ (μmol L ⁻¹)	Primary production (mmol C m ⁻³ h ⁻¹)
1: Cape Silleiro	8	26 ± 10	38 ± 23	16.88 ± 1.48	35.65 ± 0.06	0.48 ± 0.34	7.5 ± 0.4	0.67 ± 0.79	0.05 ± 0.06	0.15 ± 0.07
2: CAIBOX grid	17	42 ± 7	82 ± 20	21.84 ± 2.15	36.46 ± 0.53	0.11 ± 0.07	6.5 ± 0.4	0.12 ± 0.13	0.03 ± 0.02	0.02 ± 0.01
3: Cape Ghir	8	23 ± 6	40 ± 9	19.67 ± 1.34	36.37 ± 0.05	0.06 ± 0.04	6.7 ± 0.2	2.22 ± 3.41	0.68 ± 0.31	0.12 ± 0.14

primary production, PO₄³⁻, NO₃⁻ + NO₂⁻). The statistical analyses were performed using the SPSS program.

Results

Hydrography, primary production, and nutrient analyses—Table 1 summarizes the MLD and depth of the DCM, selected physicochemical parameters and primary production in surface waters during the three legs of the cruise. In the CAIBEX grid (leg 2), the MLD and DCM were deeper and surface waters exhibited higher temperatures, lower concentration of inorganic nutrients (PO₄³⁻, NO₃⁻ + NO₂⁻), and lower primary production compared to Cape Silleiro (leg 1) and Cape Ghir (leg 3; Table 1). The surface waters in Cape Ghir exhibited the highest concentration of inorganic nutrients (PO₄³⁻, NO₃⁻ + NO₂⁻) and primary production among the three legs of the cruise, whereas surface chlorophyll fluorescence was highest in Cape Silleiro (Table 1). Detailed hydrography of the region has been reported in Carracedo et al. (2012) and Troupin et al. (2012).

Size-fractionated N₂ fixation—Hourly N₂ fixation measured in the < 10 μm fraction using ARA in the Cape Silleiro filament system (leg 1) ranged from 0.14 to 4.57 and 0.64 to 4.03 nmol N m⁻³ h⁻¹ in the light and dark incubations, respectively. In the other filament system in Cape Ghir (leg 3), hourly N₂ fixation rates in the < 10 μm fraction using ARA ranged from 0.72 to 39.86 and 0.37 to 4.99 nmol N m⁻³ h⁻¹ in the light and dark incubations, respectively. In the higher resolution of size in the fractionated N₂ fixation measured during the large-scale survey (leg 2, CAIBOX, grid box), N₂ fixation in the < 3 μm fraction measured using ARA ranged from 0.55 to 4.61 and 0.46 to 2.76 nmol N m⁻³ h⁻¹ in the light and dark incubations, respectively. The net daily N₂ fixation in the < 3 μm fraction measured by the ¹⁵N labeling technique ranged from 12.0 to 90.0 nmol N m⁻³ d⁻¹. The rates for the < 3 μm fraction measured during the CAIBOX leg corresponded, in most cases, to approximately > 50% of the total N₂ fixation, whereas those in the 3–10 μm fraction corresponded to 0–37% of the total N₂ fixation (Figs. 1, 2). N₂ fixation in the < 3 μm fraction measured in the dark was inversely linearly correlated with primary production and dissolved O₂ and was positively linearly correlated with temperature and salinity during the CAIBOX leg (Table 2).

Size-fractionated nifH nested PCR analysis, cloning, and sequencing of clones—Results of *nifH* amplification in samples from the surface, MLD, and DCM during the three legs revealed that most (> 50%) of the *nifH* recovered was from the 0.2–3 μm fraction, ranging from 34 to 406, 10 to 421, and 48 to 810 ng in Cape Silleiro (leg 1), CAIBOX (leg 2), and Cape Ghir (leg 3), respectively (Figs. 3–5). *NifH* was also recovered from the 3–10 μm and > 10 μm fractions, but only in the surface water and at the MLD, and not in the DCM. During CAIBOX (leg 2) we found that the relative quantity (ng weight) of *nifH* amplicons that was recovered in the 0.2–3 μm fraction correlated positively (*p* < 0.05) with temperature and salinity but

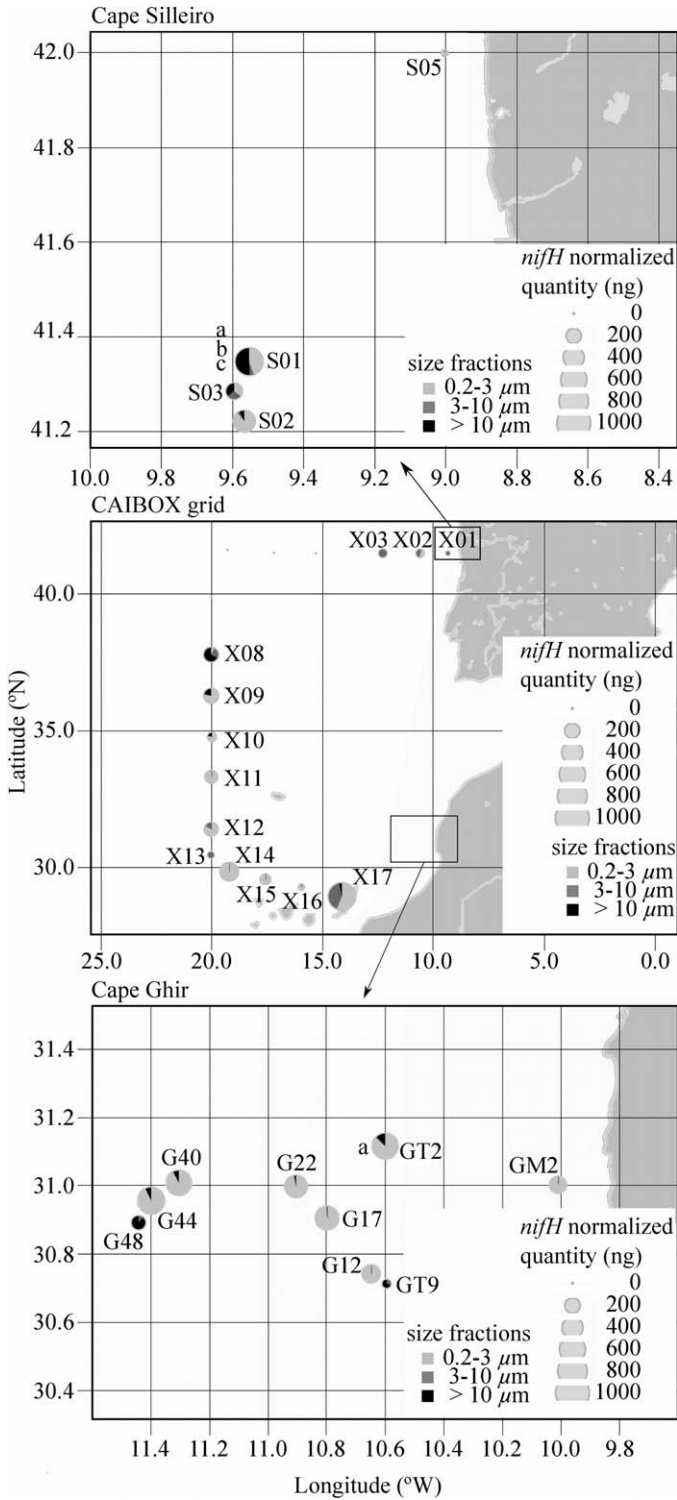


Fig. 3. Relative contribution of the different size fractions to overall quantity of *nifH* (ng) recovered in surface waters in the three legs of the cruise (Cape Silleiro, CAIBOX grid, and Cape Ghir). The sizes of the circles are proportional to the total quantity of *nifH* recovered, as indicated in the legend. Samples chosen for cloning and sequencing are indicated as 0.2–3 μm , 3–10 μm , and > 10 μm .

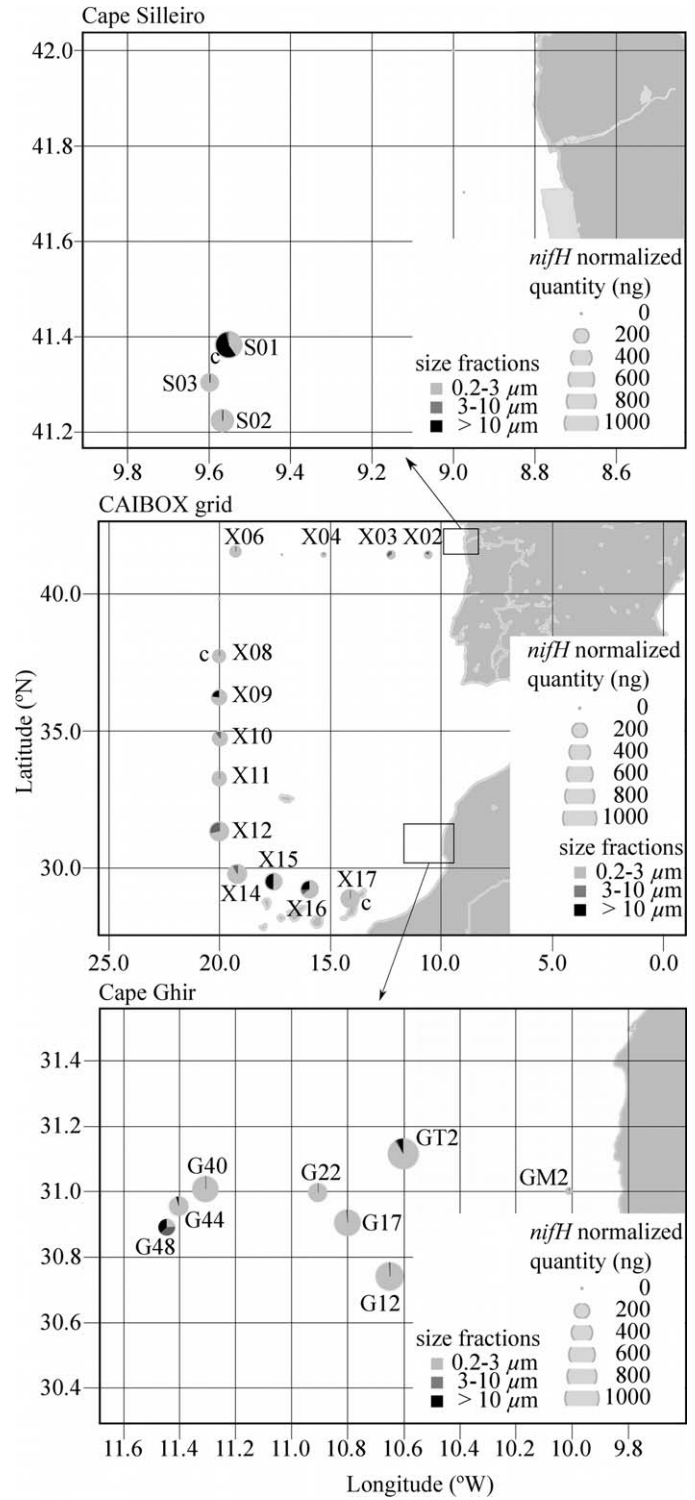


Fig. 4. Relative contribution of the different size fractions to overall quantity of *nifH* (ng) recovered at the bottom of the mixed-layer depths (MLD) in the three legs of the cruise (Cape Silleiro, CAIBOX grid, and Cape Ghir). The sizes of the circles are proportional to the total quantity of *nifH* recovered, as indicated in the legend. Samples chosen for cloning and sequencing are indicated as 0.2–3 μm , 3–10 μm , and > 10 μm .

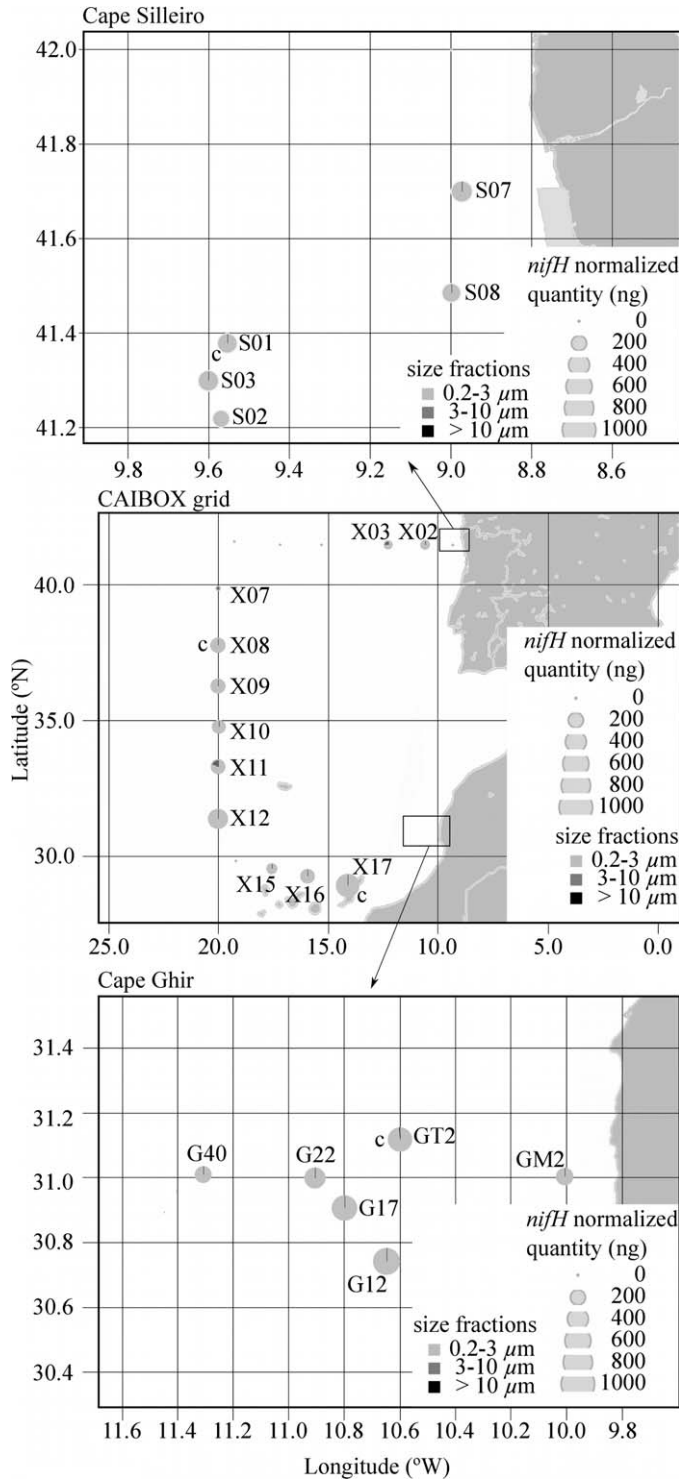
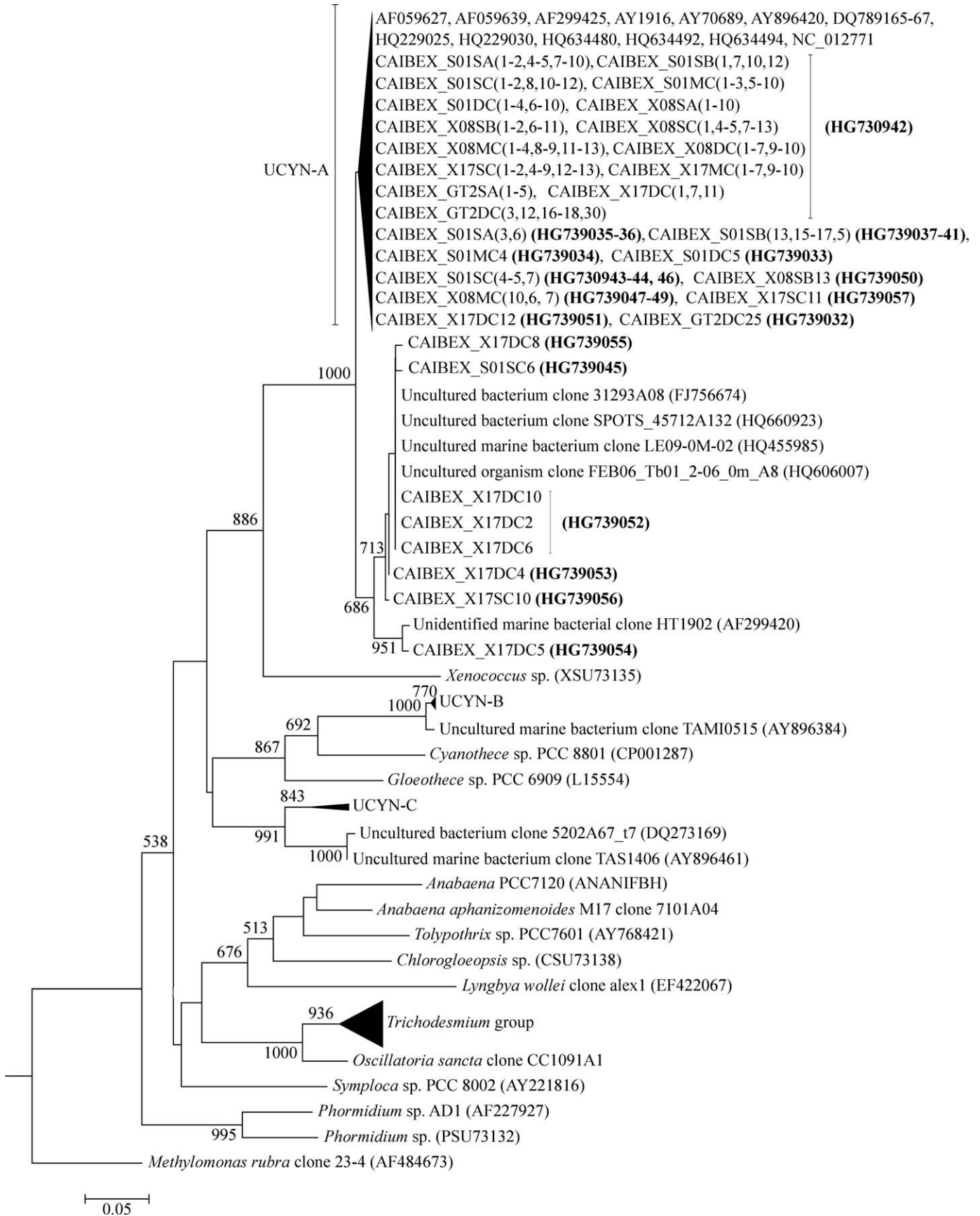


Fig. 5. Relative contribution of the different size fractions to overall quantity of *nifH* (ng) recovered in the deep chlorophyll maximum (DCM) depths in the three legs of the cruise (Cape Silleiro, CAIBOX grid, and Cape Ghir). The sizes of the circles are proportional to the total quantity of *nifH* recovered, as indicated in the legend. Samples chosen for cloning and sequencing are indicated as 0.2–3 μm , 3–10 μm , and > 10 μm .

water, MLD, and DCM, respectively (Fig. 7). The Nitro821-positive cells in the different size classes (< 1 μm , 1–3 μm , and > 3 μm) occurred as single cells, associated to particulate matter, and associated to larger cells (see Fig. 5, Benavides et al. 2013). In all cruise legs, the proportion of cells occurring as single cells was larger (reached up to 100% of all Nitro821-positive cells), followed by cells associated to particulate matter. In the two legs of the cruise (Cape Ghir and CAIBOX), those associated to larger cells were lowest in number in all size fractions (Table 3). During the Cape Silleiro leg of the cruise, the > 3 μm Nitro821-positive cells associated to larger cells were more abundant than those associated to particulate matter (Table 3). The total number of single small (< 1 μm) Nitro821-positive cells was significantly positively correlated with salinity but significantly negatively correlated with dissolved O_2 and chlorophyll fluorescence during all three cruises and at three depths (Table 2).

Discussion

The dominance of diazotrophs in the < 10 μm size fraction and their contribution to N_2 fixation observed throughout the study area in the subtropical northeast Atlantic Ocean (Benavides et al. 2011) is consistent with reports from other investigators who studied N_2 fixation in the eastern Atlantic Ocean, but at lower latitudes than in our study (Montoya et al. 2007; Goebel et al. 2010). In our study area, N_2 fixation by *Trichodesmium* represented only < 1% of the total gross N_2 fixation, and the density of these organisms was low (< 0.5 trichomes L^{-1}). N_2 fixation in the smallest fraction (< 3 μm) that we studied contributed significantly (> 50%) to the total N_2 fixation measured using both ARA and $^{15}\text{N}_2$ techniques. There may be biases in either of the two methods. On the one hand, the necessary concentration of the cells on a filter may affect the activity of the organism and may, therefore, be difficult to compare with the suspended cells in the seawater used in the $^{15}\text{N}_2$ incubations. On the other hand, ARA incubations were short and, therefore, would induce less change than the long incubations with $^{15}\text{N}_2$. Moreover, underestimation of N_2 fixation because of slow dissolution of $^{15}\text{N}_2$ gas may be another bias of this technique (Mohr et al. 2010; Großkopf et al. 2012). However, the dominance of the activity of the smallest fraction (< 3 μm) was consistently seen at all depths until the DCM. Potential diazotrophs in this smallest fraction can be cyanobacteria as well as heterotrophic bacteria, which are common in the Atlantic Ocean (Falcon et al. 2004; Langlois et al. 2005). Our PCR approaches and cell count methods focused on the diazotrophic cyanobacteria. The evidence provided by our study suggests that the predominant cyanobacterial N_2 fixers in the < 3 μm fraction most likely belonged to the small-sized UCYN-A. By far, most of the *nifH* sequences of the 150 clones obtained from samples with the highest *nifH* abundance belonged to UCYN-A. This is consistent with previous reports (Bonnet et al. 2009; Turk et al. 2011). The presence of UCYN-A *nifH* sequences in the 3–10 μm and > 10 μm fractions supports the possibility that these organisms can be attached or live in symbiosis with larger



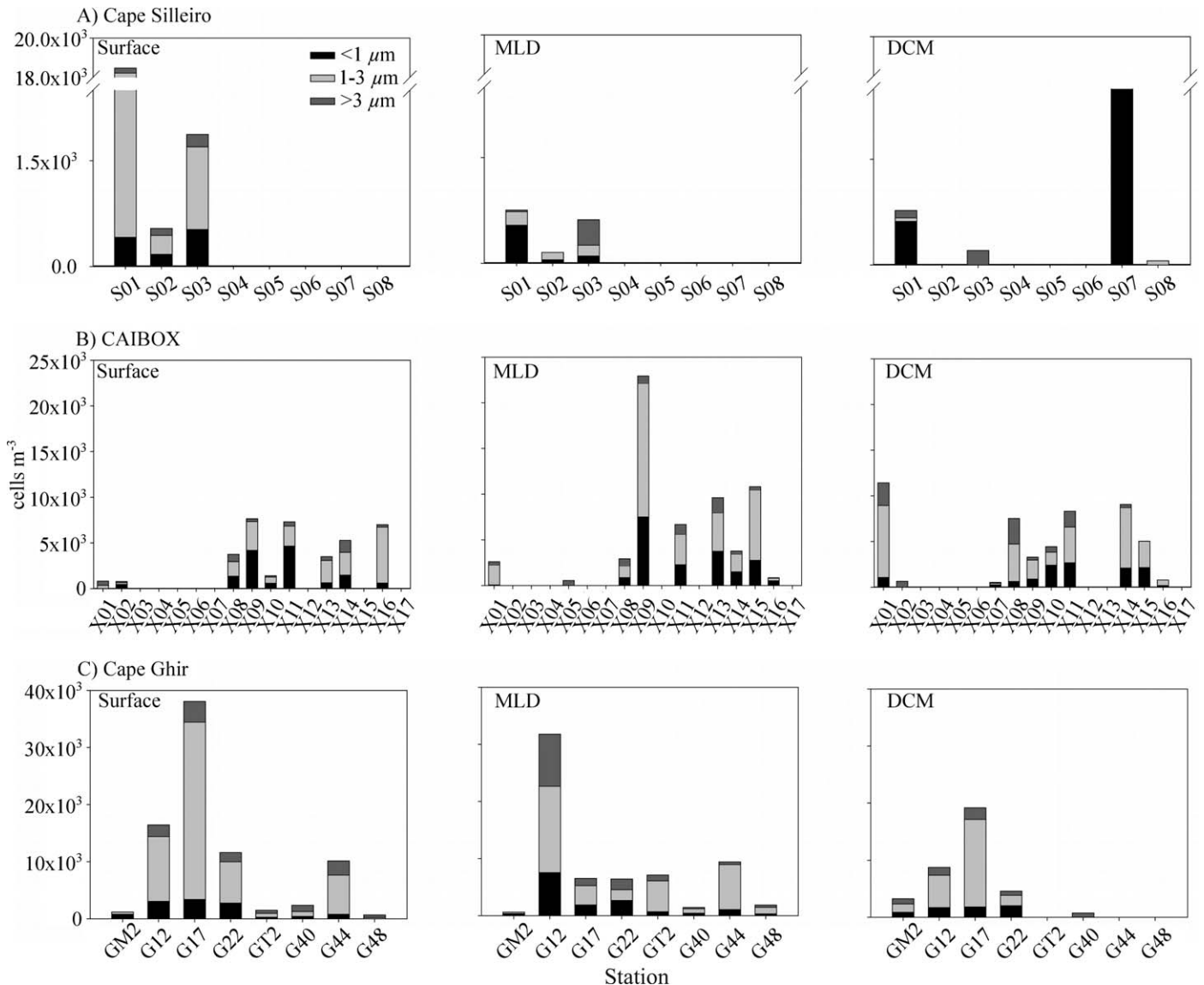


Fig. 7. Relative contribution of the different size fractions to overall Nitro821-positive cells from TSA-FISH technique in samples from the surface, at the bottom of the mixed-layer depths (MLD), and in the deep chlorophyll maximum (DCM) depths in the three legs of the cruise: (A) Cape Silleiro, (B) CAIBOX grid, and (C) Cape Ghir. The heights of the bars are the total abundance of Nitro821-positive cells.

organisms (Prymnesiophytes, Thompson et al. 2012). Moreover, UCYN-A organisms form aggregates of $> 3 \mu\text{m}$ with particulate organic material (Le Moal and Biegala 2009).

The dominance of UCYN-A in the study area is supported by the TSA-FISH counts. It is known that UCYN-A organisms are small, and, therefore, the Nitro821-positive

spherical cells $< 1 \mu\text{m}$ likely belong to these organisms (Goebel et al. 2008). The Nitro821-positive cells in the $1\text{--}3 \mu\text{m}$ size fraction may also be predominantly UCYN-A, because cells of this group of organisms may measure up to $1.5 \mu\text{m}$ (Le Moal et al. 2011). The abundances of TSA-FISH positive cells with sizes similar to UCYN-A ranged from 1.0×10^2 to $8.6 \times 10^3 \text{ L}^{-1}$. Although the abundances of these cells found

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Fig. 6. Phylogenetic tree of *nifH* sequences. Alignments based on a 199-bp DNA sequence were used to construct the neighbor-joining phylogenetic tree rooted to the *Methylomonas rubra* clone 23-4 nitrogenase iron protein (*nifH*) gene (AF484673), and relationships were bootstrapped 1000 times using CLC Sequence Viewer 6. Clone sequences obtained in this study are referred to (1) by the oceanographic cruise CAIBEX, (2) the station identification (ID), (3) the depth sampled (S = surface, M = MLD, D = DCM), (4) filter pore size (A = $10 \mu\text{m}$, B = $3 \mu\text{m}$, C = $0.22 \mu\text{m}$), and (5) clone number. GenBank accession numbers of our clone sequences are indicated in bold letters. Bootstrap values $> 50\%$ are indicated at the nodes. Scale bar = 0.05 substitutions per nucleotide position.

Table 3. Average \pm SD of cell numbers (cells m^{-3}) and percentage (in parentheses, % of cells to total of each size class) of Nitro821-positive cells found as single cells, associated with organic matter, and symbiotic with larger cells.

Leg	<i>n</i>	< 1 μm single cells	< 1 μm associated with organic matter	< 1 μm symbiotic with larger cells	1–3 μm single cells	1–3 μm associated with organic matter	1–3 μm symbiotic with larger cells	> 3 μm single cells	> 3 μm associated with organic matter	> 3 μm symbiotic with larger cells
1: Cape Silleiro										
Surface	3	312 \pm 181 (80 \pm 20)	17 \pm 30 (3 \pm 6)	17 \pm 30 (3 \pm 6)	6257 \pm 10,006 (58 \pm 52)	0 (0)	0 (0)	141 \pm 130 (65 \pm 56)	0 (0)	3 \pm 6 (2 \pm 3)
MLD	3	51 \pm 51 (67 \pm 58)	171 \pm 297 (32 \pm 55)	8 \pm 14 (2 \pm 3)	50 \pm 51 (30 \pm 34)	31 \pm 27 (18 \pm 17)	31 \pm 27 (8 \pm 17)	93 \pm 143 (57 \pm 52)	0 (0)	34 \pm 59 (10 \pm 16)
DCM	4	360 \pm 364 (51 \pm 69)	1955 \pm 2765 (49 \pm 69)	0 (0)	37 \pm 32 (67 \pm 58)	0 (0)	0 (0)	64 \pm 77 (44 \pm 51)	0 (0)	13 \pm 26 (6 \pm 12)
2: CAIBOX										
Surface	10	1399 \pm 1667 (99 \pm 1)	1 \pm 3 (0.2 \pm 0.6)	0.3 \pm 0.9 (0.02 \pm 0.07)	1821 \pm 1891 (87 \pm 27)	81 \pm 156 (11 \pm 24)	5 \pm 11 (2 \pm 4)	321 \pm 264 (83 \pm 20)	105 \pm 153 (14 \pm 18)	8 \pm 18 (2 \pm 4)
MLD	9	2179 \pm 2412 (89 \pm 35)	0 (0)	1 \pm 3 (0.1 \pm 0.3)	2220 \pm 1708 (71 \pm 39)	561 \pm 1292 (9 \pm 17)	1129 \pm 3486 (8 \pm 24)	475 \pm 390 (75 \pm 31)	176 \pm 245 (22 \pm 30)	12 \pm 27 (3 \pm 7)
DCM	9	1087 \pm 1025 (85 \pm 34)	46 \pm 137 (4 \pm 13)	0 (0)	1602 \pm 1279 (66 \pm 39)	1183 \pm 2300 (17 \pm 31)	198 \pm 593 (5 \pm 15)	852 \pm 968 (75 \pm 37)	61 \pm 125 (9 \pm 22)	84 \pm 243 (5 \pm 14)
3: Cape Ghir										
Surface	8	1407 \pm 1389 (97 \pm 9)	13 \pm 36 (3 \pm 9)	7 \pm 19 (0.2 \pm 0.7)	7212 \pm 10463 (98 \pm 4)	110 \pm 289 (2 \pm 4)	14 \pm 39 (0.2 \pm 0.5)	1328 \pm 1166 (76 \pm 35)	132 \pm 249 (10 \pm 17)	13 \pm 22 (1 \pm 1.5)
MLD	8	2006 \pm 2394 (100 \pm 0)	0 (0)	0 (0)	5261 \pm 5820 (92 \pm 13)	390 \pm 948 (7 \pm 13)	130 \pm 247 (1 \pm 3)	1895 \pm 3033 (80 \pm 35)	27 \pm 51 (6 \pm 13)	18 \pm 27 (1 \pm 2)
DCM	5	1293 \pm 845 (80 \pm 45)	0 (0)	0 (0)	2619 \pm 2263 (65 \pm 48)	2232 \pm 4991 (15 \pm 33)	0 (0)	990 \pm 292 (92 \pm 18)	0 (0)	165 \pm 370 (8 \pm 18)

in our study are mostly within the range observed at other sites in the Atlantic at lower latitudes (Langlois et al. 2008; Goebel et al. 2010; Turk et al. 2011), the highest numbers we found were much lower than those reported from the tropical region (Goebel et al. 2010; Turk et al. 2011).

The detection of UCYN-A organisms as far north as 41.5°N suggests a much wider latitudinal distribution of UCYN-A than of other groups of the diazotrophic unicellular cyanobacteria (UCYN-B and UCYN-C) and *Trichodesmium*. The distribution of *Trichodesmium* is often reported to be constrained by the 20–25°C isotherms (Capone et al. 1997). UCYN-B has not been reported in temperate regions in the North Atlantic, but has been detected at low abundances in the tropics (Foster et al. 2007; Goebel et al. 2010; Turk et al. 2011) and in subtropical regions (Langlois et al. 2008; Turk et al. 2011). UCYN-C has only been detected in the tropical region in the North Atlantic (Foster et al. 2007; Langlois et al. 2008). Here, we have detected neither UCYN-B nor UCYN-C in our libraries of the 15 samples with the highest number of *nifH* clones. Hence, they were either not present, or their abundances were too low to be detected by PCR amplification in these samples. The wider latitudinal and larger depth coverage of UCYN-A is consistent with field observations that their maximum abundances occurred at lower temperature, namely 24°C (compared, e.g., to 29°C for UCYN-B, Moisander et al. 2010). A polynomial model between UCYN-A abundance and temperature has been described in Moisander et al. (2010). These authors show that UCYN-A abundances increase with temperature up to 24°C, above which they decline. Within the temperature range of our dataset (13.1–24.2°C) the positive correlation between UCYN-A abundance and temperature agrees with the increasing part of the polynomial model. The physiological basis for the polynomial model between UCYN-A abundance and temperature is not known but may be related to the physiology of its host. Results of measurements of nitrogenase activity in size-fractionated samples suggest that N₂ fixation by UCYN-A may also be dependent on temperature. This correlation was based on N₂ fixation measured in the dark. It is not clear whether N₂ fixation in UCYN-A is under the control of a circadian clock because expression of *nifH* during a day–night cycle varies in different areas (Turk et al. 2011). Because UCYN-A lacks photosystem II (Zehr et al. 2008), nitrogenase will not be compromised by photosynthetic oxygen evolution during the day. Hence, UCYN-A *nifH* transcription in the North Pacific Ocean study was at a maximum during midday, when the rates of photosynthesis and oxygen evolution were presumably high (Church et al. 2005).

Oligotrophic conditions are generally required for the proliferation of diazotrophs such as *Trichodesmium* (Capone et al. 1997), and elevated concentrations of combined N (ammonia, nitrate, organic nitrogen) have long been considered to be a factor that would prevent N₂ fixation (Martin-Nieto et al. 1991). However, the presence and activity of Group A diazotrophic unicellular cyanobacteria in the nitrate-rich upwelling filament regions suggest that combined nitrogen did not exclude this particular group of diazotrophs. Our results are consistent with other reports

of diazotrophic unicellular cyanobacteria in nutrient-rich estuarine and coastal waters (Needoba et al. 2007; Rees et al. 2009) and in eddies in the ocean (Church et al. 2009). Another aspect that may determine the distribution and activity of unicellular diazotrophic cyanobacteria is the inverse relationship of N₂ fixation and the abundance of putative UCYN-A with chlorophyll fluorescence and total primary production, as found here. This unexpected observation contradicts the supposed positive link between phytoplankton biomass and UCYN-A abundance as suggested in Moisander et al. 2010. It has become clear that UCYN-A members rely on the transfer of fixed C from their host, the prymnesiophyte photosynthetic picoeukaryote (PPE; Thompson et al. 2012). Although most of the UCYN-A-like cells in our study were found as single cells, it is possible that these cells became detached from their loose association with their PPE hosts (i.e., prymnesiophytes) as reported by Thompson et al. (2012). These authors argued that even the gentlest filtration might disturb the loose association between UCYN-A cells and their host. Moreover, the calcareous shells of the prymnesiophytes may be damaged or dissolved by fixing reagents (such as paraformaldehyde), which may result in the detachment of the associated UCYN-A. When UCYN-A rely only on the transfer of fixed C from their host PPE, we would expect that the abundance of UCYN-A follows the number of the host cells. Interestingly, we did not find such a correlation in our flow cytometric analyses (data not shown). There may be other, hitherto unidentified, hosts of UCYN-A, but we also found the putative UCYN-A associated to particulate matter. This is consistent with previous observations (Le Moal and Biegala 2009). We suggest that this particulate matter might serve as a carbon source for UCYN-A.

The distribution and activity of diazotrophic unicellular cyanobacteria was negatively correlated with dissolved O₂. N₂ fixation is only possible when the concentration of oxygen inside the diazotrophic cell is sufficiently low because of the O₂ sensitivity of nitrogenase. When the concentration of dissolved O₂ in the seawater is low, the diffusion of oxygen into the cell will also be low; this might aid UCYN-A cells to establish the conditions that allow N₂ fixation.

This study increased the understanding of the importance of unicellular diazotrophic cyanobacteria of the UCYN-A type for the global ocean nitrogen budget. Our results support previous findings that the distribution and activity of UCYN-A may extend into temperate zones and that they may occur and actively fix N₂ even in the presence of combined nitrogen. We also presented evidence that UCYN-A-like cells may live associated with particulate organic matter in addition to having a symbiotic way of life.

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References

- ARÍSTEGUI, J., AND OTHERS. 2009. Sub-regional ecosystem variability in the Canary Current upwelling. *Prog. Oceanogr.* **83**: 33–48, doi:10.1016/j.pocean.2009.07.031
- BENAVIDES, M., N. S. R. AGAWIN, J. ARÍSTEGUI, P. FERRIOL, AND L. J. STAL. 2011. Nitrogen fixation by *Trichodesmium* and small diazotrophs in the subtropical Northeast Atlantic. *Aquat. Microb. Ecol.* **65**: 43–53, doi:10.3354/ame01534
- , ———, ———, AND S. HERNÁNDEZ-LEON. 2013. Enhancement of nitrogen fixation rates by unicellular diazotrophs vs. *Trichodesmium* after a dust deposition event in the Canary Islands. *Limnol. Oceanogr.* **58**: 267–275, doi:10.4319/lo.2013.58.1.0267
- BIEGALA, I. C., AND P. RAIMBAULT. 2008. High abundance of diazotrophic picocyanobacteria (<3 μm) in a Southwest Pacific coral lagoon. *Aquat. Microb. Ecol.* **51**: 45–53, doi:10.3354/ame01185
- BONNET, S., I. C. BIEGALA, P. DUTRIEUX, L. O. SLEMONS, AND D. G. CAPONE. 2009. Nitrogen fixation in the western equatorial Pacific: Rates, diazotrophic cyanobacterial size class distribution, and biogeochemical significance. *Global Biogeochem. Cycles* **23**: GB3012, doi:10.1029/2008GB003439
- CAPONE, D. G. 1993. Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure, p. 621–631. *In* P. F. Kemp, J. J. Cole, B. F. Sherr, and E. B. Sherr [eds.], *Handbook of methods in aquatic microbial ecology*. Lewis.
- , J. P. ZEHR, H. PAERL, B. BERGMAN, AND E. J. CARPENTER. 1997. *Trichodesmium*, a globally significant marine cyanobacterium. *Science* **276**: 1221–1229, doi:10.1126/science.276.5316.1221
- CARRACEDO, L. I., P. C. PARDO, N. VILLACIEROS-ROBINEAU, F. DE LA GRANDA, M. GILCOTO, AND F. F. PÉREZ. 2012. Temporal changes in the water mass distribution and transports along the 20°W CAIBOX section (NE Atlantic). *Ciencias Marinas* **38**: 263–286, doi:10.7773/cm.v38i1B.1793
- CHURCH, M. J., C. MAHAFFEY, R. M. LETELIER, R. LUKAS, J. P. ZEHR, AND D. M. KARL. 2009. Physical forcing of nitrogen fixation and diazotroph community structure in the North Pacific subtropical gyre. *Global Biogeochem. Cycles* **23**: GB2020, doi:10.1029/2008GB003418
- , C. M. SHORT, B. D. JENKINS, D. M. KARL, AND J. P. ZEHR. 2005. Temporal patterns of nitrogenase gene (*nifH*) expression in the oligotrophic North Pacific Ocean. *Appl. Environ. Microbiol.* **71**: 5362–5370, doi:10.1128/AEM.71.9.5362-5370.2005
- FALCON, L. I., E. J. CARPENTER, F. CIPRIANO, B. BERGMAN, AND D. G. CAPONE. 2004. N₂ fixation by unicellular bacterioplankton from the Atlantic and Pacific Oceans: Phylogeny and in situ rates. *Appl. Environ. Microbiol.* **70**: 765–770, doi:10.1128/AEM.70.2.765-770.2004
- FOSTER, R. A., D. G. CAPONE, E. J. CARPENTER, C. MAHAFFEY, A. SUBRAMANIAM, AND J. P. ZEHR. 2007. Influence of the Amazon River Plume on distributions of free-living and symbiotic cyanobacteria in the Western Tropical North Atlantic Ocean. *Limnol. Oceanogr.* **52**: 517–532, doi:10.4319/lo.2007.52.2.0517
- GALLON, J. R., AND OTHERS. 2002. Maximum rates of N₂ fixation and primary production are out of phase in a developing cyanobacterial bloom in the Baltic Sea. *Limnol. Oceanogr.* **47**: 1514–1521, doi:10.4319/lo.2002.47.5.1514
- GOEBEL, N. L., C. A. EDWARDS, B. J. CARTER, K. M. ACHILLES, AND J. P. ZEHR. 2008. Growth and carbon content of three different-sized diazotrophic cyanobacteria observed in the subtropical North Pacific. *J. Phycol.* **44**: 1212–1220, doi:10.1111/j.1529-8817.2008.00581.x
- , AND OTHERS. 2010. Abundance and distribution of major groups of diazotrophic cyanobacteria and their potential contribution to N₂ fixation in the tropical Atlantic Ocean. *Environ. Microbiol.* **12**: 3272–3289, doi:10.1111/j.1462-2920.2010.02303.x
- GROßKOPF, T., AND OTHERS. 2012. Doubling of marine dinitrogen fixation rates based on direct measurements. *Nature* **488**: 361–364, doi:10.1038/nature11338
- HAMA, T., T. MIYAZAKI, Y. OGAWA, T. IWAKUMA, M. TAKAHASHI, A. OTSUKI, AND S. ICHIMURA. 1983. Measurement of photosynthetic production of a marine phytoplankton population using a stable ¹³C isotope. *Mar. Biol.* **73**: 31–36, doi:10.1007/BF00396282
- HANSEN, H., AND F. KOROLEFF. 1999. Determination of nutrients, p. 150–228. *In* K. Grasshoff, K. Kremling, and M. Ehrhardt [eds.], *Methods of seawater analysis*. Wiley-VCH.
- JENSEN, B. B., AND COX, R. P. 1983. Direct measurements of steady-state kinetics of cyanobacterial N₂ uptake by membrane-leak mass spectrometry and comparisons between nitrogen fixation and acetylene reduction. *Appl. Environ. Microbiol.* **45**: 1331–1337, http://www.ncbi.nlm.nih.gov/pmc/articles/PMC242459/
- LANE, D. J. 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow [eds.], *Nucleic acid techniques in bacterial systematics*. John Wiley and Sons.
- LANGLOIS, R. J., D. HUMMER, AND J. LA ROCHE. 2008. Abundances and distributions of the dominant *nifH* phylotypes in the northern Atlantic Ocean. *Appl. Environ. Microbiol.* **74**: 1922–1931, doi:10.1128/AEM.01720-07
- , J. LA ROCHE, AND P. A. RAAB. 2005. Diazotrophic diversity and distribution in the tropical and subtropical Atlantic Ocean. *Appl. Environ. Microbiol.* **71**: 7910–7919, doi:10.1128/AEM.71.12.7910-7919.2005
- LE MOAL, M., AND I. C. BIEGALA. 2009. Diazotrophic unicellular cyanobacteria in the northwestern Mediterranean Sea: A seasonal cycle. *Limnol. Oceanogr.* **54**: 845–855, doi:10.4319/lo.2009.54.3.0845
- , H. COLLIN, AND I. C. BIEGALA. 2011. Intriguing diversity among diazotrophic picoplankton along a Mediterranean transect: A dominance of rhizobia. *Biogeosciences* **8**: 827–840, doi:10.5194/bg-8-827-2011
- LUO, Y. W., AND OTHERS. 2012. Database of diazotrophs in global ocean: Abundance, biomass and nitrogen fixation rates. *Earth Syst. Sci. Data* **4**: 47–73, doi:10.5194/essd-4-47-2012
- MARTIN-NIETO, J., A. HERRERO, AND E. FLORES. 1991. Control of nitrogenase mRNA levels by products of nitrate assimilation in the cyanobacterium *Anabaena* sp. strain PCC-7120. *Plant Physiol.* **97**: 825–828, doi:10.1104/pp.97.2.825
- MAZARD, S. L., N. J. FULLER, K. M. ORCUTT, O. BRIDLE, AND D. J. SCANLAN. 2004. PCR analysis of the distribution of unicellular cyanobacterial diazotrophs in the Arabian Sea. *Appl. Environ. Microbiol.* **70**: 7355–7364, doi:10.1128/AEM.70.12.7355-7364.2004
- MOHR, W., T. GROßKOPF, D. W. R. WALLACE, AND J. LA ROCHE. 2010. Methodological underestimation of oceanic nitrogen fixation rates. *PLoS ONE* **5**: e12583, doi:10.1371/journal.pone.0012583

- MOISANDER, P. H., R. A. BEINART, I. HEWSON, A. E. WHITE, K. S. JOHNSON, C. A. CARLSON, J. P. MONTOYA, AND J. P. ZEHR. 2010. Unicellular cyanobacterial distributions broaden the oceanic N₂ fixation domain. *Science* **327**: 1512–1514, doi:10.1126/science.1185468
- MONTOYA, J. P., C. M. HOLL, J. P. ZEHR, A. HANSEN, T. A. VILLAREAL, AND D. CAPONE. 2004. High rates of N₂ fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. *Nature* **430**: 1027–1031, doi:10.1038/nature02824
- , M. VOSS, AND D. CAPONE. 2007. Spatial variation in N₂-fixation rate and diazotroph activity in the tropical Atlantic. *Biogeosciences* **4**: 369–376, doi:10.5194/bg-4-369-2007
- , ———, P. KÄHLER, AND D. CAPONE. 1996. A simple, high-precision, high-sensitivity tracer assay for N₂ fixation. *Appl. Environ. Microbiol.* **62**: 986–993.
- NEEDOBA, J. A., R. A. FOSTER, C. SAKAMOTO, J. P. ZEHR, AND K. S. JOHNSON. 2007. Nitrogen fixation by unicellular diazotrophic cyanobacteria in the temperate oligotrophic North Pacific Ocean. *Limnol. Oceanogr.* **52**: 1317–1327, doi:10.4319/lo.2007.52.4.1317
- NOGALES, B., M. M. AGUILÓ-FERRETTJANS, C. MARTIN-CARDONA, J. LALUCAT, AND R. BOSCH. 2007. Bacterial diversity, composition and dynamics in and around recreational coastal areas. *Environ. Microb.* **9**: 1913–1929, doi:10.1111/j.1462-2920.2007.01308.x
- REES, A. P., J. A. GILBERT, AND B. A. KELLY-GERREYN. 2009. Nitrogen fixation in the western English Channel (NE Atlantic Ocean). *Mar. Ecol. Prog. Ser.* **374**: 7–12, doi:10.3354/meps07771
- STAAL, M., S. TE LINTEL HEKKERT, F. HARREN, AND L. J. STAL. 2001. Nitrogenase activity in cyanobacteria measured by the acetylene reduction assay: A comparison between batch incubation and on-line monitoring. *Environ. Microbiol.* **3**: 343–351, doi:10.1046/j.1462-2920.2001.00201.x
- STAL, L. J. 1988. Nitrogen fixation in cyanobacterial mats. *Methods Enzymol.* **167**: 474–490.
- THOMPSON, A. W., AND OTHERS. 2012. Unicellular cyanobacterium symbiotic with a single-celled eukaryotic alga. *Science* **337**: 1546–1550, doi:10.1126/science.1222700
- TRIPP, H. J., AND OTHERS. 2010. Metabolic streamlining in an open-ocean nitrogen-fixing cyanobacterium. *Nature* **464**: 90–94, doi:10.1038/nature08786
- TROUPIN, C., E. MASON, J. M. BECKERS, AND P. SANGRÀ. 2012. Generation of the Cape Ghir upwelling filament: A numerical study. *Ocean Model.* **41**: 1–15, doi:10.1016/j.ocemod.2011.09.001
- TURK, K. A., AND OTHERS. 2011. Nitrogen fixation and nitrogenase (*nifH*) expression in tropical waters of the eastern North Atlantic. *ISME J.* **5**: 1201–1212, doi:10.1038/ismej.2010.205
- ZANI, S., M. T. MELLON, J. L. COLLIER, AND J. P. ZEHR. 2000. Expression of *nifH* genes in natural microbial assemblages in Lake George, NY detected with RT-PCR. *Appl. Environ. Microbiol.* **66**: 3119–3124, doi:10.1128/AEM.66.7.3119-3124.2000
- ZEHR, J. P., AND L. A. McREYNOLDS. 1989. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Appl. Environ. Microbiol.* **55**: 2522–2526.
- , M. T. MELLON, AND S. ZANI. 1998. New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (*nifH*) genes. *Appl. Environ. Microbiol.* **64**: 3444–3450.
- , AND OTHERS. 2008. Globally distributed uncultivated oceanic N₂-fixing cyanobacteria lack oxygenic photosystem II. *Science* **322**: 1110–1112, doi:10.1126/science.1165340
- ZWIRGLMAIER, K. 2005. Fluorescence in situ hybridization (FISH)—the next generation. *FEMS Microbiol. Lett.* **246**: 151–158, doi:10.1016/j.femsle.2005.04.015

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