



## Evidence of high N<sub>2</sub> fixation rates in the temperate northeast Atlantic

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**Abstract.** Diazotrophic activity and primary production (PP) were investigated along two transects (Belgica BG2014/14 and GEOVIDE cruises) off the western Iberian Margin and the Bay of Biscay in May 2014. Substantial N<sub>2</sub> fixation activity was observed at 8 of the 10 stations sampled, ranging overall from 81 to 384  $\mu\text{mol N m}^{-2} \text{d}^{-1}$  (0.7 to 8.2  $\text{nmol N L}^{-1} \text{d}^{-1}$ ), with two sites close to the Iberian Margin situated between 38.8 and 40.7   N yielding rates reaching up to 1355 and 1533  $\mu\text{mol N m}^{-2} \text{d}^{-1}$ . Primary production was relatively lower along the Iberian Margin, with rates ranging from 33 to 59  $\text{mmol C m}^{-2} \text{d}^{-1}$ , while it increased towards the northwest away from the peninsula, reaching as high as 135  $\text{mmol C m}^{-2} \text{d}^{-1}$ . In agreement with the area-averaged Chl *a* satellite data contemporaneous with our study period, our results revealed that post-bloom conditions prevailed at most sites, while at the northwesternmost station the bloom was still ongoing. When converted to carbon uptake using Redfield stoichiometry, N<sub>2</sub> fixation could support 1 % to 3 % of daily PP in the euphotic layer at most sites, except at the two most active sites where this contribution to daily PP could reach up to 25 %. At the two sites where N<sub>2</sub> fixation activity was the highest,

the prymnesiophyte–symbiont *Candidatus Atelocyanobacterium thalassa* (UCYN-A) dominated the *nifH* sequence pool, while the remaining recovered sequences belonged to non-cyanobacterial phylotypes. At all the other sites, however, the recovered *nifH* sequences were exclusively assigned phylogenetically to non-cyanobacterial phylotypes. The intense N<sub>2</sub> fixation activities recorded at the time of our study were likely promoted by the availability of phytoplankton-derived organic matter produced during the spring bloom, as evidenced by the significant surface particulate organic carbon concentrations. Also, the presence of excess phosphorus signature in surface waters seemed to contribute to sustaining N<sub>2</sub> fixation, particularly at the sites with extreme activities. These results provide a mechanistic understanding of the unexpectedly high N<sub>2</sub> fixation in productive waters of the temperate North Atlantic and highlight the importance of N<sub>2</sub> fixation for future assessment of the global N inventory.

## 1 Introduction

Dinitrogen (N<sub>2</sub>) fixation is the major pathway of nitrogen (N) input to the global ocean and thereby contributes to sustaining oceanic primary productivity (Falkowski, 1997). The conversion by N<sub>2</sub>-fixing micro-organisms (diazotrophs) of dissolved N<sub>2</sub> gas into bioavailable nitrogen also contributes to new production in the euphotic layer and, as such, to the subsequent sequestration of atmospheric carbon dioxide into the deep ocean (Gruber, 2008). Estimating the overall contribution of N<sub>2</sub> fixation to carbon sequestration in the ocean requires an assessment of the global marine N<sub>2</sub> fixation.

Until recently, most studies of N<sub>2</sub> fixation have focused on the tropical and subtropical regions of the global ocean, with few attempts to measure N<sub>2</sub> fixation at higher latitudes, with the exception of enclosed brackish seas (Ohlendorf et al., 2000; Luo et al., 2012; Farnelid et al., 2013). The intense research efforts in the low-latitude regions stem from the observable presence of cyanobacterial diazotrophs such as the diatom–diazotroph association (DDA) and the colony-forming filamentous *Trichodesmium* (Capone, 1997; Capone et al., 2005; Foster et al., 2007). *Trichodesmium*, in particular, was long considered as the most active diazotroph in the global ocean. It has mostly been reported in tropical and subtropical oligotrophic oceanic waters which are thought to represent the optimal environment for its growth and N<sub>2</sub>-fixing activity (Dore et al., 2002; Breitbarth et al., 2007; Montoya et al., 2007; Needoba et al., 2007; Moore et al., 2009; Fernández et al., 2010; Snow et al., 2015). In low-latitude regions, warm-stratified surface waters depleted in dissolved inorganic nitrogen (DIN) are assumed to give a competitive advantage to diazotrophs over other phytoplankton since only they can draw N from the unlimited dissolved N<sub>2</sub> pool for their biosynthesis. As such, past estimates of global annual N<sub>2</sub> fixation were mainly based on information gathered from tropical and subtropical regions, while higher-latitude areas have been poorly explored for diazotrophic activity (Luo et al., 2012).

Studies using genetic approaches targeting the *nifH* gene encoding the nitrogenase enzyme, essential for diazotrophy, have shown the presence of diverse diazotrophs throughout the world's oceans, extending their ecological niche (Farnelid et al., 2011; Cabello et al., 2015; Langlois et al., 2015). Small diazotrophs such as unicellular diazotrophic cyanobacteria (UCYN classified in groups A, B and C) and non-cyanobacterial diazotrophs, mostly heterotrophic bacteria (e.g. Alpha- and Gammaproteobacteria), have been observed over a wide range of depths and latitudes, thereby expanding the potential for diazotrophy to a much broader geographic scale (Langlois et al., 2005, 2008; Krupke et al., 2014; Cabello et al., 2015). The discovery of a methodological bias associated with the commonly used <sup>15</sup>N<sub>2</sub> bubble-addition technique (Mohr et al., 2010) and the presence of an abundant diazotrophic community in high-latitude regions actively fixing N<sub>2</sub> (Needoba et al., 2007; Rees et al., 2009;

Blais et al., 2012; Mulholland et al., 2012; Shiozaki et al., 2015) indicate that more efforts are needed to better constrain oceanic N<sub>2</sub> fixation and diazotrophic diversity at higher latitudes.

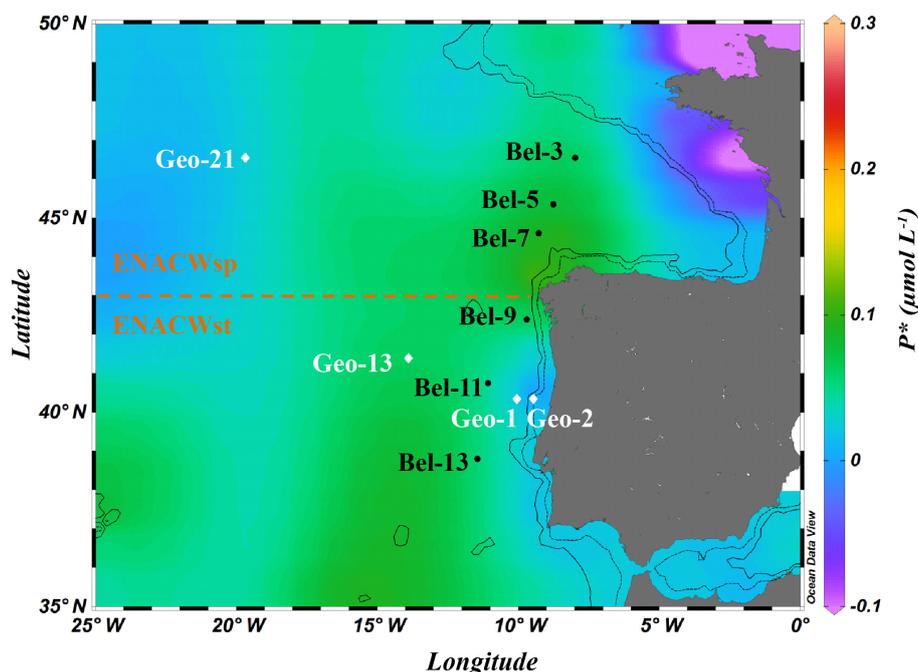
In the northeast Atlantic, the large input of iron-rich Saharan-dust-alleviating dissolved iron (dFe) limitation of the nitrogenase activity (Fe being a co-factor of the N<sub>2</sub>-fixing enzyme) (Raven, 1988; Howard and Rees, 1996; Mills et al., 2004; Snow et al., 2015) and the upwelling of subsurface waters with low DIN (dissolved inorganic nitrogen) to phosphate ratios make this region highly favourable for N<sub>2</sub> fixation activity (Deutsch et al., 2007; Moore et al., 2009). In addition, the eastern North Atlantic has been observed to harbour a highly active and particularly diverse diazotrophic community (Langlois et al., 2008; Moore et al., 2009; Großkopf et al., 2012; Ratten et al., 2015; Fonseca-Batista et al., 2017) not only in the tropical and subtropical regions but also in the temperate Iberian region which was reported to be a hotspot for the globally important prymnesiophyte–UCYN-A symbiotic associations (Cabello et al., 2015). Earlier studies in the Iberian open waters investigated diazotrophic activity either under stratified water column conditions of boreal summer and autumn (Moore et al., 2009; Benavides et al., 2011; Snow et al., 2015; Fonseca-Batista et al., 2017) or during the winter convection period (Rijkenberg et al., 2011; Agawin et al., 2014). Here, we present N<sub>2</sub> fixation rate measurements and the taxonomic affiliation of the diazotrophic community from two consecutive campaigns, carried out in the northeast sector of the Atlantic Ocean in May 2014, during and after the spring bloom.

## 2 Material and methods

### 2.1 Site description and sample collection

Field experiments were conducted during two nearly simultaneous cruises in May 2014. The Belgica BG2014/14 cruise (21–30 May 2014, R/V *Belgica*) investigated the Bay of Biscay and the western Iberian Margin. In parallel, the GEOVIDE expedition in the framework of the international GEOTRACES programme (GA01 section, 16 May to 29 June 2014, R/V *Pourquoi pas?*) sailed from the Portuguese shelf area towards Greenland and ended in Newfoundland, Canada (<https://doi.org/10.17600/14000200>). N<sub>2</sub> fixation activities were determined at 10 stations within the Iberian Basin, among which four sites were investigated during the GEOVIDE cruise (stations Geo-1, Geo-2, Geo-13 and Geo-21) and six sites during the BG2014/14 cruise (stations Bel-3, Bel-5, Bel-7, Bel-9, Bel-11 and Bel-13; Fig. 1).

All sampling sites were located within the Iberian Basin Portugal Current System (PCS) (Ambar and Fiúza, 1994), which is influenced by highly fluctuating wind stresses (Frouin et al., 1990). The predominant upper-layer water mass in this basin is the eastern North Atlantic central wa-



**Figure 1.** Location of sampling stations during the Belgica BG2014/14 (black labels) and GEOVIDE (white labels) cruises (May 2014) superimposed on a map of the seasonal average phosphate excess ( $P^* = [\text{PO}_4^{3-}] - [\text{NO}_3^-]/16$ ) at 20 m (April to June for the period from 1955 to 2012; World Ocean Atlas 2013; Garcia et al., 2013). Areas of dominance of the eastern North Atlantic central waters of subpolar (ENACWsp) and subtropical (ENACWst) origin are separated by a dashed horizontal line. Dashed and solid black contour lines illustrate 500 and 1500 m isobaths, respectively; Schlitzer, R., Ocean Data View.

ter (ENACW), a winter-mode water, which according to Fiúza (1984) consists of two components (see  $\theta$ -S diagrams in Fig. S1 in the Supplement): (i) the lighter, relatively warm ( $>14^\circ\text{C}$ ) and salty (salinity  $>35.6$ ) ENACWst formed in the subtropical Azores Front region ( $\sim 35^\circ\text{N}$ ) when Azores mode water is subducted as a result of strong evaporation and winter cooling; and (ii) the colder and less saline ENACWsp, underlying the ENACWst, formed in the subpolar eastern North Atlantic (north of  $43^\circ\text{N}$ ) through winter cooling and deep convection (McCartney and Talley, 1982). The spatial distribution of these central waters allowed the categorization of the sampling sites into two groups: (i) ENACWsp stations north of  $43^\circ\text{N}$  (Bel-3, Bel-5, Bel-7 and Geo-21), only affected by the ENACWsp (Fig. S1a, b), and (ii) ENACWst stations, south of  $43^\circ\text{N}$ , characterized by an upper layer influenced by the ENACWst and a subsurface layer, by the ENACWsp (Fig. S1a, b). Most of the ENACWst stations were open-ocean sites (Bel-9, Bel-11, Bel-13 and Geo-13), while two stations were in proximity of the Iberian shelf (Geo-1 and Geo-2) (Tonnard et al., 2018).

Temperature, salinity and photosynthetically active radiation (PAR) profiles down to 1500 m depth were obtained using a conductivity–temperature–depth (CTD) sensor (SBE 09 and SBE 911+, during the BG2014/14 and GEOVIDE cruises, respectively) fitted to the rosette frames. For all biogeochemical measurements, seawater samples were col-

lected with Niskin bottles attached to the rosette and closed at specific depths in the upper 200 m. In particular, for stable isotope incubation experiments, seawater was collected in 4.5 L acid-cleaned polycarbonate (PC) bottles from four depths corresponding to 54 %, 13 %, 3 % and 0.2 % of surface PAR at stations Bel-3, Bel-5, Bel-7, Bel-9, Bel-11 and Geo-2. At stations Geo-1, Geo-13 and Geo-21, two additional depths corresponding to 25 % and 1 % of surface PAR were also sampled for the same purpose.

## 2.2 Nutrient measurements

Ammonium ( $\text{NH}_4^+$ ) concentrations were measured on-board during both cruises, while nitrate plus nitrite ( $\text{NO}_3^- + \text{NO}_2^-$ ) concentrations were measured on-board only during the GEOVIDE expedition. During the BG2014/14 cruise, samples for  $\text{NO}_3^- + \text{NO}_2^-$  and phosphate ( $\text{PO}_4^{3-}$ ) measurements were filtered ( $0.2\ \mu\text{m}$ ) and stored at  $-20^\circ\text{C}$  until analysis at the home-based laboratory.  $\text{PO}_4^{3-}$  data are not available for the GEOVIDE cruise.

Nutrient concentrations were determined using the conventional fluorometric (for  $\text{NH}_4^+$ ) (Holmes et al., 1999) and colorimetric methods (for the other nutrients) (Grasshoff et al., 1983) with detection limits (DLs) of  $64\ \text{nmol L}^{-1}$  ( $\text{NH}_4^+$ ),  $90\ \text{nmol L}^{-1}$  ( $\text{NO}_3^- + \text{NO}_2^-$ ) and  $60\ \text{nmol L}^{-1}$  ( $\text{PO}_4^{3-}$ ). For the BG2014/14 cruise, chlorophyll *a* (Chl *a*) concentrations were determined according to Yentsch and Menzel (1963).

Briefly, 250 mL of seawater were filtered onto Whatman GF/F glass microfiber filters (0.7 µm nominal pore size), followed by pigment extraction in 90 % acetone, centrifugation and fluorescence measurement using a Shimadzu RF-150 fluorometer. For the GEOVIDE cruise, Chl *a* concentrations were measured as described in Ras et al. (2008). Briefly, filters samples were extracted in 100 % methanol, disrupted by sonification and clarified by vacuum filtration through Whatman GF/F filters. The extracts were analysed by high-performance liquid chromatography (HPLC Agilent Technologies 1200).

### 2.3 <sup>15</sup>N<sub>2</sub> fixation and <sup>13</sup>C-HCO<sub>3</sub><sup>-</sup> uptake rates

N<sub>2</sub> fixation and primary production (PP) were determined simultaneously from the same incubation sample at each depth in duplicate, using the <sup>15</sup>N-N<sub>2</sub> dissolution method (Großkopf et al., 2012) and <sup>13</sup>C-NaHCO<sub>3</sub> tracer addition technique (Hama et al., 1983), respectively. Details concerning the applied <sup>15</sup>N<sub>2</sub> dissolution method can be found in Fonseca-Batista et al. (2017). Briefly, <sup>15</sup>N<sub>2</sub>-enriched seawater was prepared by degassing prefiltered (0.2 µm) low-nutrient seawater, under acid-clean conditions using a peristaltic pump slowly circulating (100 mL min<sup>-1</sup>) the seawater through two degassing membrane contactor systems (MiniModule, Liqui-Cel) in series, held under high vacuum (50 mbar). The degassed water was directly transferred into 2 L gastight Tedlar bags (Sigma-Aldrich) fitted with a septum through which 30 mL of pure <sup>15</sup>N<sub>2</sub> gas (98 <sup>15</sup>N atom %, Eurisotop, lot number 23/051301) were injected before the bags were shaken 24 h for tracer equilibration. This <sup>15</sup>N<sub>2</sub> gas batch was previously shown to be free of <sup>15</sup>N-labelled contaminants such as nitrate, nitrite, ammonium and nitrous oxide (Fonseca-Batista et al., 2017). Each PC incubation bottle was partially filled with sampled seawater, then amended with 250 mL of <sup>15</sup>N<sub>2</sub>-enriched seawater and spiked with 3 mL of <sup>13</sup>C-labelled dissolved inorganic carbon (DIC; 200 mmol L<sup>-1</sup> solution of NaH<sup>13</sup>CO<sub>3</sub>, 99 %, Eurisotop). The <sup>13</sup>C-DIC added to a 4.5 L incubation bottle results in a ~ 6.5 % increment of the initial DIC content, considered equal to the average oceanic DIC concentration (~ 2000 µmol kg<sup>-1</sup>; Zeebe and Wolf-Gladrow, 2003). This allows sufficient tracer enrichment for a sensitive detection in the particulate organic carbon (POC) pool as a result of incorporation (Hama et al., 1983). Finally, each incubation bottle was topped off with the original seawater sample. Samples were then incubated for 24 h in on-deck incubators circulated with surface seawater and wrapped with neutral density screens (Rosco) simulating the in situ irradiance conditions. After incubation, water was transferred under helium pressure from each PC bottle into triplicate 12 mL gastight Exetainer vials (Labco) poisoned (100 µL of saturated HgCl<sub>2</sub> solution) and pre-flushed with helium for the determination of the <sup>15</sup>N and <sup>13</sup>C atom % enrichments of the dissolved N<sub>2</sub> (in duplicate) and DIC pools. The remaining incubated sample was filtered onto pre-

combusted MGFs (glass microfiber filters, 0.7 µm nominal pore size, Sartorius), which were subsequently dried at 60 °C and stored at room temperature. The natural concentration and isotopic composition of POC and particulate nitrogen (PN) were assessed by filtering immediately after sampling an additional 4.5 L of non-spiked seawater from each depth. All samples were measured for POC and PN concentrations and isotopic compositions using an elemental analyser (EuroVector Euro EA 3000) coupled to an isotope ratio mass spectrometer (IRMS; Delta V Plus, Thermo Scientific) and calibrated against international certified reference materials (CRMs): IAEA-N1 and IAEA-305B for N and IAEA-CH6 and IAEA-309B for C. The isotopic composition of the DIC and dissolved N<sub>2</sub> pools was determined using a gas bench system coupled to an IRMS (Nu Instruments Perspective). Exetainer vials were first injected with He to create a 4 mL headspace and then equilibrated on a rotatory shaker: for 12 h after phosphoric acid addition (100 µL, 99 %, Sigma-Aldrich) for DIC analyses and only for an hour without acid addition for N<sub>2</sub> analyses. DIC measurements were corrected according to Miyajima et al. (1995) and <sup>15</sup>N<sub>2</sub> enrichments were calibrated with atmospheric N<sub>2</sub>. N<sub>2</sub> fixation and carbon uptake volumetric rates were computed as shown in Eq. (1):

$$\text{N}_2 \text{ or HCO}_3^- \text{ uptake rate (nmol L}^{-1} \text{ d}^{-1} \text{ or } \mu\text{mol m}^{-3} \text{ d}^{-1}) = \frac{A_{\text{PN or POC}}^{\text{final}} - A_{\text{PN or POC}}^{t=0}}{A_{\text{N}_2 \text{ or DIC}} - A_{\text{PN or POC}}^{t=0}} \times \frac{[\text{PN or POC}]}{\Delta t}, \quad (1)$$

where  $A_{\text{PN or POC}}$  represents the <sup>15</sup>N or <sup>13</sup>C atom % excess of PN or POC, respectively, at the beginning ( $t = 0$ ) and end (final) of the incubation, while  $A_{\text{N}_2 \text{ or DIC}}$  represents the <sup>15</sup>N or <sup>13</sup>C atom % excess of the dissolved inorganic pool (N<sub>2</sub> or DIC); and  $\Delta t$  represents the incubation period.

Depth-integrated rates were calculated by non-uniform gridding trapezoidal integration for each station. The DLs, defined as the minimal detectable uptake rates, were determined as detailed in Fonseca-Batista et al. (2017). To do so, the minimal acceptable <sup>15</sup>N or <sup>13</sup>C enrichment of PN or POC after incubation (Montoya et al., 1996) is considered to be equal to the natural isotopic composition, specific to each sampled depth, plus 3 times the uncertainty obtained for N and C isotopic analysis of CRMs. All remaining experiment-specific terms are then used to recalculate the minimum detectable uptake. Carbon uptake rates were always above their specific DL, while N<sub>2</sub> fixation was not detectable at any of the four depths of stations Bel-3 and Bel-5, nor at Bel-9 at 120 m, Bel-11 at 45 m and Geo-21 at 18 m (see Table S1).

### 2.4 DNA sampling and *nifH* diversity analysis

During the BG2014/14 and GEOVIDE cruises, water samples were also collected for DNA extraction and *nifH* sequencing at the stations where N<sub>2</sub> fixation rate measurements were carried out. Overall, 2 L of seawater samples were vacuum filtered (20 to 30 kPa) through sterile 0.2 µm 47 mm

membrane filters (cellulose acetate Sartorius-type 111 for BG2014/14; Millipore's Isopore – GTTP04700 for GEOVIDE) subsequently placed in Cryovials directly flash deep frozen in liquid nitrogen. At the land-based laboratory, samples were transferred to a  $-80^{\circ}\text{C}$  freezer until nucleic acid extraction.

For the BG2014/14 samples, DNA was extracted from the samples using the Power Water DNA Isolation kit (MOBIO) and checked for integrity by agarose gel electrophoresis. The amplification of *nifH* sequences was performed on 3–50 ng  $\mu\text{L}^{-1}$  environmental DNA samples using one unit of Taq polymerase (5PRIME), by nested PCR according to Zani et al. (2000) and Langlois et al. (2005). Amplicons of the predicted 359 bp size observed by gel electrophoresis were cloned using the pGEM-T Easy cloning kit (PROMEGA) according to the manufacturer's instructions. A total of 103 clones were sequenced by the Sanger technique (GATC, Marseille).

For the GEOVIDE samples, DNA was extracted using the QIAGEN DNeasy Plant Mini Kit as instructed by the manufacture, with a modified step to improve cell lysis. This step consisted of an incubation at  $52^{\circ}\text{C}$  on an orbital shaker for 1 h (300 rpm) with 50  $\mu\text{L}$  of lysozyme solution (5 mg  $\text{mL}^{-1}$  in TE buffer), 45  $\mu\text{L}$  of Proteinase K solution (20 mg  $\text{mL}^{-1}$  in Milli-Q PCR-grade water) and 400  $\mu\text{L}$  of AP1 lysis buffer from the QIAGEN DNeasy Plant Mini Kit. DNA concentration and purity were assessed with NanoDrop 2000 and then stored at  $-80^{\circ}\text{C}$ . The DNA samples were screened for the presence of the *nifH* gene as described in Langlois et al. (2005). Samples that tested positive were further prepared for next-generation sequencing on an Illumina MiSeq platform using primers that included the *nifH1* and *nifH2* primers (Zani et al., 2000; Langlois et al., 2005; Ratten, 2017) attached to Illumina adaptors and barcodes for multiplexing in the Illumina MiSeq instrument. Next-generation sequencing was carried out at the Integrated Microbiome Resource (IMR) of the Centre for Comparative Genomics and Evolutionary Bioinformatics (CGEB) at Dalhousie University (Halifax, Canada). Raw Illumina paired-end reads of *nifH* were preprocessed using the QIIME pipeline (Quantitative Insights Into Microbial Ecology; Caporaso et al., 2010) following the IMR workflow ([https://github.com/mlangill/microbiome\\_helper/wiki/16S-standard-operating-procedure](https://github.com/mlangill/microbiome_helper/wiki/16S-standard-operating-procedure), last access: 1 September 2018; Comeau et al., 2017). The 28 OTUs for the *nifH* genes presented in this study were assembled based on 96 % identity of sequence reads.

DNA alignments were performed using the Molecular Evolutionary Genetics Analysis software (MEGA 7.0) (Kumar et al., 2016) and *nifH* operational taxonomic units (*nifH*-OTUs) were defined with a maximum 5 % divergence cut-off. DNA sequences were translated into amino acid sequences; then, *nifH* evolutionary distances considered as the number of amino acid substitutions per site were computed using the Poisson correction method (Nei, 1987). All posi-

tions containing gaps and missing data were eliminated (see phylogenetic tree in Fig. 6). One representative sequence of each *nifH*-OTU was deposited in GenBank under the accession numbers referenced from KY579322 to KY579337, for the Belgica DNA samples and referenced from MH974781 to MH974795 for the GEOVIDE Iberian samples.

## 2.5 Statistical analysis

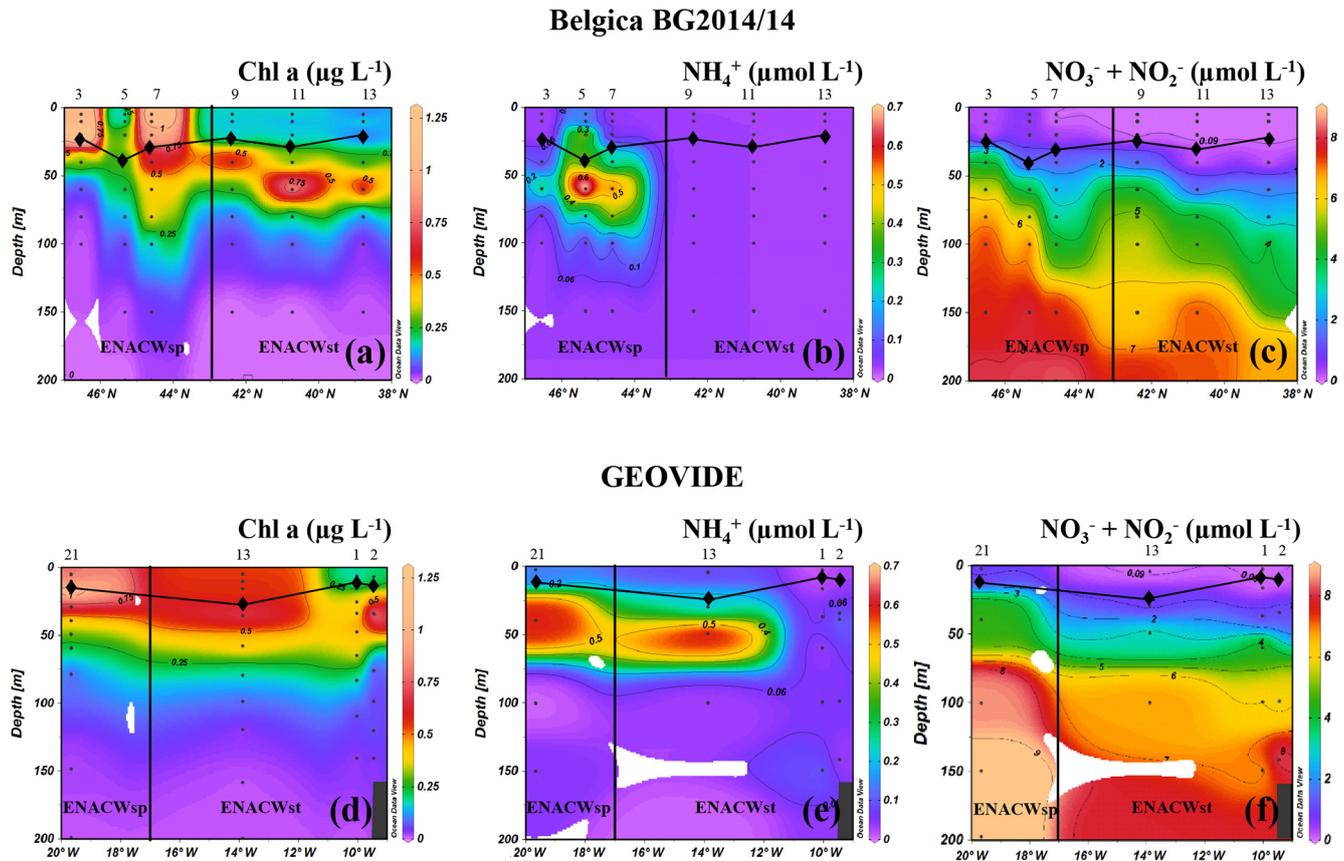
The relationship between N<sub>2</sub> fixation activities and ambient physical and chemical properties was examined using SigmaPlot (Systat Software, San Jose, CA) by computing Spearman rank correlation coefficients linking depth-integrated rates and volumetric rates of N<sub>2</sub> fixation and primary production to environmental variables. These ambient variables were either averaged or integrated over the euphotic layer, or considered as discrete measurements. These variables include temperature, salinity, Chl *a*,  $\text{NH}_4^+$ ,  $\text{NO}_3^- + \text{NO}_2^-$ , phosphorus excess ( $\text{P}^* = [\text{PO}_4^{3-}] - [\text{NO}_3^- + \text{NO}_2^-]/16$ ) derived from in situ nutrient measurements and climatological data (Garcia et al., 2013), dissolved iron concentrations determined for the GEOVIDE cruise (Tonnard et al., 2018) and satellite-derived dust deposition fluxes at the time of our study (Giovanni online data system). When nutrient concentrations were below the DL, we used the DL value to run the correlation test. In addition, we ran a principal component analysis (PCA) using XLSTAT 2017 (Addinsoft, Paris, France, 2017) to get an overview of the interconnection between all the latter key variables with N<sub>2</sub> fixation at the time of our study. The output of the PCA is discussed in Sect. 4.3.

## 3 Results

### 3.1 Ambient environmental settings

Surface waters of all the ENACWst stations showed a relatively strong stratification resulting from the progressive spring heating, with sea surface temperature (SST) ranging from 15.3 (Geo-13) to 17.2  $^{\circ}\text{C}$  (Bel-13). At the surface, nutrients were depleted ( $\text{NO}_3^- + \text{NO}_2^- < 0.09 \mu\text{M}$  in the upper 20 m; Fig. 2c, f) and Chl *a* concentrations were low ( $< 0.25 \mu\text{g L}^{-1}$ ; Fig. 2a, d) but showed a subsurface maximum (between 0.5 and 0.75  $\mu\text{g L}^{-1}$  at approximately 50 m), a common feature for oligotrophic open-ocean waters. Amongst the ENACWst stations, station Geo-13 had a slightly higher nutrient content ( $\text{NO}_3^- + \text{NO}_2^- = 0.7 \mu\text{M}$ ) in the lower mixed layer depth (MLD) and a higher Chl *a* concentration ( $> 0.5 \mu\text{g L}^{-1}$  in the upper 35 m).

Surface waters at ENACWsp stations were less stratified (SST between 14.0 and 14.5  $^{\circ}\text{C}$ ), were nutrient replete (surface  $\text{NO}_3^- + \text{NO}_2^-$  ranging from 0.3 to 0.8  $\mu\text{M}$ ) and had a higher phytoplankton biomass (Chl *a* between 0.7 to 1.2  $\mu\text{g L}^{-1}$  in the upper 30 m except for station Bel-5). Highest Chl *a* values were observed at station Bel-7 (44.6  $^{\circ}\text{N}$ ,



**Figure 2.** Spatial distribution of Chl *a* (a, d), NH<sub>4</sub><sup>+</sup> (b, e) and NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> (c, f) concentrations along the Belgica BG2014/14 (a, b, c) and GEOVIDE (d, e, f) cruise tracks. Station numbers are indicated above the sections. The vertical black line represents the boundary between areas with dominance of eastern North Atlantic waters of subpolar (ENACWsp) and subtropical (ENACWst) origin. Mixed layer depth (MLD, black lines connecting diamonds) was estimated using a temperature threshold criterion of 0.2 °C relative to the temperature at 10 m (de Boyer Montégut et al., 2004); Schlitzer, R., Ocean Data View.

9.3° W), which appeared to be located within an anticyclonic mesoscale eddy as evidenced by the downwelling structure detected in the Chl *a* and NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> profiles (Fig. 2a, c) at this location (as well as T and S sections; data not shown).

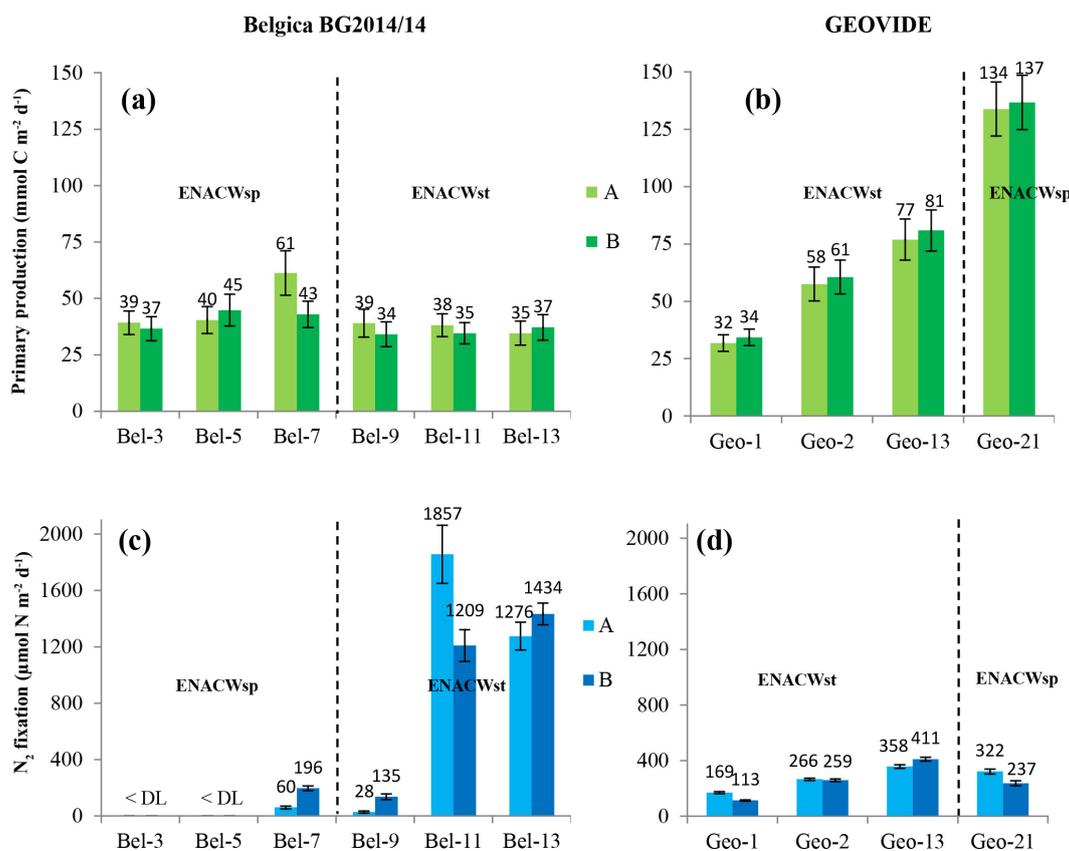
### 3.2 Primary production and satellite-based Chl *a* observations

PP, estimated through the incorporation of enriched bicarbonate (<sup>13</sup>C-NaHCO<sub>3</sub>) into the POC pool, illustrated volumetric rates ranging from 7 to 3500 µmol C m<sup>-3</sup> d<sup>-1</sup> (see Table S1) and euphotic-layer-integrated rates ranging from 32 to 137 mmol C m<sup>-2</sup> d<sup>-1</sup> (Fig. 3a, b and Table S2). PP was relatively homogenous in the Bay of Biscay (stations Bel-3, Bel-5 and Bel-7) and along the Iberian Margin (Bel-9, Bel-11, Bel-13 and Geo-1) with average rates ranging from 33 to 43 mmol C m<sup>-2</sup> d<sup>-1</sup>, except for station Bel-7, where it was slightly higher (52 mmol C m<sup>-2</sup> d<sup>-1</sup>; Fig. 3a, b and Table S2), likely due to the presence of an anticyclonic mesoscale structure at this location. PP increased westwards away from the Iberian Peninsula, reaching highest values at

stations Geo-13 and Geo-21 (79 and 135 mmol C m<sup>-2</sup> d<sup>-1</sup>, respectively; Fig. 3b), but also slightly higher on the Portuguese shelf (reaching 59 mmol C m<sup>-2</sup> d<sup>-1</sup> at Geo-2). These results are in the range of past measurements in this region for the same period of the year, ranging from 19 to 103 mmol C m<sup>-2</sup> d<sup>-1</sup> (Marañón et al., 2000; Fernández et al., 2005; Poulton et al., 2006; Fonseca-Batista et al., 2017). Area-averaged Chl *a* derived from satellite imagery for a time period overlapping with ours (Giovanni online data system; Fig. 4a, b) revealed that post-bloom conditions prevailed at most sites (Bel-3 to Bel-13 and Geo-1 to Geo-13), while bloom conditions were still ongoing at station Geo-21 at the time of our study.

### 3.3 N<sub>2</sub> fixation and dominant diazotrophs at the sampling sites

Volumetric N<sub>2</sub> fixation rates were above the DL at 8 of the 10 stations sampled in this study (Bel-3 and Bel-5 being below the DL) and ranged from 0.7 to 65.4 nmol N L<sup>-1</sup> d<sup>-1</sup>



**Figure 3.** Spatial distribution ( $\pm$ SD) of depth-integrated rates of primary production (**a, b**) (duplicates are in light and dark green bars with the corresponding values in  $\text{mmol C m}^{-2} \text{d}^{-1}$ ); N<sub>2</sub> fixation (**c, d**) (duplicates are in light and dark blue bars with the corresponding values in  $\mu\text{mol N m}^{-2} \text{d}^{-1}$ ) determined during the Belgica BG2014/14 (**a, c**) and GEOVIDE (**b, d**) cruises. Error bars represent the propagated measurement uncertainty of all parameters used to compute volumetric uptake rates.

(see Table S1), with areal rates ranging between 81 and  $1533 \mu\text{mol N m}^{-2} \text{d}^{-1}$  (Fig. 3c, d and Table S2).

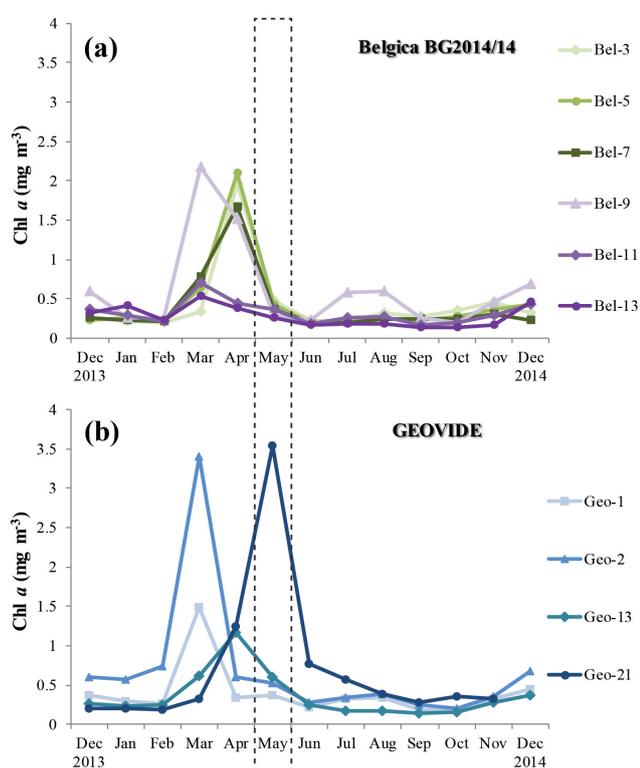
We observed intense N<sub>2</sub> fixation activities at the two sites (Bel-11 and Bel-13) most affected by ENACWst (Fig. S1). At stations Bel-11 and Bel-13, volumetric rates of N<sub>2</sub> fixation ranged from 2.4 to  $65.4 \text{ nmol N L}^{-1} \text{d}^{-1}$ , with highest rates found at surface level ( $65.4$  and  $45.0 \text{ nmol N L}^{-1} \text{d}^{-1}$ , respectively), while areal rates averaged  $1533$  and  $1355 \mu\text{mol N m}^{-2} \text{d}^{-1}$ , respectively. N<sub>2</sub> fixation was detected at all four GEOVIDE stations. Shelf-influenced (Geo-1 and Geo-2) and open-ocean (Geo-13) ENACWst sites, geographically close to Bel-11 and Bel-13, also displayed high N<sub>2</sub> fixation activities, with volumetric rates ranging from  $1.0$  to  $7.1 \text{ nmol N L}^{-1} \text{d}^{-1}$  (Table S1), while depth-integrated rates averaged  $141$ ,  $262$  and  $384 \mu\text{mol N m}^{-2} \text{d}^{-1}$ , respectively (Fig. 3c, d and Table S2). Significant N<sub>2</sub> fixation rates were also measured at stations that exhibited the highest primary production rates, including Bel-7, Geo-13 and Geo-21 (Fig. 3). We computed the relative contribution of N<sub>2</sub> fixation to PP by converting N<sub>2</sub> fixation rates to carbon uptake using either the Redfield ratio of 6.6 or the determined median POC / PN ratio for natural

particles (equivalent to the mean value of  $6.3 \pm 1.1$ ,  $\pm$ SD,  $n = 46$ ; Table 1). N<sub>2</sub> fixation contributed to less than 2 % of PP at the ENACWsp sites Bel-7 and Geo-21 and between 3 % and 28 % of PP at the ENACWst sites, except for station Bel-9 where it supported about 1 % of PP.

Screening of the *nifH* genes from DNA samples collected during the BG2014/14 cruise returned positive *nifH* presence at stations Bel-11 and Bel-13, which displayed the largest areal N<sub>2</sub> fixation rates. Cloning of the *nifH* amplicons found in surface waters (54 % PAR level where volumetric rates of N<sub>2</sub> fixation were the highest) yielded 103 *nifH* sequences. No successful *nifH* amplifications were obtained at the other Belgica stations or depths where diazotrophic activities were lower or undetectable. All the clones ( $n = 41$ ) recovered from station Bel-11 were taxonomically assigned to a single OTU that had 99 % identity at the nucleotide level and 100 % similarity at the amino acid level with the symbiotic diazotrophic cyanobacteria UCYN-A1 or *Candidatus Atelocyanobacterium thalassa*, first characterized from station ALOHA in the North Pacific (Figs. 5a and 6) (Thompson et al., 2012). While the UCYN-A OTU also dominated the clones recovered from station Bel-13, 14 ad-

**Table 1.** Relative contribution (%) of N<sub>2</sub> fixation to PP.

Province	Station	Latitude (° N)	Longitude (° E)	N <sub>2</sub> fixation contribution to PP (%) (Redfield 6.6 ratio)	SD	N <sub>2</sub> fixation contribution to PP (%) (mean POC / PN ratio of 6.3 ± 1.1)	SD
ENACW <sub>sp</sub>	Bel-3	46.5	-8.0	0	–	0	–
	Bel-5	45.3	-8.8	0	–	0	–
	Bel-7	44.6	-9.3	2	0.4	1	0.4
	Geo-21	46.5	-19.7	1	0.02	1	0.0
ENACW <sub>st</sub>	Bel-9	42.4	-9.7	1	0.1	1	0.1
	Bel-11	40.7	-11.1	28	1.9	25	1.8
	Bel-13	38.8	-11.4	25	1.3	23	1.2
	Geo-1	40.3	-10.0	3	0.2	3	0.1
	Geo-2	40.3	-9.5	3	0.1	3	0.1
	Geo-13	41.4	-13.9	3	0.1	3	0.1

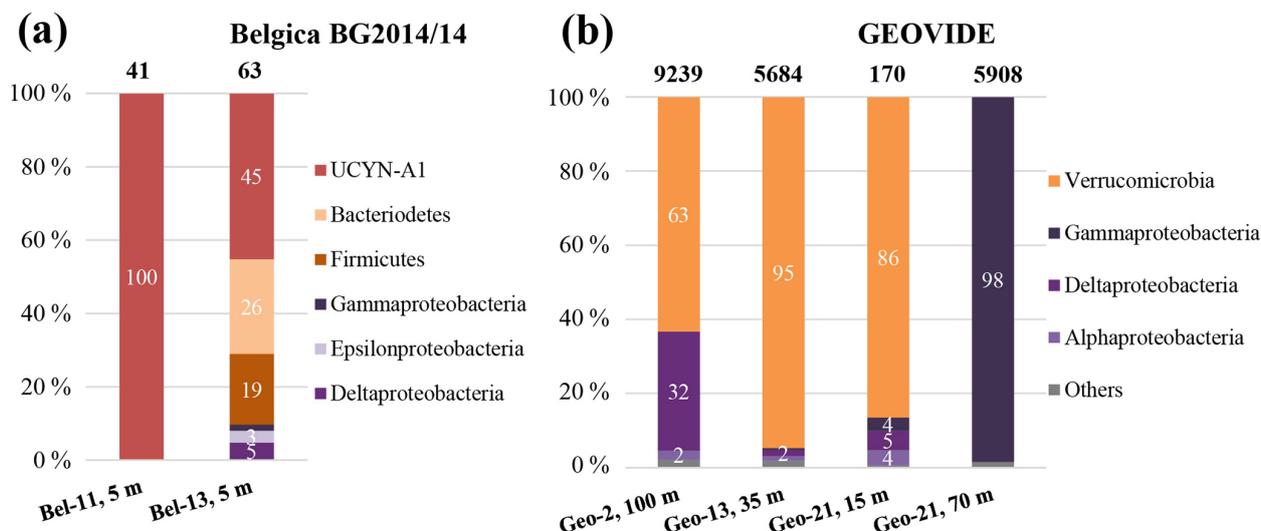
**Figure 4.** Time series of area-averaged chlorophyll *a* concentration ( $\text{mg m}^{-3}$ ) registered by Aqua MODIS satellite (Giovanni online satellite data system) between December 2013 and 2014 for the  $0.5^\circ \times 0.5^\circ$  grid surrounding the different stations during the (a) Belgica BG2014/14 and (b) GEOVIDE cruises. The dashed box highlights the sampling period for both cruises (May 2014).

ditional *nifH* phylotypes affiliated with non-cyanobacterial diazotrophs were also recovered at that station (Figs. 5a and 6). Among these 15 OTUs, represented by a total of 62 sequenced clones, 45.2% of the sequences were affil-

iated with UCYN-A1 (identical to those found at Bel-11) and the rest with heterotrophic bacteria, with 25.8% affiliated with Bacteroidetes, 19.3% with Firmicutes and 9.7% with Proteobacteria (Gamma-, Epsilon- and Deltaproteobacteria; Figs. 5a and 6). For the GEOVIDE cruise, *nifH* screening returned positive *nifH* presence at stations Geo-2, Geo-13 and Geo-21. Next-generation sequencing of these amplicons yielded in total 21 001 reads, with a range of 170 to 9239 *nifH* amplicons per sample, belonging exclusively to non-cyanobacterial diazotrophs, with the major affiliation to Verrucomicrobia, and Gamma-, Delta- and Alphaproteobacteria, representing 54%, 28%, 15% and 1% of total *nifH* amplicons, respectively (Figs. 5b and 6). Members of a clade that has recently been characterized from the TARA expedition through metagenome assembled genomes of marine heterotrophic diazotrophs (Delmont et al., 2018) were found among the Gammaproteobacteria OTU types that dominated the community at station Geo-21.

### 3.4 Relationship between N<sub>2</sub> fixation rates and environmental variables

N<sub>2</sub> fixation activities were measured in surface waters characterized by relatively low SST (12.5–17.3 °C) and a wide range of dissolved inorganic nitrogen (DIN) concentrations ( $\text{NO}_3^- + \text{NO}_2^-$  from <0.1 to 7.6  $\mu\text{M}$ ). Water-column-integrated N<sub>2</sub> fixation tended to increase with average surface water salinity ( $n = 10$ ,  $p < 0.05$ , Table S3) but was inversely correlated with satellite-based dust deposition in May 2014, the month during which our sampling took place ( $n = 10$ ,  $p < 0.01$ ). Volumetric rates of N<sub>2</sub> fixation tended to increase with temperature ( $n = 46$ ,  $p < 0.01$ , Table S4) and excess phosphorus concentration (only available for Belgica-studied sites,  $n = 24$ ,  $p < 0.01$ ) while being negatively correlated with nitrate plus nitrite concentration ( $n = 46$ ,  $p < 0.01$ ).



**Figure 5.** Diversity of *nifH* sequences during (a) the Belgica BG2014/14 cruise (successfully recovered only at stations Bel-11 and Bel-13, 5 m) and (b) the GEOVIDE cruise (stations Geo-2, 100 m; Geo-13, 35 m; and Geo-21, 15 and 70 m). The total numbers of recovered sequences are indicated on top of the bars, and the exact percentage represented by each group is shown inside the bars.

## 4 Discussion

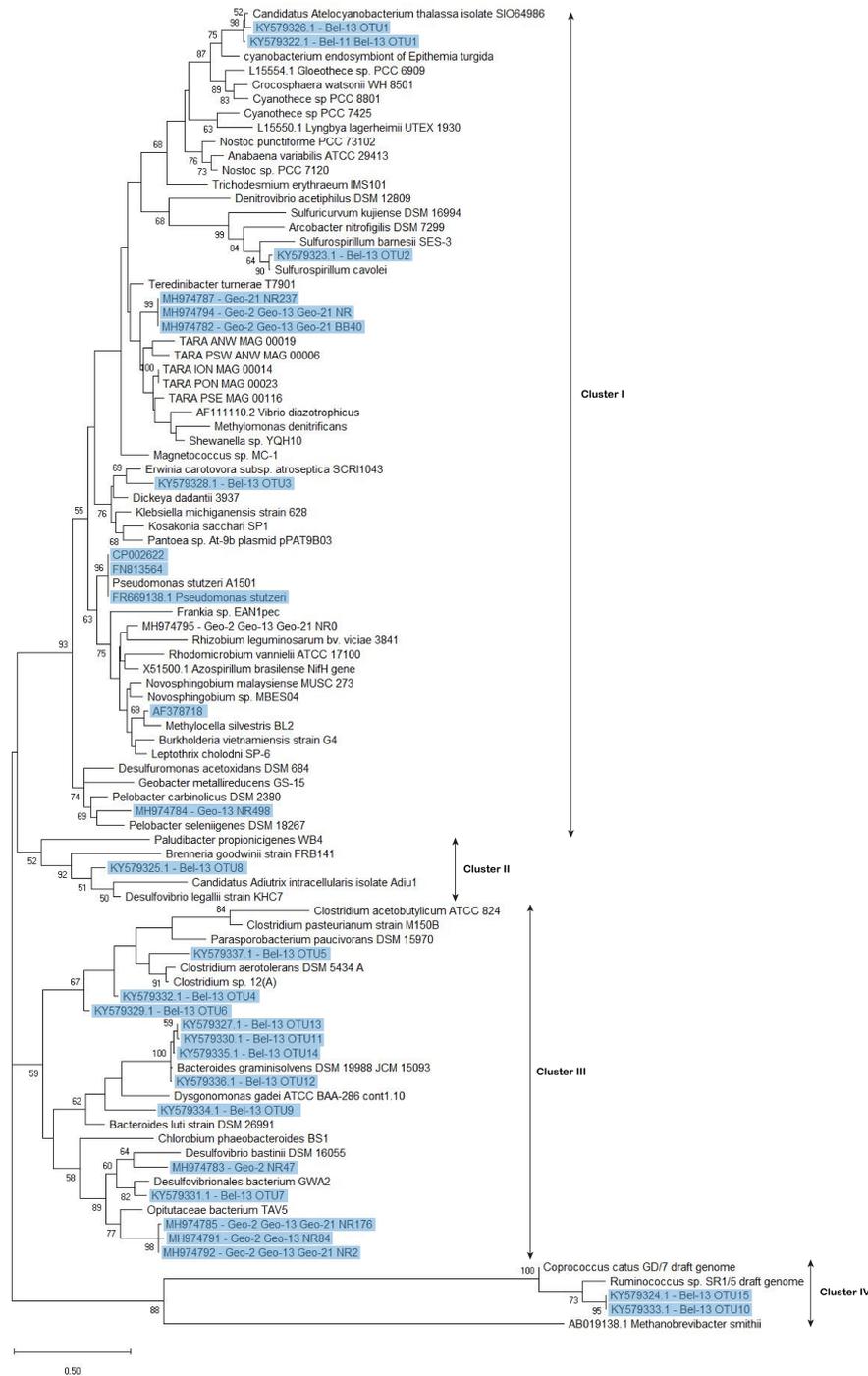
During two quasi-simultaneous expeditions to the Iberian Basin and the Bay of Biscay in May 2014 (38.8–46.5° N), we observed N<sub>2</sub> fixation activity in surface waters of most visited stations (except for the two northernmost sites in the Bay of Biscay). Our results are in support of other recent studies that have observed diazotrophic communities and significant N<sub>2</sub> fixation rates in marine environments departing from the previously established belief that diazotrophs are preferentially associated with warm oceanic water and low fixed-nitrogen concentrations (Needoba et al., 2007; Rees et al., 2009; Blais et al., 2012; Mulholland et al., 2012; Shiozaki et al., 2015). Although there is growing evidence that diazotrophs and their activity can extend geographically to temperate coastal and shelf-influenced regions, there still exist very few rate measurements at higher latitudes, especially in open waters. In the following sections, we shall (1) discuss the significance of N<sub>2</sub> fixation in the Iberian Basin as well as its relation to primary productivity pattern and extend our view to the whole Atlantic Ocean, (2) provide information on the taxonomic affiliation of diazotrophs present at the time of our study and (3) explore potential environmental conditions that may have supported this unexpectedly high diazotrophic activity in the Iberian Basin.

### 4.1 Significance of N<sub>2</sub> fixation in the temperate ocean

In the present study, we found surprisingly high N<sub>2</sub> fixation activities at most of the studied sites. Rates were exceptionally elevated at two open-ocean stations located between 38.8 and 40.7° N at about 11° W (averaging 1533 and 1355  $\mu\text{mol N m}^{-2} \text{d}^{-1}$  at stations Bel-11 and Bel-13, respec-

tively; Fig. 3c, d, and Tables S1 and S2). Although N<sub>2</sub> fixation was not detected in the central Bay of Biscay (stations Bel-3 and Bel-5), rates recorded at all the other sites were relatively high, not only in shelf-influenced areas (141 and 262  $\mu\text{mol N m}^{-2} \text{d}^{-1}$  at stations Geo-1 and Geo-2, respectively) but also in the open ocean (average activities between 81 and 384  $\mu\text{mol N m}^{-2} \text{d}^{-1}$  at stations Bel-7, Bel-9, Geo-13 and Geo-21).

By fuelling the bioavailable nitrogen pool, N<sub>2</sub> fixation may support marine PP, but the extent of this contribution needs to be established for areas outside tropical and subtropical regions. PP rates measured here are of similar range if not slightly higher than those reported in earlier investigations in the northeast Atlantic from subtropical to temperate waters (32 to 137  $\text{mmol C m}^{-2} \text{d}^{-1}$  relative to 19 to 103  $\text{mmol C m}^{-2} \text{d}^{-1}$ ) (Marañón et al., 2000; Fernández et al., 2005; Poulton et al., 2006; Fonseca-Batista et al., 2017). However, the contribution of N<sub>2</sub> fixation to PP in the present work (1%–28% of PP) reached values twice as high as those reported in other studies for the tropical and subtropical northeast Atlantic (contributions to PP ranging from <1% to 12%) (Voss et al., 2004; Rijkenberg et al., 2011; Fonseca-Batista et al., 2017). This observation further questions the accepted premise that oligotrophic surface waters of tropical and subtropical regions are the key environment where diazotrophic activity significantly supports marine primary productivity (Capone et al., 2005; Luo et al., 2014). Nevertheless, it is important to keep in mind that our computation relies on the assumption that only photoautotrophic diazotrophs contribute to bulk N<sub>2</sub> fixation, which may not always be the case, particularly in the present study, where mostly heterotrophic diazotrophs were observed. However,



**Figure 6.** Phylogenetic tree of *nifH*-predicted amino acid sequences generated using the maximum likelihood method of the Kimura 2 parameter model (Kimura, 1980) via the Molecular Evolutionary Genetics Analysis software (MEGA 7.0) (Kumar et al., 2016). Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.4038)). All sequences recovered from DNA samples, including those previously identified and the newly recovered ones (with  $\geq 95\%$  similarity at the nucleotide level with representative clones) are highlighted in blue. For the *nifH* sequences recovered from the GEOVIDE cruise, only those contributing to the cumulative 98% of recovered sequences were included in this tree. Bootstrap support values ( $\geq 50\%$ ) for 100 replications are shown at nodes. The scale bar indicates the number of sequence substitutions per site. The archaean *Methanobrevibacter smithii* was used as an outgroup. Accession numbers for published sequences used to construct the phylogenetic tree are given.

it is likely that all the recently fixed-nitrogen ultimately becomes available for the whole marine autotrophic community.

Previous studies in the open waters of the Iberian Basin (35–50° N, east of 25° W) reported relatively lower N<sub>2</sub> fixation rates (from <0.1 to 140 μmol N m<sup>-2</sup> d<sup>-1</sup>), regardless of whether the bubble-addition method (Montoya et al., 1996) or the dissolution method (Mohr et al., 2010; Großkopf et al., 2012) was used. However, these studies were carried out largely outside the bloom period, either during the late growing season (summer and autumn) (Moore et al., 2009; Benavides et al., 2011; Snow et al., 2015; Riou et al., 2016; Fonseca-Batista et al., 2017) or during winter (Rijkenberg et al., 2011; Agawin et al., 2014). In contrast, the present study took place in spring, during or just at the end of the vernal phytoplankton bloom. Differences in timing of these various studies and, to a lesser extent, in methodologies (bubble-addition versus dissolution method) may explain the discrepancies in diazotrophic activity observed between our study and earlier works. Yet, the 20-month survey by Moreira-Coello et al. (2017) in nitrogen-rich temperate coastal waters in the southern Bay of Biscay, covering the seasonal spring bloom and upwelling pulses, did not reveal significant N<sub>2</sub> fixation activities: from 0.1 to 1.6 μmol N m<sup>-2</sup> d<sup>-1</sup> (up to 3 orders of magnitude lower than those reported here). However, unlike our study, this work was carried out not only using the bubble-addition method but also in an inner coastal system, as opposed to the mainly open waters investigated here, making it difficult to predict which variable or combination of variables caused the difference observed between the two studies.

Our maximal values recorded at stations Bel-11 and Bel-13 are 1 order of magnitude higher than maximal N<sub>2</sub> fixation rates reported further south for the eastern tropical and subtropical North Atlantic (reaching up to 360–424 μmol N m<sup>-2</sup> d<sup>-1</sup>) (Großkopf et al., 2012; Subramaniam et al., 2013; Fonseca-Batista et al., 2017). Besides these two highly active sites, N<sub>2</sub> fixation rates at the other studied locations (ranging between 81 and 384 μmol N m<sup>-2</sup> d<sup>-1</sup>) were still in the upper range of values reported for the whole eastern Atlantic region. Yet, conditions favouring N<sub>2</sub> fixation are commonly believed to be met in tropical and subtropical regions where highest activities have mostly been measured, particularly in the eastern North Atlantic (e.g. higher seawater temperature, DIN limiting concentrations, excess phosphorus supply through eastern boundary upwelling systems) (Capone et al., 2005; Deutsch et al., 2007; Luo et al., 2014; Fonseca-Batista et al., 2017).

In the Atlantic Ocean, very high N<sub>2</sub> fixation rates up to ~1000 μmol N m<sup>-2</sup> d<sup>-1</sup>, as observed here, have only been reported for temperate coastal waters of the northwest Atlantic (up to 838 μmol N m<sup>-2</sup> d<sup>-1</sup>) (Mulholland et al., 2012) and for tropical shelf-influenced and mesohaline waters of the Caribbean and Amazon River plume (maximal rates ranging between 898 and 1600 μmol N m<sup>-2</sup> d<sup>-1</sup>) (Capone et al.,

2005; Montoya et al., 2007; Subramaniam et al., 2008). Shelf and mesohaline areas have indeed been shown to harbour considerable N<sub>2</sub> fixation activity, not only in tropical regions (Montoya et al., 2007; Subramaniam et al., 2008) but also in waters extending from temperate to polar areas (Rees et al., 2009; Blais et al., 2012; Mulholland et al., 2012; Shiozaki et al., 2015). Yet, the environmental conditions leading to the high N<sub>2</sub> fixation rates in these regions are currently not well understood. For tropical mesohaline systems, the conditions proposed to drive such an intense diazotrophic activity include the occurrence of highly competitive diatom–diazotroph associations and the influence of excess phosphorus input (i.e. excess relative to the canonical Redfield P/N ratio; expressed as P\*) from the Amazon River (Subramaniam et al., 2008). However, such conditions of excess P were not observed in previous studies carried out in high-latitude shelf regions with elevated N<sub>2</sub> fixation activities (Blais et al., 2012; Mulholland et al., 2012; Shiozaki et al., 2015), nor were they distinctly apparent in the present study (see Sect. 4.3). In addition, while tropical mesohaline regions are characterized by the predominance of diatom–diazotroph associations (and filamentous *Trichodesmium* spp.), in temperate shelf areas the diazotrophic community is reported to be essentially dominated by UCYN-A and heterotrophic bacteria (Rees et al., 2009; Blais et al., 2012; Mulholland et al., 2012; Agawin et al., 2014; Shiozaki et al., 2015; Moreira-Coello et al., 2017).

#### 4.2 Features of the diazotrophic community composition in the temperate North Atlantic

Our qualitative assessment of *nifH* diversity revealed a predominance of UCYN-A symbionts, only at the two stations with the highest surface N<sub>2</sub> fixation rates (up to 65.4 and 45.0 nmol N L<sup>-1</sup> d<sup>-1</sup> at Bel-11 and Bel-13, respectively; Table S1), while the remaining *nifH* sequences recovered belonged to heterotrophic diazotrophs, at Bel-13 as well as at all the other sites where *nifH* genes could be detected. No *Trichodesmium nifH* sequences were recovered from either BG2014/14 or GEOVIDE DNA samples, and the absence of the filamentous cyanobacteria was also confirmed by the CHEMTAX analysis of phytoplankton pigments (Manon Tonnard, personal communication, 2018). Previous work in temperate regions of the global ocean, including the Iberian Margin, also reported that highest N<sub>2</sub> fixation activities were predominantly related to the presence of UCYN-A symbionts, followed by heterotrophic bacteria, while *Trichodesmium* filaments were low or undetectable (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012; Agawin et al., 2014; Shiozaki et al., 2015; Moreira-Coello et al., 2017).

UCYN-A (in particular from the UCYN-A1 clade) symbionts were shown to live in symbioses with single-celled prymnesiophyte algae (Thompson et al., 2012). This symbiotic association, considered obligate, has been reported to be

particularly abundant in the central and eastern basin of the North Atlantic (Rees et al., 2009; Krupke et al., 2014; Cabello et al., 2015; Martínez-Pérez et al., 2016).

Besides UCYN-A, all the remaining *nifH* sequences recovered from both cruises, although obtained through different approaches, belonged to non-cyanobacterial diazotrophs. The phylogenetic tree (Fig. 6) showed that the non-cyanobacterial diazotrophs clustered with (1) Verrucomicrobia, a phylum yet poorly known that includes aerobic to microaerophilic methanotrophs groups, found in a variety of environments (Khadem et al., 2010; Wertz et al., 2012), (2) anaerobic bacteria, obligate or facultative, mostly affiliated with Cluster III phylotypes of functional nitrogenase (e.g. Bacteroidetes, Firmicutes, Proteobacteria) and lastly (3) phylotypes from Clusters I, II and IV (e.g. Proteobacteria and Firmicutes). Among the Cluster III phylotypes, Bacteroidetes are commonly encountered in the marine environment and are known as specialized degraders of organic matter that preferably grow attached to particles or algal cells (Fernández-Gómez et al., 2013). N<sub>2</sub> fixation activity has previously been reported in five Bacteroidetes strains including *Bacteroides graminisolvens*, *Paludibacter propionigenes* and *Dysgonomonas gadei* (Inoue et al., 2015), which are the closest cultured relatives of the *nifH*-OTUs detected at station Bel-13 (Fig. 6). Anaerobic Cluster III phylotypes have been previously recovered from different ocean basins (Church et al., 2005; Langlois et al., 2005, 2008; Man-Aharonovich et al., 2007; Rees et al., 2009; Halm et al., 2012; Mulholland et al., 2012). These diazotrophs were suggested to benefit from anoxic microzones found within marine snow particles or zooplankton guts to fix N<sub>2</sub> thereby avoiding oxygenic inhibition of their nitrogenase enzyme (Braun et al., 1999; Church et al., 2005; Scavotto et al., 2015). Therefore, the bloom to early post-bloom conditions, prevailing during our study, were likely beneficial for the development of diazotrophic groups that depend on the availability of detrital organic matter or on the association with grazing zooplankton. In contrast, at the northernmost Geo-21 station, we observed a dominance of Gammaproteobacteria phylotypes belonging to a recently identified clade of marine diazotrophs within the Oceanospirillales (Delmont et al., 2018).

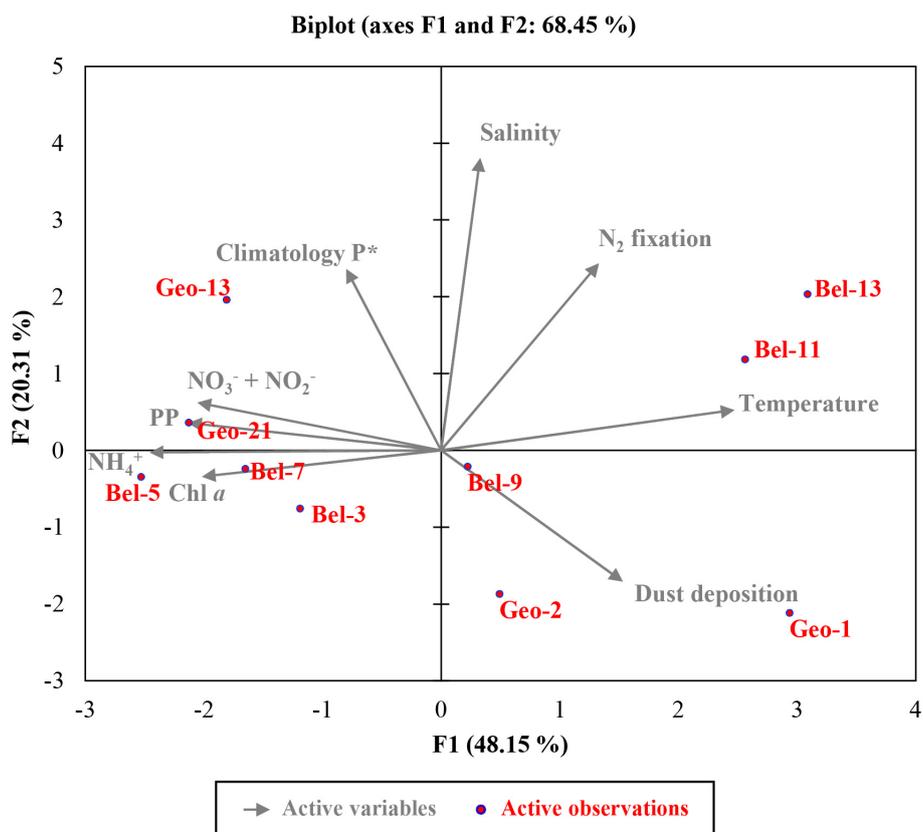
These observations tend to strengthen the idea that not only UCYN-A (Cabello et al., 2015; Martínez-Pérez et al., 2016) but also non-cyanobacterial diazotrophs (Halm et al., 2012; Shiozaki et al., 2014; Langlois et al., 2015) play a substantial role in oceanic N<sub>2</sub> fixation. Although it is possible to assign a broad taxonomic affiliation to classify the *nifH* genes, very little is known with respect to their physiology, their role in the ecosystem and the factors controlling their distribution, due to the lack of representative whole genome sequences and environmentally relevant strains available for experimentation (Bombar et al., 2016). While the widespread distribution of UCYN-A and non-cyanobacterial diazotrophs has been reported, their contribution to in situ activity remains poorly quantified.

### 4.3 Key environmental drivers of N<sub>2</sub> fixation

Environmental conditions that promote autotrophic and heterotrophic N<sub>2</sub> fixation activity in the ocean are currently not well understood (Luo et al., 2014). While heterotrophic diazotrophs would not be directly affected by the commonly recognized environmental controls of autotrophic diazotrophy such as solar radiation, seawater temperature and DIN, as they possess fundamentally different ecologies, the molecular and cellular processes for sustaining N<sub>2</sub> fixation activity would nevertheless require a supply of dFe and P (Raven, 1988; Howard and Rees, 1996; Mills et al., 2004; Snow et al., 2015). Besides the need for these critical inorganic nutrients, heterotrophic N<sub>2</sub> fixation was also recently shown to be highly dependent on the availability of organic matter (Bonnet et al., 2013; Rahav et al., 2013, 2016; Loescher et al., 2014).

Findings from the GEOVIDE cruise tend to support the hypothesis of a stimulating effect of organic matter availability on N<sub>2</sub> fixation activity at the time of our study. Lemaitre et al. (2018) report that surface waters (upper 100–120 m) of the Iberian Basin (stations Geo-1 and Geo-13) and the West European Basin (Geo-21) carried significant POC loads (POC of 166, 171 and 411 mmol C m<sup>-2</sup>, respectively) with a dominant fraction of small size POC (the 1–53 μm size fraction; 75 %, 92 % and 64 % of the total POC, respectively). Smaller cells, usually being slow-sinking particles, are more easily remineralized in surface waters (Villa-Alfageme et al., 2016). This is confirmed by the very low export efficiency (only 3 % to 4 % of euphotic layer integrated PP) observed at stations Geo-13 and Geo-21, suggesting an efficient shallow remineralization (Lemaitre et al., 2018). This availability of organic matter in the upper layers likely contributed to supplying remineralized P (organic P being generally more labile than other organic nutrients; Vidal et al., 1999, 2003) and to enhancing the residence time of dFe originating from atmospheric deposition due to the formation of organic ligands (Jickells, 1999; de Baar and de Jong, 2001; Sarthou et al., 2003).

P\* values from the BG2014/14 cruise (Table S1) and the climatological P\* data for the Iberian Basin (Garcia et al., 2013) do not exhibit a clear PO<sub>4</sub><sup>3-</sup> excess in the region (P\* ranging from -0.1 to 0.1 μmol L<sup>-1</sup>; Fig. 1 and Tables S1 and S2). Nevertheless, Spearman rank correlations indicate that volumetric N<sub>2</sub> fixation rates were significantly correlated with the BG2014/14 shipboard P\* values ( $n = 24$ ,  $p < 0.01$ , Table S4), with stations Bel-11 and Bel-13 weighing heavily in this correlation. Without the data from these two sites (data not shown), the correlation between in situ P\* and N<sub>2</sub> fixation rates is no longer significant ( $n = 16$ ,  $p = 0.163$ ), while P\* becomes highly correlated with PP and Chl *a* ( $n = 16$ ,  $p = 0.0257$  and  $0.016$ , respectively). This suggests that the effect of P\* on N<sub>2</sub> fixation, although not clearly evident from absolute values, was most important at stations Bel-11 and Bel-13 but nonetheless existent at the other sites (Bel-7 and



**Figure 7.** Euclidean distance biplot illustrating the axis loadings for the two main PCA components based on the Spearman rank correlation matrix shown in Table S3. Variables taken into account include depth-integrated rates of N<sub>2</sub> fixation and PP, average phosphate excess at 20 m depth surrounding each sampled site recovered from World Ocean Atlas 2013 climatology data between April and June from 1955 to 2012 (Garcia et al., 2013); satellite average dust deposition (dry plus wet) derived during April 2014 (Giovanni online data system, NASA Goddard Earth Sciences Data and Information Services Center) and ambient variables (temperature, salinity and nutrient data). Coloured dots in the biplot represent the projection of the different stations. Axis 1 has high negative loadings for PP, Chl *a*, NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup>, and high positive loadings for temperature and N<sub>2</sub> fixation rates, with values of  $-0.812$ ,  $-0.768$ ,  $-0.936$ ,  $-0.783$ ,  $0.942$  and  $0.506$ , respectively (see Table S5). Axis 2 has high positive loadings of  $0.584$ ,  $0.943$  and  $0.602$  for climatological P\*, salinity and N<sub>2</sub> fixation rates, respectively. PCA analysis was run in XLSTAT 2017 (Addinsoft, Paris, France, 2017).

Bel-9). The occurrence of N<sub>2</sub> fixation in oligotrophic waters displaying weak P\* values, depleted in DIN and PO<sub>4</sub><sup>3-</sup> but replete in dFe, might in fact reflect the direct use by diazotrophs of dissolved organic phosphorus (DOP). Indeed, according to Landolfi et al. (2015) diazotrophy ensures the supply of additional N and energy for the enzymatic mineralization of DOP (synthesis of extracellular alkaline phosphatase). Therefore, a likely enhanced DOP release towards the end of the spring bloom may have contributed to sustaining N<sub>2</sub> fixation in the studied region. Such DOP utilization has indeed been reported for various marine organisms, particularly diazotrophic cyanobacteria (Dyhrman et al., 2006; Dyhrman and Haley, 2006) and bacterial communities (Luo et al., 2009).

Supply routes of dFe to surface waters of the investigated area relied on lateral advection from the continental shelf (stations Geo-1 and Geo-2) (Tonnard et al., 2018), vertical mixing due to post-winter convection (Thuróczy et al., 2010;

Rijkenberg et al., 2012; García-Ibáñez et al., 2015) and/or atmospheric dust deposition (dry plus wet). Atmospheric deposition may have been particularly important for the area of stations Bel-11 and Bel-13 receiving warm and saline surface waters from the subtropics.

Atmospheric aerosol deposition determined during the GEOVIDE cruise (Shelley et al., 2017), as well as the satellite-based dust deposition (dry plus wet) averaged over the month of May 2014 (Fig. S3b; Giovanni online satellite data system, NASA Goddard Earth Sciences Data and Information Services Center), reveal rather weak dust loadings over the investigated region, resulting in areal N<sub>2</sub> fixation rates being actually inversely correlated with the satellite-based average dust input ( $p < 0.01$ ; Table S3). In contrast, satellite-based dust deposition (dry plus wet) averaged over the month of April 2014 (i.e. preceding the timing of sampling) indicates high fluxes over the subtropical waters located south of the studied region (Fig. S3a;). The  $\theta$ -S di-

agrams at stations Bel-11 and Bel-13 (and to a lesser extent at Geo-13; Fig. S1) illustrate the presence of very warm and saline waters, which were advected from the subtropics as suggested by the satellite SST images (Fig. S2). We thus argue that advection of surface waters from south of the study area represented a source of atmospherically derived dFe and contributed to driving the high N<sub>2</sub> fixation activity recorded at stations Bel-11 and Bel-13. This resulted in N<sub>2</sub> fixation rates there being positively (although weakly) correlated ( $p = 0.45$ ; Table S3) with the April average dust input.

For the central Bay of Biscay, where N<sub>2</sub> fixation was below the DL (stations Bel-3 and Bel-5), dust deposition in April 2014 was also the lowest, suggesting that N<sub>2</sub> fixation there might have been limited by dFe availability. Indeed, at stations Bel-3 and Bel-5 diazotrophic activity in surface waters was boosted following dFe amendments ( $>25 \text{ nmol N L}^{-1} \text{ d}^{-1}$ ; Li et al., 2018).

Thus, the enhanced N<sub>2</sub> fixation activity at stations Bel-11 and Bel-13, as compared to the other sites, was likely stimulated by the combined effects of the presence of highly competitive prymnesiophyte–UCYN-A symbionts, organic matter as a source of DOP, positive P\* signatures and advection of subtropical surface waters enriched in dFe.

These statements are further supported by the outcome of a multivariate statistical analysis, providing a comprehensive view of the environmental features influencing N<sub>2</sub> fixation. A principal component analysis (PCA; Fig. 7 and Tables S2 and S5) generated two components (or axes) explaining 68 % of the system's variability. Axis 1 illustrates the productivity of the system, or more precisely the oligotrophic state towards which it was evolving. Axis 1 is defined by a strong positive relation with surface temperature (reflecting the onset of stratification, particularly for stations Bel-11 and Bel-13; Fig. 7) and an inverse relation with PP and associated variables (Chl *a*, NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup>), which reflects the prevailing post-bloom conditions of the system. Sites characterized by a moderate (Bel-3 and Bel-5) to high (Bel-7, Geo-21 and to a lesser extent Geo-13) PP appear indeed tightly linked to these PP-associated variables as illustrated in Fig. 7. Axis 2 is defined by the positive relation with surface salinity and P\* (Fig. 7) and reflects the advection of surface waters of subtropical origin for stations Bel-11, Bel-13 and Geo-13. For stations Geo-1 and Geo-2, the inverse relation with surface salinity (Fig. 7) is interpreted to reflect fluvial inputs (Tonnard et al., 2018). Finally, this statistical analysis indicates that N<sub>2</sub> fixation activity was likely influenced by the two PCA components, tentatively identified as productivity (Axis 1) and surface water advection (Axis 2) from the shelf and the subtropical region.

## 5 Conclusions

The present work highlights the occurrence of elevated N<sub>2</sub> fixation activities ( $81\text{--}1533 \mu\text{mol N m}^{-2} \text{ d}^{-1}$ ) in spring 2014

in open waters of the temperate eastern North Atlantic, off the Iberian Peninsula. These rates exceed those reported by others for the Iberian Basin but which were largely obtained outside the bloom period (from  $<0.1$  to  $140 \mu\text{mol N m}^{-2} \text{ d}^{-1}$ ). In contrast, we did not detect any N<sub>2</sub> fixation activity in the central Bay of Biscay. At sites where significant N<sub>2</sub> fixation activity was measured, rates were similar to or up to an order of magnitude larger than values reported for the eastern tropical and subtropical North Atlantic, regions commonly believed to represent the main areas harbouring oceanic N<sub>2</sub> fixation for the eastern Atlantic. Assuming that the carbon versus nitrogen requirements by these N<sub>2</sub> fixers obeyed the Redfield stoichiometry, N<sub>2</sub> fixation was found to contribute 1 %–3 % of the euphotic layer daily PP and even up to 23 %–25 % at the sites where N<sub>2</sub> fixation activities were the highest. The prymnesiophyte–symbiont *Candidatus Atelocyanobacterium thalassa* (UCYN-A) contributed the most to the *nifH* sequences recovered at the two sites where N<sub>2</sub> fixation activity was the highest, while the remaining sequences belonged exclusively to heterotrophic bacteria. We speculate that the unexpectedly high N<sub>2</sub> fixation activity recorded at the time of our study was sustained by (i) organic matter availability in these open waters, resulting from the prevailing vernal bloom to post-bloom conditions, in combination with (ii) excess phosphorus signatures which appeared to be tightly related to diazotrophic activity particularly at the two most active sites. Yet, these observations and hypotheses rely on the availability of dFe with evidence for input from shelf waters and pulsed atmospheric dust deposition being a significant source of iron. Further studies are required to investigate this possible link between N<sub>2</sub> fixation activity and phytoplankton bloom under iron-replete conditions in the studied region and similar environments, as these would be required to be considered in future assessment of global N<sub>2</sub> fixation.

*Data availability.* The data associated with the paper are available from the corresponding author upon request.

*Supplement.* The supplement related to this article is available online at: <https://doi.org/10.5194/bg-16-999-2019-supplement>.

*Author contributions.* VR, VM, FP, GS, ME, JR, LC and FrD contributed to mounting the research project and contributed to funding this work. DFB, XL, VR, FF, NL, HP, FP, GS, LC and FrD were involved in the collection of seawater samples and/or carrying out the on-board incubation experiments during the Belgica BG2014/14 and GEOVIDE cruises. DFB, XL, FID, FF and FrD were involved in the processing of stable isotope incubation experiments (N<sub>2</sub> fixation and primary production), while XL, NB, NL, MT, MG, HP, FP and LC were involved in nutrient, trace element and particulate organic pool assessments. DFB, VR, VM, SG and JL were involved

in the analysis and treatment of biomolecular data. DFB drafted the manuscript, which was edited by all authors.

*Competing interests.* The authors declare that they have no conflict of interest.

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