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Evidence of high N₂ fixation rates in the temperate Northeast Atlantic

3

4 Debany Fonseca-Batista^{1,2}, Xuefeng Li^{1,3}, Virginie Riou⁴, Valérie Michotey⁴, Florian Deman¹,
5 François Fripiat⁵, Sophie Guasco⁴, Natacha Brion¹, Nolwenn Lemaitre^{1,6,7}, Manon Tonnard^{6,8},
6 Morgane Gallinari⁶, Hélène Planquette⁶, Frédéric Planchon⁶, Géraldine Sarthou⁶, Marc Elskens¹,
7 Julie LaRoche², Lei Chou³, Frank Dehairs¹
8
9 ¹ Analytical, Environmental and Geo-Chemistry, Earth System Sciences Research Group, Vrije Universiteit Brussel,
1050 Brussels, Belgium

- ² Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4R2
- 12 ³ Service de Biogéochimie et Modélisation du Système Terre, Université Libre de Bruxelles, 1050 Brussels, Belgium
- 13 ⁴ Aix-Marseille Univ, Université de Toulon, CNRS, IRD, MIO, Marseille, France
- ⁵ Max Planck Institute for Chemistry, Climate Geochemistry Department, 55128 Mainz, Germany
- ⁶ Laboratoire des Sciences de l'Environnement MARin CNRS UMR 6539 Institut Universitaire Européen de la
 Mer, 29280 Plouzané, France
- ⁷ Department of Earth Sciences, Institute of Geochemistry and Petrology, ETH-Zürich, 8092 Zürich, Switzerland
- 18 ⁸ Institute for Marine and Antarctic Studies, University of Tasmania, Hobart, TAS 7001, Australia
- 19

20 Correspondence to: Debany Fonseca P. Batista (dbatista8@hotmail.com)

Abstract. Diazotrophic activity and primary production (PP) were investigated along two transects (Belgica 21 BG2014/14 and GEOVIDE cruises) off the western Iberian Margin and the Bay of Biscay in May 2014. Substantial 22 N_2 fixation activity was observed at 8 of the 10 stations sampled, ranging overall from 81 to 384 µmol N m⁻² d⁻¹ (0.7 23 to 8.2 nmol N L⁻¹ d⁻¹), with two sites close to the Iberian Margin situated between 38.8° N and 40.7° N yielding rates 24 reaching up to 1355 and 1533 µmol N m⁻² d⁻¹. Primary production was relatively lower along the Iberian Margin with 25 26 rates ranging from 33 to 59 mmol C m⁻² d⁻¹, while it increased towards the northwest away from the Peninsula, reaching as high as 135 mmol C m⁻² d⁻¹. In agreement with the area-averaged Chl a satellite data contemporaneous 27 28 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 29 stoichiometry, N_2 fixation could support 1 to 3% of daily PP in the euphotic layer at most sites, except at the two 30 most active sites where this contribution to daily PP could reach up to 25%. At the two sites where N_2 fixation 31 activity was the highest, the prymnesiophyte-symbiont Candidatus Atelocyanobacterium thalassa (UCYN-A) 32 dominated the *nifH* sequence pool, while the remaining recovered sequences belonged to non-cyanobacterial 33 phylotypes. At all the other sites however, the recovered *nifH* sequences were exclusively assigned phylogenetically 34 35 to non-cyanobacterial phylotypes. The intense N_2 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 36 evidenced by the significant surface particulate organic carbon concentrations. Also, the presence of excess 37 phosphorus signature in surface waters seemed to contribute to sustaining N_2 fixation, particularly at the sites with 38 39 extreme activities. These results provide a mechanistic understanding of the unexpectedly high N_2 fixation in productive waters of the temperate North Atlantic, and highlight the importance of N_2 fixation for future assessment 40 41 of global N inventory.

43 **1 Introduction**

Dinitrogen (N_2) 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 N₂-fixing micro-organisms (diazotrophs) of dissolved N₂ 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 N₂ fixation to carbon sequestration in the ocean requires an assessment of the

49 global marine N_2 fixation.

50 Until recently most studies of N₂ fixation have focused on the tropical and subtropical regions of the global ocean, with few attempts to measure N₂ fixation at higher latitudes, with the exception of enclosed brackish seas 51 52 (Ohlendieck et al., 2000; Luo et al., 2012; Farnelid et al., 2013). The intense research efforts in the low latitude 53 regions stem for the observable presence of cyanobacterial diazotrophs such as the diatom-diazotroph association 54 (DDA) and the colony-forming filamentous Trichodesmium (Capone, 1997; Capone et al., 2005; Foster et al., 2007). 55 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 56 57 environment for its growth and N₂-fixing activity (Dore et al., 2002; Breitbarth et al., 2007; Montoya et al., 2007; 58 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 59 60 to diazotrophs over other phytoplankton since only they can draw N from the unlimited dissolved N₂ pool for their biosynthesis. As such, past estimates of global annual N₂ fixation were mainly based on information gathered from 61 tropical and subtropical regions, while higher latitude areas have been poorly explored for diazotrophic activity (Luo 62 63 et al., 2012).

Studies using genetic approaches targeting the *nifH* gene encoding the nitrogenase enzyme, essential for diazotrophy, 64 65 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 66 67 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, 68 69 thereby expanding the potential for diazotrophy to a much broader geographic scale (Langlois et al., 2005, 2008; 70 Krupke et al., 2014; Cabello et al., 2015). The discovery of a methodological bias associated to the commonly used 71 15 N₂ bubble-addition technique (Mohr et al., 2010) and the presence of an abundant diazotrophic community in high 72 latitude regions actively fixing N₂ (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_2 fixation and diazotrophic 73 74 diversity at higher latitudes. In the Northeast Atlantic, the large input of iron-rich Saharan dust alleviating dissolved iron (dFe) limitation of the 75

nitrogenase activity (Fe being a co-factor of the N₂-fixing enzyme) (Raven, 1988; Howard & Rees, 1996; Mills et al., 76 77 2004; Snow et al., 2015) and the upwelling of subsurface waters with low DIN (dissolved inorganic nitrogen) to 78 phosphate ratios, make this region highly favorable for N₂ fixation activity (Deutsch et al., 2007; Moore et al., 2009). 79 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; 80 81 Fonseca-Batista et al., 2017) not only in the tropical and subtropical regions but also in the temperate Iberian region 82 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 83 stratified water column conditions of boreal summer and autumn (Moore et al., 2009; Benavides et al., 2011; Snow et 84

- 85 al., 2015; Fonseca-Batista et al., 2017) or during the winter convection period (Rijkenberg et al., 2011; Agawin et al.,
- 86 2014). Here, we present N₂ fixation rate measurements and the taxonomic affiliation of the diazotrophic community
- 87 from two consecutive campaigns carried out in the Northeast sector of the Atlantic Ocean in May 2014, during and
- 88 after the spring bloom.

89 2 Material and Methods

90 2.1 Site description and sample collection

91 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, 92 the GEOVIDE expedition in the framework of the international GEOTRACES program (GA01 section, May 16 to 93 June 29 2014, R/V Pourquoi pas?) sailed from the Portuguese shelf area towards Greenland and ended in 94 Newfoundland, Canada (http://dx.doi.org/10.17600/14000200). N₂ fixation activities were determined at ten stations 95 within the Iberian Basin, among which four sites were investigated during the GEOVIDE cruise (stations Geo-1, 96 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 97 98 and Bel-13; Fig. 1).

- 99 All sampling sites were located within the Iberian Basin Portugal Current System (PCS) (Ambar and Fiúza, 1994) 100 which is influenced by highly fluctuating wind stresses (Frouin et al., 1990). The predominant upper layer water mass 101 in this basin is the Eastern North Atlantic Central Water (ENACW), a winter-mode water, which according to Fiúza 102 (1984) consists of two components (see θ /S diagrams in Supporting Information Fig. S1): (i) the lighter, relatively 103 warm (> 14 °C) and salty (salinity > 35.6) ENACWst formed in the subtropical Azores Front region (~35° N) when 104 Azores Mode Water is subducted as a result of strong evaporation and winter cooling; and (ii) the colder and less 105 saline ENACWsp, underlying the ENACWst, formed in the subpolar eastern North Atlantic (north of 43° N) through winter cooling and deep convection (McCartney and Talley, 1982). The spatial distribution of these Central Waters 106 107 allowed the categorization of the sampling sites into 2 groups: (i) ENACWsp stations north of 43° N (Bel-3, Bel-5, 108 Bel-7, and Geo-21) only affected by the ENACWsp (Fig. S1a, b) and (ii) ENACWst stations, south of 43° N, 109 characterized by an upper layer influenced by the ENACWst and an 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 110 111 were in proximity of the Iberian shelf (Geo-1 and Geo-2) (Tonnard et al., 2018). 112 Temperature, salinity and photosynthetically active radiation (PAR) profiles down to 1500 m depth were obtained
- 112 reinpenduce, samily and photosyntheterally derive radiation (1710) profiles down to 1500 in depth were obtained 113 using a conductivity-temperature-depth (CTD) sensor (SBE 09 and SBE 911+, during the BG2014/14 and GEOVIDE 114 cruises, respectively) fitted to the rosette frames. For all biogeochemical measurements, seawater samples were 115 collected with Niskin bottles attached to the rosette and closed at specific depths in the upper 200 m. In particular, for 116 stable isotope incubation experiments seawater was collected in 4.5 L acid-cleaned polycarbonate (PC) bottles from 117 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, 118 and Geo-2. At stations Geo-1, Geo-13 and Geo-21, two additional depths corresponding to 25% and 1% of surface 119 PAR were also sampled for the same purpose.
- 120

121 2.2 Nutrient measurements

122 Ammonium (NH_4^+) concentrations were measured on board during both cruises, while nitrate + nitrite ($NO_3^- + NO_2^-$)

123 concentrations were measured on board only during the GEOVIDE expedition. During the BG2014/14 cruise,

samples for NO₃⁻ + NO₂⁻ and phosphate (PO₄³⁻) measurements were filtered (0.2 μ m) and stored at -20 °C until analysis at the home-based laboratory. PO₄³⁻ data are not available for the GEOVIDE cruise.

126 Nutrient concentrations were determined using the conventional fluorometric (for NH4⁺) (Holmes et al., 1999) and

- 127 colorimetric methods (for the other nutrients) (Grasshoff et al., 1983) with detection limits (DL) of 64 nmol L⁻¹
- 128 (NH₄⁺), 90 nmol L⁻¹ (NO₃⁻ + NO₂⁻) and 60 nmol L⁻¹ (PO₄³⁻). For the BG2014/14 cruise, chlorophyll *a* (Chl *a*)
- 129 concentrations were determined according to Yentsch and Menzel (1963). Briefly, 250 mL of seawater was filtered
- 130 onto Whatman GF/F glass microfiber filters (0.7 µm nominal pore size), followed by pigment extraction in 90%
- 131 acetone, centrifugation and fluorescence measurement using a Shimadzu RF-150 fluorometer. For the GEOVIDE
- 132 cruise, Chl *a* concentrations were measured as described in Ras et al. (2008). Briefly, filters samples were extracted
- 133 in 100% methanol, disrupted by sonification, and clarified by vacuum filtration through Whatman GF/F filters. The
- 134 extracts were analysed by high-performance liquid chromatography (HPLC Agilent Technologies 1200).

135 2.3 ¹⁵N₂ fixation and ¹³C-HCO₃⁻ uptake rates

136 N₂ fixation and primary production (PP) were determined simultaneously from the same incubation sample at each depth in duplicate, using the ¹⁵N-N₂ dissolution method (Großkopf et al., 2012) and ¹³C-NaHCO₃ tracer addition 137 technique (Hama et al., 1983), respectively. Details concerning the applied ¹⁵N₂ dissolution method can be found in 138 Fonseca-Batista et al. (2017). Briefly, ¹⁵N₂-enriched seawater was prepared by degassing prefiltered (0.2 µm) low 139 nutrient seawater, under acid-clean conditions using a peristaltic pump slowly circulating (100 mL min⁻¹) the 140 141 seawater through two degassing membrane contactor systems (MiniModule, Liqui-Cel) in series, held under high 142 vacuum (50 mbar). The degassed water was directly transferred into 2 L gastight Tedlar bags (Sigma-Aldrich) fitted 143 with a septum through which 30 mL of pure ¹⁵N₂ gas (98 ¹⁵N atom%, Eurisotop, lot number 23/051301) was injected before the bags were shaken 24 hours for tracer equilibration. This ${}^{15}N_2$ gas batch was previously shown to be free of 144 ¹⁵N-labelled contaminants such as nitrate, nitrite, ammonium and nitrous oxide (Fonseca-Batista et al., 2017). Each 145 PC incubation bottle was partially filled with sampled seawater, then amended with 250 mL of ¹⁵N₂-enriched 146 seawater and spiked with 3 mL of ¹³C-labelled dissolved inorganic carbon (DIC; 200 mmol L⁻¹ solution of 147 NaH13CO3, 99%, Eurisotop). The 13C-DIC added to a 4.5 L incubation bottle results in a ~6.5% increment of the 148 149 initial DIC content, considered equal to the average oceanic DIC concentration (~2000 µmol kg⁻¹; Zeebe and Wolf-150 Gladrow, 2003). This allows sufficient tracer enrichment for a sensitive detection in the particulate organic carbon 151 (POC) pool as a result of incorporation (Hama et al., 1983). Finally, each incubation bottle was topped off with the 152 original seawater sample. Samples were then incubated for 24 hours in on-deck incubators circulated with surface 153 seawater and wrapped with neutral density screens (Rosco) simulating the in situ irradiance conditions. After 154 incubation, water was transferred under helium pressure from each PC bottle into triplicate 12 mL gastight Exetainer 155 vials (Labco) poisoned (100 µL of saturated HgCl₂ solution) and pre-flushed with helium for the determination of the 15 N and 13 C atom% enrichments of the dissolved N₂ (in duplicate) and DIC pools. The remaining incubated sample 156 was filtered onto pre-combusted MGF filters (glass microfiber filters, 0.7 µm nominal pore size, Sartorius), which 157 158 were subsequently dried at 60 °C and stored at room temperature. The natural concentration and isotopic composition 159 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 160 161 compositions using an elemental analyser (EuroVector Euro EA 3000) coupled to an isotope ratio mass spectrometer 162 (Delta V Plus, Thermo Scientific) and calibrated against international certified reference materials (CRM): IAEA-N1 and IAEA-305B for N and IAEA-CH6 and IAEA-309B for C. The isotopic composition of the DIC and dissolved N₂ 163 pools was determined using a gas bench system coupled to an IRMS (Nu Instruments Perspective). Exetainers vials 164

- were first injected with He to create a 4 mL headspace and then equilibrated on a rotatory shaker: for 12 hours after phosphoric acid addition (100 μ L, 99%, Sigma-Aldrich) for DIC analyses and only for an hour without acid addition for N₂ analyses. DIC measurements were corrected according to Miyajima et al. (1995) and ¹⁵N₂ enrichments were calibrated with atmospheric N₂. N₂ fixation and carbon uptake volumetric rates were computed as shown in Equation 1:
- 170 $N_2 \text{ or HCO}_3^- \text{ uptake } rate (\text{nmol } L^{-1}d^{-1}\text{ or } \mu\text{mol } m^{-3}d^{-1}) = \frac{A_{PN \text{ or } POC}^{final} A_{PN \text{ or } POC}^{t=0}}{A_{N_2 \text{ or } DIC} A_{PN \text{ or } POC}^{t=0}} \times \frac{[PN \text{ or } POC]}{\Delta t}$ (1)
- where $A_{PN or POC}$ represents the ¹⁵N or ¹³C atom% excess of PN or POC, respectively, at the beginning (t =0) and end (final) of the incubation, while $A_{N_2 or DIC}$ represents the ¹⁵N or ¹³C atom% excess of the dissolved inorganic pool (N₂ or DIC); and Δt represents the incubation period.
- 174 Depth-integrated rates were calculated by non-uniform gridding trapezoidal integration for each station. The DL, 175 defined as the minimal detectable uptake rates were determined as detailed in Fonseca-Batista et al. (2017). To do so, 176 the minimal acceptable ¹⁵N or ¹³C enrichment of PN or POC after incubation (Montoya et al., 1996) is considered to 177 be equal to the natural isotopic composition, specific to each sampled depth, plus three times the uncertainty obtained 178 for N and C isotopic analysis of CRM. All remaining experiment-specific terms are then used to recalculate the 179 minimum detectable uptake. Carbon uptake rates were always above their specific DL, while N₂ fixation was not 180 detectable at any of the four depths of stations Bel-3 and Bel-5, nor at Bel-9 120 m, Bel-11 45 m and Geo-21 18 m 181 (see Supporting Information Table S1).
- 182

183 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 N₂ fixation rate measurements were carried out. Two liters of seawater samples were vacuum filtered (20 to 30 kPa) through sterile 0.2 μ m 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 °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 μ 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).

- 196 For the GEOVIDE samples, DNA was extracted using the QIAGEN DNeasy Plant Mini Kit as instructed by the 197 manufacture, with a modified step to improve cell lysis. This step consisted of an incubation at 52 °C on an orbital shaker for 1 hour (300 rpm) with 50 μ L of lysozyme solution (5 mg mL⁻¹ in TE buffer), 45 μ L of Proteinase K 198 199 solution (20 mg mL⁻¹ in MilliQ PCR grade water) and 400 µL of AP1 lysis buffer from the QIAGEN DNeasy Plant 200 Mini Kit. DNA concentration and purity were assessed with NanoDrop 2000 and then stored at -80 °C. The DNA 201 samples were screened for the presence of the nifH gene as described in Langlois et al. (2005). Samples that tested 202 positive were further prepared for next generation sequencing on an Illumina MiSeq platform using primers that included the nifH1/2 primers (Langlois et al., 2005; Ratten, 2017) attached to Illumina adaptors and barcodes for 203
- 204 multiplexing in the Illumina MiSeq instrument. Next generation sequencing was carried out at the Integrated

Microbiome Resource (IMR) of the Centre for Comparative and Evolutionary Biology (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; Comeau et al., 2017). The 28 OTUs for the *nifH* genes presented in this study were assembled based on 96% identity of sequence reads.

210 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-

212 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
positions containing gaps and missing data were eliminated (see phylogenetic tree in Fig. 6). One representative

215 sequence of each *nifH*-OTU was deposited in GenBank under the accession numbers referenced from KY579322 to

216 KY579337, for the Belgica DNA samples and referenced from MH974781 to MH974795 for the GEOVIDE Iberian

217 samples.

218

219 2.5 Statistical analysis

The relationship between N_2 fixation activities and ambient physical and chemical properties was examined, using 220 221 SigmaPlot (Systat Software, San Jose, CA) by computing Spearman rank correlation coefficients linking depth-222 integrated rates and volumetric rates of N₂ fixation and primary production to environmental variables. These 223 ambient variables were either averaged or integrated over the euphotic layer, or considered as discrete measurements. 224 These variables include temperature, salinity, Chl a, NH_4^+ , $NO_3^-+NO_2^-$, phosphorus excess ($P^* = [PO_4^{3-}] - [NO_3^-+NO_2^-]$ 225 +NO₂⁻] / 16) derived from in situ nutrient measurements and climatological data (Garcia et al., 2013), dissolved iron 226 concentrations determined for the GEOVIDE cruise (Tonnard et al., 2018) and satellite-derived dust deposition fluxes 227 at the time of our study (Giovanni online data system). When nutrient concentrations were below the DL we used the 228 DL value to run the correlation test. In addition, we ran a principal component analysis (PCA) using XLSTAT 2017 229 (Addinsoft, Paris, France, 2017) to get an overview of the interconnection between all the latter key variables with N_2 230 fixation at the time of our study. The output of the PCA are discussed in section 4.3.

231 3 Results

232 **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 °C (Bel-13). At the surface, nutrients were depleted ($NO_3^- + NO_2^- < 0.09 \mu$ M in the upper 20 m; Fig. 2c, f) and Chl *a* concentrations were low (< 0.25 µg L⁻¹; Fig. 2a, d) but showed a subsurface maximum (between 0.5 and 0.75 µg L⁻¹ at approximately 50 m), a

237 common feature for oligotrophic open ocean waters. Amongst the ENACWst stations, station Geo-13 had a slightly

- 238 higher nutrient content (NO₃⁻ + NO₂⁻ = 0.7 μ M) in the lower mixed layer depth (MLD) and a higher Chl a
- 239 concentration (> $0.5 \ \mu g \ L^{-1}$ in the upper 35 m).
- 240 Surface waters at ENACWsp stations were less stratified (SST between 14.0 and 14.5 °C), were nutrient replete
- 241 (surface $NO_3^- + NO_2^-$ ranging from 0.3 to 0.8 μ M) and had a higher phytoplankton biomass (Chl *a* between 0.7 to 1.2
- 242 μg L⁻¹ in the upper 30 m except for station Bel-5). Highest Chl *a* values were observed at station Bel-7 (44.6° N, 9.3°
- 243 W), which appeared to be located within an anticyclonic mesoscale eddy as evidenced by the downwelling structure

244 detected in the Chl *a* and $NO_3^- + NO_2^-$ profiles (Fig. 2a, c) at this location (as well as T and S sections, data not 245 shown).

246

247 **3.2 Primary production and satellite-based Chl** *a* observations

Primary production (PP), estimated through the incorporation of enriched bicarbonate (13C-NaHCO₃) into the POC 248 pool, illustrated volumetric rates ranging from 7 to 3500 µmol C m⁻³ d⁻¹ (see Supporting Information Table S1) and 249 euphotic layer integrated rates ranging from 32 to 137 mmol C m⁻² d⁻¹ (Fig. 3a, b, and Supporting Information Table 250 S2). PP was relatively homogenous in the Bay of Biscay (stations Bel-3, Bel-5 and Bel-7) and along the Iberian 251 Margin (Bel-9, Bel-11, Bel-13 and Geo-1) with average rates ranging from 33 to 43 mmol C m⁻² d⁻¹, except for 252 station Bel-7 where it was slightly higher (52 mmol C m⁻² d⁻¹; Fig. 3a, b, and Table S2), likely due to the presence of 253 254 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⁻² d⁻¹, respectively; Fig. 3b), but also 255 slightly higher on the Portuguese shelf (reaching 59 mmol C m⁻² d⁻¹ at Geo-2). These results are in the range of past 256 measurements in this region for the same period of the year, ranging from 19 to 103 mmol C m⁻² d⁻¹ (Marañón et al., 257 258 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-259 260 bloom conditions prevailed at most sites (Bel-3 to Bel-13 and Geo-1 to Geo-13) while bloom conditions were still 261 ongoing at station Geo-21 at the time of our study.

262

263 3.3 N₂ fixation and dominant diazotrophs at the sampling sites

Volumetric N₂ 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⁻¹ d⁻¹ (see Table S1), with areal rates ranging between 81 and 1533 µmol N m⁻² d⁻¹ (Fig. 3c, d, and Table S2).

- We observed intense N₂ fixation activities at the two sites (Bel-11 and Bel-13) most affected by ENACWst (Fig. S1). 267 At stations Bel-11 and Bel-13, volumetric rates of N₂ fixation ranged from 2.4 to 65.4 nmol N L⁻¹ d⁻¹, with highest 268 rates found at surface level (65.4 and 45.0 nmol N L⁻¹ d⁻¹, respectively), while areal rates averaged 1533 and 1355 269 270 µmol N m⁻² d⁻¹, respectively. N₂ fixation was detected at all four GEOVIDE stations. Shelf-influenced (Geo-1 and 271 Geo-2) and open ocean (Geo-13) ENACWst sites, geographically close to Bel-11 and Bel-13, also displayed high N2 fixation activities with volumetric rates ranging from 1.0 to 7.1 nmol N L⁻¹ d⁻¹ (Table S1) while depth-integrated rates 272 averaged 141, 262 and 384 µmol N m⁻² d⁻¹, respectively (Fig. 3c, d, and Table S2). Significant N₂ fixation rates were 273 274 also measured at stations that exhibited the highest primary production rates, including Bel-7, Geo-13 and Geo-21 275 (Fig. 3). We computed the relative contribution of N_2 fixation to PP by converting N_2 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 276 mean value of $6.3 \pm 1.1, \pm$ SD, n = 46; Table 1). N₂ fixation contributed to less than 2% of PP at the ENACWsp sites 277 Bel-7 and Geo-21 and between 3 to 28% of PP at the ENACWst sites, except for station Bel-9 where it supported 278 279 about 1% of PP. 280 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 that displayed the largest areal N_2 fixation rates. Cloning of the *nifH* amplicons found in surface waters (54% PAR level where volumetric rates of N_2 fixation were the highest) yielded
- 283 103 nifH sequences. No successful nifH amplifications were obtained at the other Belgica stations or depths where

284 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 285 286 amino acid level with the symbiotic diazotrophic cyanobacteria UCYN-A1 or Candidatus Atelocyanobacterium 287 thalassa, first characterized from station ALOHA in the North Pacific (Fig. 5a and 6) (Thompson et al., 2012). While 288 the UCYN-A OTU also dominated the clones recovered from station Bel-13, fourteen additional *nifH* phylotypes 289 affiliated with non-cyanobacterial diazotrophs were also recovered at that station (Fig. 5a and 6). Among these 15 290 OTUs, represented by a total of 62 sequenced clones, 45.2% of the sequences were affiliated to UCYN-A1 (identical 291 to those found at Bel-11), and the rest to heterotrophic bacteria with 25.8% affiliated to Bacteroidetes, 19.3% to 292 Firmicutes and 9.7% to Proteobacteria (Gamma-, Epsilon- and Delta-proteobacteria; Fig. 5a and 6). For the 293 GEOVIDE cruise, nifH screening returned positive nifH presence at stations Geo-2, Geo-13 and Geo-21. Next 294 generation sequencing of these amplicons yielded in total 21001 reads, with a range of 170 to 9239 nifH amplicons 295 per sample, belonging exclusively to non-cyanobacterial diazotrophs, with the major affiliation to Verrucomicrobia, 296 and Gamma-, Delta- and Alpha-proteobacteria, representing 54, 28, 15 and 1% of total nifH amplicons, respectively 297 (Fig. 5b and 6). Members of a clade that has recently been characterized from the TARA expedition through 298 metagenome assembled genomes of marine heterotrophic diazotrophs (Delmont et al., 2018), were found among the 299 Gammaproteobacteria OTU types that dominated the community at station Geo-21.

300

301 3.4 Relationship between N₂ fixation rates and environmental variables

N₂ 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 (NO₃⁻ + NO₂⁻ from < 0.1 to 7.6 μ M). Water column integrated N₂ fixation tended to increase with average surface water salinity (n = 10, *p* < 0.05, Table S3) but was inversely correlated to satellite-based dust deposition in May 2014, the month during which our sampling took place (n = 10, *p* < 0.01). Volumetric rates of N₂ 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 to nitrate plus nitrite concentration (n = 46, *p* < 0.01).

309 4 Discussion

310 During two quasi-simultaneous expeditions to the Iberian Basin and the Bay of Biscay in May 2014 (38.8–46.5° N), 311 we observed N₂ fixation activity in surface waters of most visited stations (except for the two northernmost sites in 312 the Bay of Biscay). Our results are in support of other recent studies that have observed diazotrophic communities 313 and significant N_2 fixation rates in marine environments departing from the previously established belief that 314 diazotrophs are preferentially associated with warm oceanic water and low fixed-nitrogen concentrations (Needoba et 315 al., 2007; Rees et al., 2009; Blais et al., 2012; Mulholland et al., 2012; Shiozaki et al., 2015). Although there is 316 growing evidence that diazotrophs and their activity can extend geographically to temperate coastal and shelfinfluenced regions, there still exist very few rate measurements at higher latitudes, especially in open waters. In the 317 following sections we shall (1) discuss the significance of N_2 fixation in the Iberian Basin as well as its relation to 318 319 primary productivity pattern and extend our view to the whole Atlantic Ocean, (2) provide information on the 320 taxonomic affiliation of diazotrophs present at the time of our study, and (3) explore potential environmental 321 conditions that may have supported this unexpectedly high diazotrophic activity in the Iberian Basin.

323 4.1 Significance of N₂ fixation in the temperate ocean

In the present study, we found surprisingly high N_2 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 μ mol N m⁻² d⁻¹ at stations Bel-11 and Bel-13, respectively; Fig. 3c, d, and Tables S1 and S2). Although N₂
- 327 fixation was not detected in the central Bay of Biscay (stations Bel-3 and Bel-5), rates recorded at all the other sites
- 328 were relatively high, not only in shelf-influenced areas (141 and 262 µmol N m⁻² d⁻¹ at stations Geo-1 and Geo-2,
- 329 respectively) but also in the open ocean (average activities between 81 and 384 µmol N m⁻² d⁻¹ at stations Bel-7, Bel-
- 330 9, Geo-13 and Geo-21).
- By fuelling the bioavailable nitrogen pool, N_2 fixation may support marine primary production (PP), but the extent of 331 332 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 333 subtropical to temperate waters (32 to 137 mmol C m⁻² d⁻¹ relative to 19 to 103 mmol C m⁻² d⁻¹) (Marañón et al., 334 2000; Fernández et al., 2005; Poulton et al., 2006; Fonseca-Batista et al., 2017). However, the contribution of N₂ 335 fixation to PP in the present work (1–28% of PP) reached values twice as high as those reported in other studies for 336 337 the tropical and subtropical northeast Atlantic (contributions to PP ranging from < 1% to 12%) (Voss et al., 2004; 338 Rijkenberg et al., 2011; Fonseca-Batista et al., 2017). This observation further questions the accepted premise that 339 oligotrophic surface waters of tropical and subtropical regions are the key environment where diazotrophic activity 340 significantly supports marine primary productivity (Capone et al., 2005; Luo et al., 2014). Nevertheless, it is 341 important to keep in mind that our computation relies on the assumption that only photoautotrophic diazotrophs 342 contribute to bulk N₂ fixation, which may not always be the case, particularly in the present study, where mostly 343 heterotrophic diazotrophs were observed. However, it is likely that all the recently fixed-nitrogen ultimately becomes 344 available for the whole marine autotrophic community.

Previous studies in the open waters of the Iberian Basin ($35-50^\circ$ N, east of 25° W) reported relatively lower N₂ 345 fixation rates (from < 0.1 to 140 µmol N m⁻² d⁻¹), regardless of whether the bubble-addition method (Montoya et al., 346 347 1996) or the dissolution method (Mohr et al., 2010; Großkopf et al., 2012) was used. However, these studies were 348 carried out largely outside the bloom period, either during the late growing season (summer and autumn) (Moore et 349 al., 2009; Benavides et al., 2011; Snow et al., 2015; Riou et al., 2016; Fonseca-Batista et al., 2017) or during winter 350 (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 351 352 methodologies (bubble-addition versus dissolution method) may explain the discrepancies in diazotrophic activity 353 observed between our study and earlier works. Yet, the 20 months survey by Moreira-Coello et al. (2017) in nitrogenrich temperate coastal waters in the southern Bay of Biscay, covering the seasonal spring bloom and upwelling 354 pulses, did not reveal significant N₂ fixation activities: from 0.1 to 1.6 μ mol N m⁻² d⁻¹ (up to 3 orders of magnitude 355 356 lower than those reported here). However, unlike our study, this work was carried out not only using the bubble-357 addition method but also in an inner coastal system, as opposed to the mainly open waters investigated here, making 358 it difficult to predict which variable or combination of variables caused the difference observed between the two 359 studies.

360 Our maximal values recorded at stations Bel-11 and Bel-13 are one order of magnitude higher than maximal N₂ 361 fixation rates reported further south for the eastern tropical and subtropical North Atlantic (reaching up to 360-424362 µmol N m⁻² d⁻¹) (Großkopf et al., 2012; Subramaniam et al., 2013; Fonseca-Batista et al., 2017). Besides these two 363 highly active sites, N₂ fixation rates at the other studied locations (ranging between 81 and 384 µmol N m⁻² d⁻¹) were

364 still in the upper range of values reported for the whole eastern Atlantic region. Yet, conditions favouring N₂ 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 N₂ fixation rates up to ~1000 µmol N m⁻² d⁻¹ as observed here, have only been 369 reported for temperate coastal waters of the Northwest Atlantic (up to 838 µmol N m⁻² d⁻¹) (Mulholland et al., 2012) 370 371 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⁻² d⁻¹) (Capone et al., 2005; Montova et al., 2007; Subramaniam et al., 372 373 2008). Shelf and mesohaline areas have indeed been shown to harbour considerable N_2 fixation activity, not only in 374 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 375 376 conditions leading to the high N₂ fixation rates in these regions are currently not well understood. For tropical 377 mesohaline systems, the conditions proposed to drive such an intense diazotrophic activity include the occurrence of 378 highly competitive diatom-diazotrophs associations and the influence of excess phosphorus input (i.e., excess relative 379 to the canonical Redfield P/N ratio; expressed as P*) from the Amazon River (Subramaniam et al., 2008). However, 380 such conditions of excess P were not observed in previous studies carried out in high latitude shelf regions with elevated N₂ fixation activities (Blais et al., 2012; Mulholland et al., 2012; Shiozaki et al., 2015), nor was it distinctly 381 382 apparent in the present study (see section 4.3). In addition, while tropical mesohaline regions are characterized by the 383 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., 384 385 2009; Blais et al., 2012; Mulholland et al., 2012; Agawin et al., 2014; Shiozaki et al., 2015; Moreira-Coello et al., 386 2017).

387

388 4.2 Features of the diazotrophic community composition in the temperate North Atlantic

389 Our qualitative assessment of nifH diversity revealed a predominance of UCYN-A symbionts, only at the two stations 390 with the highest surface N₂ fixation rates (up to 65.4 and 45.0 nmol N L⁻¹ d⁻¹ 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 391 392 at all the other sites where *nifH* genes could be detected. No *Trichodesmium nifH* sequences were recovered from 393 either BG2014/14 or GEOVIDE DNA samples, and the absence of the filamentous cyanobacteria was also confirmed by the CHEMTAX analysis of phytoplankton pigments (M. Tonnard, personal communication, January 2018). 394 395 Previous work in temperate regions of the global ocean, including the Iberian Margin also reported that highest N₂ 396 fixation activities were predominantly related to the presence of UCYN-A symbionts, followed by heterotrophic 397 bacteria, while Trichodesmium filaments were low or undetectable (Needoba et al., 2007; Rees et al., 2009; 398 Mulholland et al., 2012; Agawin et al., 2014; Shiozaki et al., 2015; Moreira-Coello et al., 2017).

399 UCYN-A (in particular from the UCYN-A1 clade) were shown to live in symbioses with single-celled400 prymnesiophyte algae (Thompson et al., 2012). This symbiotic association, considered obligate, has been reported to

401 be particularly abundant in the central and eastern basin of the North Atlantic (Rees et al., 2009; Krupke et al., 2014;

402 Cabello et al., 2015; Martínez-Pérez et al., 2016).

403 Besides UCYN-A, all the remaining *nifH* sequences recovered from both cruises, although obtained through different

404 approaches, belonged to non-cyanobacterial diazotrophs. The phylogenetic tree (Fig. 6) showed that the non-

405 cyanobacterial diazotrophs clustered with (1) Verrucomicrobia, a phylum yet poorly known that includes aerobic to

406 microaerophilic methanotrophs groups, found in a variety of environments (Khadem et al., 2010; Wertz et al., 2012), 407 (2) anaerobic bacteria, obligate or facultative, mostly affiliated to Cluster III phylotypes of functional nitrogenase 408 (e.g., Bacteriodetes, Firmicutes, Proteobacteria) and lastly (3) phylotypes from Clusters I, II, and IV (e.g., 409 Proteobacteria and Firmicutes). Among the Cluster III phylotypes, Bacteroidetes are commonly encountered in the 410 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). N₂ fixation activity has previously been reported in five 411 412 Bacteroidetes strains including Bacteroides graminisolvens, Paludibacter propionicigenes and Dysgonomonas gadei 413 (Inoue et al., 2015) which are the closest cultured relatives of the nifH-OTUs detected at station Bel-13 (Fig. 6). 414 Anaerobic Cluster III phylotypes have been previously recovered from different ocean basins (Church et al., 2005; 415 Langlois et al., 2005, 2008; Man-Aharonovich et al., 2007; Rees et al., 2009; Halm et al., 2012; Mulholland et al., 416 2012). These diazotrophs were suggested to benefit from anoxic microzones found within marine snow particles or 417 zooplankton guts to fix N_2 thereby avoiding oxygenic inhibition of their nitrogenase enzyme (Braun et al., 1999; 418 Church et al., 2005; Scavotto et al., 2015). Therefore, the bloom to early post-bloom conditions, prevailing during our 419 study, were likely beneficial for the development of diazotrophic groups that depend on the availability of detrital 420 organic matter or on the association with grazing zooplankton. In contrast, at the northernmost Geo-21 station, we 421 observed a dominance of Gammaproteobacteria phylotypes belonging to a recently identified clade of marine 422 diazotrophs within the Oceanospirillales (Delmont et al., 2018). 423 These observations tend to strengthen the idea that not only UCYN-A (Cabello et al., 2015; Martínez-Pérez et al., 424 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_2 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 noncyanobacterial diazotrophs has been reported, their contribution to in situ activity remains poorly quantified.

430

431 **4.3 Key environmental drivers of N₂ fixation**

Environmental conditions that promote autotrophic and heterotrophic N_2 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_2 fixation activity would nevertheless require a supply of dFe and P (Raven, 1988; Howard & Rees, 1996; Mills et al., 2004; Snow et al., 2015). Besides the need for these critical inorganic nutrients, heterotrophic N_2 fixation was also recently shown to be highly dependent on the availability of organic matter (Bonnet et al., 2013;

439 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 N₂ 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⁻², respectively) with a dominant fraction of small size POC (the 1–53 μ m 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

- 447 13 and Geo-21, suggesting an efficient shallow remineralisation (Lemaitre et al., 2018). This availability of organic
- 448 matter in the upper layers likely contributed to supplying remineralized P (organic P being generally more labile than 449 other organic nutrients; Vidal et al., 1999, 2003) and to enhancing the residence time of dFe originating from 450 atmospheric deposition due to the formation of organic ligands (Jickells, 1999; de Baar and de Jong, 2001; Sarthou et 451 al., 2003).
- P* values from the BG2014/14 cruise (Table S1) and the climatological P* data for the Iberian Basin (Garcia et al., 452 2013) do not exhibit a clear PO₄³⁻ excess in the region (P* ranging from -0.1 to 0.1 µmol L⁻¹; Fig. 1 and Tables S1 453 454 and S2). Nevertheless, Spearman rank correlations indicate that volumetric N_2 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-13 455 weighing heavily in this correlation. Without the data from these two sites (data not shown), the correlation between 456 in situ P* and N₂ fixation rates is no longer significant (n= 16, p = 0.163), while P* becomes highly correlated with 457 458 PP and Chl a (n = 16, p = 0.0257 and 0.016, respectively). This suggests that the effect of P* on N₂ fixation, although 459 not clearly evident from absolute values, was most important at stations Bel-11 and Bel-13 but nonetheless existent at 460 the other sites (Bel-7 and Bel-9). The occurrence of N₂ fixation in oligotrophic waters displaying weak P* values, depleted in DIN and PO₄³⁻ but replete in dFe might in fact reflect the direct use by diazotrophs of dissolved organic 461 462 phosphorus (DOP). Indeed, according to Landolfi et al. (2015) diazotrophy ensures the supply of additional N and 463 energy for the enzymatic mineralization of DOP (synthesis of extracellular alkaline phosphatase). Therefore, a likely 464 enhanced DOP release towards the end of the spring bloom may have contributed to sustaining N_2 fixation in the studied region. Such DOP utilization has indeed been reported for various marine organisms, particularly 465 diazotrophic cyanobacteria (Dyhrman et al., 2006; Dyhrman & Haley, 2006) and bacterial communities (Luo et al., 466 467 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 + 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.

- 473 Atmospheric aerosol deposition determined during the GEOVIDE cruise (Shelley et al., 2017), as well as the 474 satellite-based dust deposition (dry + wet) averaged over the month of May 2014 (Fig. S3b; Giovanni online satellite 475 data system, NASA Goddard Earth Sciences Data and Information Services Center), reveal rather weak dust loadings 476 over the investigated region, resulting in areal N₂ fixation rates being actually inversely correlated to the satellite-477 based average dust input (p < 0.01, Table S3). In contrast, satellite-based dust deposition (dry + wet) averaged over 478 the month of April 2014 (i.e. preceding the timing of sampling) indicates high fluxes over the subtropical waters 479 located south of the studied region (Fig. S3a;). The θ /S diagrams at stations Bel-11 and Bel-13 (and to a lesser extent 480 at Geo-13; Fig. S1) illustrate the presence of very warm and saline waters, which were advected from the subtropics 481 as suggested by the satellite SST images (Fig. S2). We thus argue that advection of surface waters from south of the 482 study area represented a source of atmospherically derived dFe and contributed to driving the high N₂ fixation 483 activity recorded at stations Bel-11 and Bel-13. This resulted in N_2 fixation rates there being positively (although weakly) correlated (p = 0.45, Table S3) with the April average dust input. 484
- 485 For the central Bay of Biscay, where N_2 fixation was below the DL (stations Bel-3 and Bel-5), dust deposition in
- 486 April 2014 was also the lowest, suggesting that N_2 fixation there might have been limited by dFe availability. Indeed,
- 487 at stations Bel-3 and Bel-5 diazotrophic activity in surface waters was boosted following dFe amendments (> 25
- 488 nmol N $L^{-1} d^{-1}$; Li et al., 2018).

Thus, the enhanced N_2 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.

493 These statements are further supported by the outcome of a multivariate statistical analysis, providing a comprehensive view of the environmental features influencing N2 fixation. A principal component analysis (PCA; 494 495 Fig. 7 and Tables S2 and S5) generated two components (or axes) explaining 68% of the system's variability. Axis 1 496 illustrates the productivity of the system, or more precisely the oligotrophic state towards which it was evolving. Axis 497 1 is defined by a strong positive relation with surface temperature (reflecting the onset of stratification, particularly 498 for stations Bel-11 and Bel-13; Fig. 7) and an inverse relation with PP and associated variables (Chl a, NH₄⁺, NO₃⁻ + 499 NO_2), which reflects the prevailing post-bloom conditions of the system. Sites characterized by a moderate (Bel-3 500 and Bel-5) to high (Bel-7, Geo-21 and to a lesser extent Geo-13) PP appear indeed tightly linked to these PP-501 associated variables as illustrated in Fig. 7. Axis 2 is defined by the positive relation with surface salinity and P* (Fig. 502 7) and reflects the advection of surface waters of subtropical origin for stations Bel-11, Bel-13 and Geo-13. For 503 stations Geo-1 and Geo-2, the inverse relation with surface salinity (Fig. 7) is interpreted to reflect fluvial inputs 504 (Tonnard et al., 2018). Finally, this statistical analysis indicates that N_2 fixation activity was likely influenced by the 505 two PCA components, tentatively identified as productivity (axis 1) and surface water advection (axis 2) from the 506 shelf and the subtropical region.

507 5 Conclusions

The present work highlights the occurrence of elevated N2 fixation activities (81-1533 µmol N m⁻² d⁻¹) in spring 2014 508 509 in open waters of the temperate eastern North Atlantic, off the Iberian Peninsula. These rates exceed those reported 510 by others for the Iberian Basin, but which were largely obtained outside the bloom period (from < 0.1 to 140 µmol N $m^{-2} d^{-1}$). In contrast, we did not detect any N₂ fixation activity in the central Bay of Biscay. At sites where significant 511 512 N₂ 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 513 514 harbouring oceanic N_2 fixation for the eastern Atlantic. Assuming that the carbon versus nitrogen requirements by 515 these N_2 fixers obeyed the Redfield stoichiometry, N_2 fixation was found to contribute 1–3% of the euphotic layer daily PP and even up to 23-25% at the sites where N₂ fixation activities were the highest. The prymnesiophyte-516 517 symbiont Candidatus Atelocyanobacterium thalassa (UCYN-A) contributed the most to the nifH sequences recovered 518 at the two sites where N_2 fixation activity were the highest, while the remaining sequences belonged exclusively to 519 heterotrophic bacteria. We speculate that the unexpectedly high N₂ fixation activity recorded at the time of our study 520 was sustained by (i) organic matter availability in these open waters, resulting from the prevailing vernal bloom to 521 post-bloom conditions, in combination with (ii) excess phosphorus signatures which appeared to be tightly related to 522 diazotrophic activity particularly at the two most active sites. Yet these observations and hypotheses rely on the 523 availability of dFe with evidence for input from shelf waters and pulsed atmospheric dust deposition being a 524 significant source of iron. Further studies are required to investigate this possible link between N_2 fixation activity and phytoplankton bloom under iron-replete conditions in the studied region and similar environments, as these 525 526 would require to be considered in future assessment of global N₂ fixation.

⁵²⁸ Data availability. The data associated with the paper are available from the corresponding author upon request.

- 529
- 530 The Supplement related to this article is available.
- 531

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

533

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- 841 Table

Table 1: Relative contribution (%) of N2 fixation to Primary Production (PP).

Province	Station	Latitude (° N)	Longitude (° E)	N ₂ fixation contribution to PP (%) (Redfield 6.6 ratio)	SD	N_2 fixation contribution to PP (%) (mean POC/PN ratio of 6.3 ± 1.1)	SD
ENACWsp	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
ENACWst	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



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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 ($P^* = [PO_4^{3-}] - [NO_3^{-}] / 16$) 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 horizontal dashed line. Black dashed and solid contour lines illustrate 500 m and 1500 m isobaths, respectively. (Schlitzer, R., Ocean Data View).



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Figure 2: Spatial distribution of Chl *a* (a, d), NH_4^+ (b, e) and $NO_3^- + NO_2^-$ (c, f) concentrations along the Belgica BG2014/14 (upper panels) and GEOVIDE (lower panels) 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).



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871 Figure 3: Spatial distribution (± SD) of depth-integrated rates of primary production (a, b) (duplicates are in light and dark green bars with the corresponding values in mmol C m⁻² d⁻¹); N₂ fixation (c, d) (duplicates are in light and 872 dark blue bars with the corresponding values in µmol N m⁻² d⁻¹) determined during the Belgica BG2014/14 (a, c) and 873 874 GEOVIDE (b, d) cruises. Error bars represent the propagated measurement uncertainty of all parameters used to 875 compute volumetric uptake rates.

Bel-11 Bel-13

0

Geo-1

Geo-2

Geo-13

Geo-21

60

Bel-7

Bel-9

< DL

Bel-5

< DL

Bel-3







Figure 4: Time series of area-averaged chlorophyll a concentration (mg m⁻³) registered by Aqua MODIS satellite (Giovanni online satellite data system) between December 2013 and December 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).





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886 Figure 5: Diversity of *nifH* sequences during (a) the Belgica BG2014/14 cruise (successfully recovered only at

and 70 m. The total numbers of recovered sequences are indicated on top of the bars, and the exact percentage

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

889 represented by each group is shown inside the bars.



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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

- 893 (MEGA 7.0) (Kumar et al., 2016). Initial tree(s) for the heuristic search were obtained automatically by applying
- 894 Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite
- 895 Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma
- 896 distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4038)). All
- 897 sequences recovered from DNA samples, including those previously identified and the newly recovered ones (with \geq
- 898 95% similarity at the nucleotide level with representative clones) are highlighted in blue. For the nifH sequences
- 899 recovered from the GEOVIDE cruise, only those contributing to the cumulative 98% of recovered sequences were
- 900 included in this tree. Bootstrap support values (\geq 50%) for 100 replications are shown at nodes. The scale bar
- 901 indicates the number of sequence substitutions per site. The archaean Methanobrevibacter smithii was used as an
- 902 outgroup. Accession numbers for published sequences used to construct the phylogenetic tree are given.





906 Figure 7: Euclidean distance biplot illustrating the axis loadings for the two main PCA components based on the 907 Spearman rank correlation matrix shown in Table S3. Variables taken into account include depth-integrated rates of 908 N_2 fixation and primary production (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., 909 910 2013); satellite average dust deposition (dry + wet) derived during April 2014 (Giovanni online data system, NASA 911 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 912 loadings for PP, Chl a, NH_4^+ and $NO_3^- + NO_2^-$, and high positive loadings for temperature and N_2 fixation rates, with 913 values of -0.812, -0.768, -0.936, -0.783, 0.942 and 0.506, respectively (see Table S5). Axis 2 has high positive 914 915 loadings of 0.584, 0.943 and 0.602 for climatological P*, salinity and N₂ fixation rates, respectively. PCA analysis 916 was run in XLSTAT 2017 (Addinsoft, Paris, France, 2017).