

Evidence of high N₂ fixation rates in productive waters of 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. We report
substantial N₂ fixation activities at 8 of the 10 stations sampled, ranging overall between 81 and 384 μmol N m⁻² d⁻¹
25 (0.7 to 8.2 nmol N L⁻¹ d⁻¹), with two sites close to the Iberian Margin between 38.8° N and 40.7° N yielding rates
reaching up to 1355 and 1533 μmol N m⁻² d⁻¹ (65 and 45 nmol N L⁻¹ d⁻¹ at surface level, respectively). Although
diazotrophic activity was not detected at two northern stations in the central Bay of Biscay, when converted to carbon
uptake using Redfield stoichiometry, N₂ fixation at the eight other sites generally contributed to 1–3% of euphotic
layer daily PP, and up to 25% and 23%, respectively at the two most active sites. In the Atlantic Ocean, N₂ fixation
30 rates exceeding 1000 μmol N m⁻² d⁻¹ have previously only been reported in the temperate and tropical western North
Atlantic waters having coastal, shelf or mesohaline characteristics, as opposed to the mostly open ocean conditions
studied here. At the two sites where N₂ fixation activity was the highest; *nifH* sequences assigned to the
prymnesiophyte-symbiont *Candidatus Atelocyanobacterium thalassa* (UCYN-A) dominated the *nifH* sequence pool
recovered from DNA samples, while the remaining sequences, as for all the ones recovered from the other sites,
35 belonged exclusively to non-cyanobacterial phylotypes. Previous studies in the Iberian Basin have systematically
reported lower N₂ fixation rates (from < 0.1 to 140 μmol N m⁻² d⁻¹), as compared to those found in the present study,
and this regardless of whether the bubble-addition method or the dissolution method were applied. Earlier studies in
the Iberian region were conducted largely outside the bloom period, unlike the present work which was carried out in
spring, yet in all cases the assessment of *nifH* gene diversity, suggests a predominance of UCYN-A and non-
40 cyanobacterial diazotrophs. We support that the unexpectedly high N₂ fixation activities recorded at the time of our
study were promoted by the availability of phytoplankton-derived organic matter produced during the spring bloom,
as evidenced by the significant surface particulate organic carbon concentrations, and by the presence of excess
phosphorus signature in surface waters, particularly at the sites with extreme activities. Our findings stress the need

for a more detailed monitoring of oceanic N₂ fixation in productive waters of the temperate North Atlantic to better
45 constrain the basin-scale nitrogen input to the ocean inventory.

1 Introduction

Dinitrogen (N₂) 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
50 (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
global marine N₂ fixation.

Until recently most studies of N₂ fixation have focused on the tropical and subtropical regions of the global ocean,
55 with few attempts to measure N₂ fixation at higher latitudes, with the exception of enclosed brackish seas
(Ohlendieck et al., 2000; Luo et al., 2012; Farnelid et al., 2013). The intense research effort 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, long considered as the most active diazotroph in the global ocean, has mostly been
60 reported in oligotrophic tropical and subtropical oceanic waters, thought to represent the optimal environment for its
growth and N₂-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 N₂ pool for their biosynthesis. As such,
65 past estimates of global annual N₂ 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
70 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 depth and latitude, 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 to the commonly used ¹⁵N₂ bubble-
addition technique (Mohr et al., 2010) and the presence of an abundant diazotrophic community in high latitude
75 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 N₂ 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 N₂-fixing enzyme) (Raven, 1988; Howard & Rees, 1996; Mills et al.,
80 2004; Snow et al., 2015) and the upwelling of subsurface waters with low DIN (dissolved inorganic nitrogen) to
phosphate ratios, make this region highly favorable for N₂ fixation activity (Deutsch et al., 2007; Moore et al., 2009).
In addition, the northeast 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 of prymnesiophyte-UCYN-A symbiotic associations at the global ocean scale (Cabello et al., 2015). Earlier studies in the Iberian open waters investigated the diazotrophic activity either during 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 winter convection period (Rijkenberg et al., 2011; Agawin et al., 2014). In the present work, we present N₂ fixation rate measurements from two consecutive missions carried out in the Northeast sector of the Atlantic Ocean in May 2014, during and after the spring bloom. We provide evidence for the presence of a diazotrophic community and their taxonomic affiliation of the diazotrophic community by amplifying the *nifH* gene from DNA samples collected at the same stations.

2 Material and Methods

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 program (GA01 section, May 16 to June 29 2014, R/V *Pourquoi pas?*) sailed from the Portuguese shelf area towards Greenland and ended in Newfoundland, Canada (<http://dx.doi.org/10.17600/14000200>). For the latter expedition, only stations within the Iberian Basin investigated for N₂ fixation activity (stations Geo-1, 2, 13 and 21) are considered in this paper and the measurements are compared with those conducted at the six sites studied during the BG2014/14 cruise (stations Bel-3, 5, 7, 9, 11 and 13; Fig. 1).

2.1 Environmental conditions

Temperature, salinity and photosynthetically active radiation (PAR) profiles were determined using a conductivity-temperature-depth sensor (SBE 09 and SBE 911+, during the BG2014/14 and GEOVIDE cruises, respectively) fitted on a rosette equipped with either 12 or 24 Niskin bottles to sample seawater for biogeochemical measurements. Water column concentrations of ammonium (NH₄⁺) during both cruises were measured on board as well as nitrate + nitrite (NO₃⁻ + NO₂⁻) concentrations during the GEOVIDE expedition. During the BG2014/14 cruise, samples dedicated for NO₃⁻ + NO₂⁻ and phosphate (PO₄³⁻) measurements were filtered (0.2 μm) and stored at -20°C until analysis at the home-based laboratory. PO₄³⁻ data are not yet available for the GEOVIDE cruise.

Nutrient concentrations were determined using the conventional fluorometric (for NH₄⁺) (Holmes et al., 1999) and colorimetric methods (for the other nutrients) (Grasshoff et al., 1983) with detection limits (DL) of 64 nmol L⁻¹ (NH₄⁺), 90 nmol L⁻¹ (NO₃⁻ + NO₂⁻) and 60 nmol L⁻¹ (PO₄³⁻). For the BG2014/14 cruise, chlorophyll *a* (Chl *a*) concentrations were determined according to Yentsch and Menzel (1963), by filtering 250 mL of seawater sample 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.

2.2 ¹⁵N₂ fixation and ¹³C-HCO₃⁻ uptake rates

N₂ fixation and primary production (PP) were determined simultaneously in duplicate using the ¹⁵N-N₂ dissolution method (Großkopf et al., 2012) and ¹³C-NaHCO₃ tracer addition (Hama et al., 1983) techniques. Seawater samples

were collected in 4.5 L acid-cleaned polycarbonate (PC) bottles from a minimum of four depths (six at stations Geo-1, Geo-13 and Geo-21) equivalent to 54%, 13%, 3% and 0.2% of surface PAR (plus 25% and 1% PAR for Geo-1, Geo-13 and Geo-21). Details concerning the applied $^{15}\text{N}_2$ dissolution method can be found in Fonseca-Batista et al. (2017). Briefly, $^{15}\text{N}_2$ -enriched seawater was prepared by degassing prefiltered (0.2 μm) low nutrient seawater, thereafter stored in 2 L gastight Tedlar bags (Sigma-Aldrich) subsequently injected with 30 mL of pure $^{15}\text{N}_2$ gas (98 ^{15}N atom%, Eurisotop, lot number 23/051301) and left to equilibrate. This $^{15}\text{N}_2$ gas batch (Eurisotop) has previously been shown to be free of ^{15}N -labelled contaminants such as nitrate, nitrite, ammonium and nitrous oxide. Each PC incubation bottle was partially filled with sampled seawater, then amended with 250 mL of $^{15}\text{N}_2$ -enriched seawater, spiked with 3 mL of a $\text{NaH}^{13}\text{CO}_3$ solution (200 mmol L^{-1} , 99%, Eurisotop) and topped off with the original seawater sample. Samples were incubated for 24 hours in on-deck incubators circulated with surface seawater and wrapped with neutral density screens (Rosco) simulating the in situ irradiance conditions. After incubation, samples were filtered onto pre-combusted MGF filters (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 particulate organic carbon and particulate nitrogen (POC and PN) were assessed by filtering 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 analyzer (EuroVector Euro EA 3000) coupled to an isotope ratio mass spectrometer (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. N_2 fixation and carbon uptake volumetric rates were computed as described in Montoya et al. (1996), and depth-integrated rates were calculated by non-uniform gridding trapezoidal integration for each station. Minimal detectable uptake rates were determined as detailed in Fonseca-Batista et al. (2017). To do so, the minimal acceptable ^{15}N or ^{13}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, increased by three times the uncertainty obtained for N and C isotopic analysis of CRM. 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_2 fixation was undetectable for some incubations (see details in section 3.3).

2.3 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_2 fixation rate measurements were carried out, prior to incubation. 2 L volumes were vacuum filtered (20 to 30 kPa) through 0.2 μm sterile cellulose acetate filters (47 mm Sartorius type 111) 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 $\text{ng } \mu\text{L}^{-1}$ 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 directed by the 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 μL of lysozyme solution (5 mg mL^{-1} in TE buffer), 45 μL of ProteinaseK

solution (20 mg mL⁻¹ in MilliQ PCR grade water) and 400 µL 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 °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 *nifH*1/2 primers (Langlois et al., 2005; Ratten, 2017) attached to Illumina adaptors and barcodes for multiplexing in the Illumina MiSeq instrument. 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) using 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. 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 which are 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 Supporting Information Fig. S1). One 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.

3 Results

3.1 Ambient environmental settings

Sites sampled in May 2014 during the Belgica BG2014/14 and GEOVIDE cruises, 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 Water (ENACW), a winter mode water which consists of two components, according to Fiúza (1984) (see θ/S diagrams, Fig. 2): (i) the lighter, relatively warm and salty ENACW_{st} formed in the subtropical Azores Front region (~35° N) when Azores Mode Water is subducted as a result of strong evaporation and winter cooling; and (ii) the colder and less saline ENACW_{sp}, underlying the ENACW_{st}, and 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 allowed categorizing the sampling sites in 2 groups: (i) ENACW_{sp} stations north of 43° N (Bel-3, Bel-5, Bel-7, and Geo-21) only affected by the ENACW_{sp} (Fig. 2a, b) and (ii) ENACW_{st} stations, south of 43° N, characterized by the upper layer being influenced by ENACW_{st} and the subsurface layer by ENACW_{sp} (Fig. 2a, b). Most of these ENACW_{st} stations are open ocean sites (Bel-9, Bel-11, Bel-13, and Geo-13) while two are influenced by their proximity to the shelf (Geo-1 and Geo-2) (Tonnard et al., 2018).

Surface waters of all the ENACW_{st} 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). Nutrients were depleted at the surface ($\text{NO}_3^- + \text{NO}_2^- < 0.09 \mu\text{M}$ in the upper 20 m; Fig. 3c, f) and surface Chl *a* concentrations were low ($< 0.25 \mu\text{g L}^{-1}$; Fig. 3a, 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 higher Chl *a* (> 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°C), were nutrient replete (surface $\text{NO}_3^- + \text{NO}_2^-$ ranging from 0.3 to 0.8 μM) 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° N, 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 $\text{NO}_3^- + \text{NO}_2^-$ profiles (Fig. 3a, c) at this location (as well as T and S sections, data not shown).

3.2 Primary production and pigment distribution

Volumetric rates of carbon uptake ranged from 7 to 3500 $\mu\text{mol C m}^{-3} \text{d}^{-1}$ (see Supporting Information Table S1) and euphotic layer integrated rates varied from 32 to 137 $\text{mmol C m}^{-2} \text{d}^{-1}$ (Fig. 4a, b, and Supporting Information 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 $\text{mmol C m}^{-2} \text{d}^{-1}$, except at station Bel-7 where it was slightly higher (52 $\text{mmol C m}^{-2} \text{d}^{-1}$; Fig. 4a, b, and Supporting Information 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 $\text{mmol C m}^{-2} \text{d}^{-1}$, respectively; Fig. 4b) as well as closer to the shelf (reaching 59 $\text{mmol C m}^{-2} \text{d}^{-1}$ at Geo-2). These results are in the range of past measurements for the same period of the year, ranging from 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). Our observations also coincide with the area-averaged Chl *a* time series obtained from satellite data (Giovanni online data system; Fig. 4c, d) which reveal that post bloom conditions prevailed at most sites (Bel-3 to Bel-13 and Geo-1 to Geo-13) while the bloom was still ongoing at station Geo-21 at the time of our study. Higher PP rates appear to coincide with the increase, offshore and towards the shelf, of the relative abundance of diatoms, based on fucoxanthin pigment concentrations (Tonnard et al., in preparation). At the GEOVIDE sites exhibiting lowest fixed-nitrogen concentrations, Geo-1 and Geo-13, prymnesiophytes represented 30–40% of the phytoplankton community, compared to 20–35% at stations Geo-21 and Geo-2 (based on the presence of 19'-hexanoyloxyfucoxanthin pigment). Such relative abundances are in agreement with the global abundance of prymnesiophytes ($32 \pm 5\%$) proposed by Swan et al. (2016).

3.3 N₂ fixation and dominant diazotrophs at the sampling sites

Volumetric N₂ fixation rates were above the detection limit at 8 of the 10 stations sampled in this study (excluding Bel-3 and Bel-5 where rates were below the detection limit) and ranged from 0.7 to 65.4 $\text{nmol N L}^{-1} \text{d}^{-1}$ (see Supporting Information Table S1), with areal rates ranging between 81 and 1533 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ (Fig. 5a, b, and Supporting Information Table S2).

We observed intense N₂ fixation activities at the two sites (Bel-11 and Bel-13) most affected by ENACW waters of subtropical origin (Fig. 2). At stations Bel-11 and Bel-13, volumetric rates of N₂ 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. N₂ fixation was also relatively high at the most productive sites

Bel-7 and Geo-21 with volumetric rates ranging from 1.0 to 8.2 nmol N L⁻¹ d⁻¹ and areal rates averaging 128 and 279 μmol N m⁻² d⁻¹, respectively. In contrast to the Belgica sites, N₂ 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 N₂ fixation activities with volumetric rates ranging between 1.0 and 7.1 nmol N L⁻¹ d⁻¹ (Supporting Information Table S1) while depth-integrated rates averaged 141, 262 and 384 μmol N m⁻² d⁻¹, respectively (Fig. 5a, b, and Supporting Information Table S2). We computed the relative contribution of N₂ fixation to PP by converting N₂ fixation rates to carbon uptake using either a Redfield ratio of 6.6 or the determined median POC/PN ratio for natural particles (equivalent to the mean value of 6.3 ± 1.1, ± SD, n = 46; Table 1). N₂ fixation contributed to less than 2% of PP at the ENACWsp sites Bel-7 and Geo-21 and between 3 to 28% of PP at the ENACWst sites, except at 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 that displayed the largest areal N₂ fixation rates. Cloning of the positive *nifH* amplicons found in surface waters (54% PAR level where volumetric rates of N₂ fixation were 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 of the clones (n = 41) recovered from station Bel-11 were regrouped in a single OTU that had 99% similarity 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 (Fig. 5c and S1) (Thompson et al., 2012). While the UCYN-A OTU also dominated the clones recovered from station Bel-13, fourteen additional *nifH* phylotypes affiliated with non-cyanobacterial diazotrophs were also recovered at that station (Fig. 5c and S1). Among these 15 OTUs, represented by a total of 62 sequenced clones, 45.2% of the sequences were affiliated to UCYN-A1 (identical to those found at Bel-11), and the rest to heterotrophic bacteria with 25.8% affiliated to Bacteroidetes, 19.3% to Firmicutes and 9.7% to Proteobacteria (Gamma-, Epsilon- and Deltaproteobacteria; Fig. 5c and S1). For the GEOVIDE cruise, *nifH* screening returned positive *nifH* presence at stations Geo-2, Geo-13 and Geo-21. High throughput tag sequencing of the amplicons yielded in total 21001 reads, with a range of 170 to 9239 *nifH* amplicons per sample, belonging exclusively to bacterial diazotrophs, with the major affiliation to Verrucomicrobia, and Gamma-, Delta- and Alpha-proteobacteria, representing 54, 28, 15 and 1% of total *nifH* amplicons, respectively (Fig. 5d and S1). Members of a clade that has been recently characterized from the TARA expedition through metagenome reconstructed genomes of marine heterotrophic diazotrophs (Delmont et al., 2018), were found among the Gammaproteobacteria OTU types that dominated the community at station Geo-21.

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 N₂ fixation activity in surface waters of most stations (except at the two northernmost sites in the Bay of Biscay). Surface waters sampled during this study were characterized by relatively low SST (12.5–17.3°C) and a wide range of DIN concentrations (NO₃⁻ + NO₂⁻ from < 0.1 to 7.6 μM). Our results are in support of other recent studies, that have observed diazotrophic communities and significant N₂ fixation rates in marine environments that depart 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 extend

geographically to temperate coastal and shelf-influenced regions, there are still very few rate measurements at higher latitudes, especially in open waters. In the following sections (1) we discuss the significance of N₂ fixation in the Iberian Basin, its relation to primary productivity pattern and extend our view to the whole Atlantic Ocean, (2) we provide information on the taxonomic affiliation of diazotrophs that were present at the time of our study, and (3) we explore potential environmental conditions that may have supported this unexpectedly high diazotrophic activity in the Iberian Basin.

290 **4.1 Significance of N₂ fixation in the temperate ocean**

In the present study, we found surprisingly high N₂ fixation activities at most of the studied sites. Rates were exceptionally elevated at two open ocean sites located between 38.8–40.7° N at about 11° W (averaging 1533 and 1355 μmol N m⁻² d⁻¹ at stations Bel-11 and Bel-13, respectively; Fig. 5a, b, and Tables S1 and S2). Although N₂ 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 μmol N m⁻² d⁻¹ at stations Geo-1 and Geo-2, respectively) but also in the open ocean (average activities between 81–384 μmol N m⁻² d⁻¹ at stations Bel-7, Bel-9, Geo-13 and Geo-21).

By fuelling the bioavailable nitrogen pool, N₂ fixation may support marine primary production (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 works for subtropical to temperate waters of the northeast Atlantic (32 to 137 mmol C m⁻² d⁻¹ relative to 19 to 103 mmol C m⁻² d⁻¹) (Marañón et al., 2000; Fernández et al., 2005; Poulton et al., 2006; Fonseca-Batista et al., 2017). However, N₂ fixation contributions 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 general idea that oligotrophic surface waters of tropical and subtropical regions are the key environment where marine primary productivity is significantly supported by diazotrophic activity (Capone et al., 2005; Luo et al., 2014). Nevertheless, it is important to keep in mind that this computation relies on the assumption that only photoautotrophic diazotrophs contribute to bulk N₂ fixation, which is not always the case, particularly in the present study, where mostly heterotrophic diazotrophs were observed. However, 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 N₂ fixation rates (from < 0.1 to 140 μmol N m⁻² d⁻¹), regardless of whether the bubble-addition method (Montoya et al., 1996) or the dissolution method (Mohr et al., 2010; Großkopf et al., 2012) were used. However, these studies were carried out largely outside the bloom period, either during the late growth 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, different methodologies (bubble-addition versus dissolution method) may explain the discrepancies in diazotrophic activity observed between our study and earlier works. Yet, the 20 months 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 revealed significant N₂ fixation activities: from 0.1 to 1.6 μmol N m⁻² d⁻¹ (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 studied here, making it difficult to predict which variable or combination of variables caused the difference in observations between both studies.

Our maximal values recorded at stations Bel-11 and Bel-13 are one order of magnitude higher than maximal N_2 fixation rates reported further south for the eastern tropical and subtropical North Atlantic (reaching up to 360–424 $\mu\text{mol N m}^{-2} \text{d}^{-1}$) (Großkopf et al., 2012; Subramaniam et al., 2013; Fonseca-Batista et al., 2017). Besides these two highly active sites, N_2 fixation rates at the other studied locations (ranging between 81–384 $\mu\text{mol N m}^{-2} \text{d}^{-1}$) were still in the upper range of values reported for the whole eastern Atlantic border. Yet, conditions favouring N_2 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_2 fixation rates up to $\sim 1000 \mu\text{mol N m}^{-2} \text{d}^{-1}$ as observed here, have only been reported for temperate coastal waters of the Northwest Atlantic (up to 838 $\mu\text{mol N m}^{-2} \text{d}^{-1}$) (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 $\mu\text{mol N m}^{-2} \text{d}^{-1}$) (Capone et al., 2005; Montoya et al., 2007; Subramaniam et al., 2008). Shelf and mesohaline areas have indeed been shown to harbour considerable N_2 fixation activity, not only in tropical regions (Montoya et al., 2007; Subramaniam et al. 2008) but also in areas from temperate to polar regions (Rees et al., 2009; Blais et al., 2012; Mulholland et al., 2012; Shiozaki et al., 2015). Yet, the environmental conditions that lead to the high N_2 fixation rates in those 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-diazotrophs 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_2 fixation activities (Blais et al., 2012; Mulholland et al., 2012; Shiozaki et al., 2015), nor was it distinctly apparent in the present study (see section 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 highest recorded surface N_2 fixation rates (up to 65.4 and 45.0 $\text{nmol N L}^{-1} \text{d}^{-1}$ at Bel-11 and Bel-13, respectively; Table S1) while the remaining *nifH* sequences recovered belonged to heterotrophic diazotrophs, at Bel-13 and also 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 a CHEMTAX analysis of phytoplankton pigments (Tonnard et al., in preparation). Previous work in temperate regions of the global ocean, including the Iberian Margin also reported that highest N_2 fixation activities were predominantly related to the presence of UCYN-A cells, 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 cells (in particular from the UCYN-A1 clade) were shown to live in mutualistic symbioses with single-celled prymnesiophyte algae (Thompson et al., 2012). This symbiotic association, considered as an 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. These bacterial diazotrophs consisted, as indicated by their closest known representatives, (1) of Verrucomicrobia, a phylum yet poorly known that includes aerobic to microaerophile methanotrophs groups, found in a variety of environments (Khadem et al., 2010; Wertz et al., 2012), (2) of anaerobic bacteria, obligate or facultative, mostly affiliated to Cluster III phylotypes of functional nitrogenase (e.g., Bacteroidetes, Firmicutes, Proteobacteria) and finally (3) of 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). N₂ fixation activity has previously been reported in five Bacteroidetes strains including *Bacteroides graminisolvens*, *Paludibacter propionicigenes* and *Dysgonomonas gadei* (Inoue et al., 2015) which are the closest cultured relatives of the *nifH*-OTUs detected at station Bel-13 (Fig. S1). 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 N₂ 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 to the development of diazotrophic groups that depend on the availability of detrital organic matter or the association with grazing zooplankton. In contrast, at the northern most Geo-21 station, we observed a particularly high relative contribution of Gammaproteobacteria belonging to a recently identified clade of marine diazotrophs within the Oceanospirillales (Delmont et al., 2018).

These observations tend to strengthen the idea of a substantial role played not only by UCYN-A (Cabello et al., 2015; Martínez-Pérez et al., 2016) but also by non-cyanobacteria (Halm et al., 2012; Shiozaki et al., 2014; Langlois et al., 2015) in oceanic N₂ fixation. Although it is possible to assign a broad taxonomic affiliation to classify the *nifH* genes, we know very little with respect to their physiology, their role in the ecosystem and the factors that control their distribution largely due to the lack of representative whole genome sequences and environmentally relevant strains available for experimentation (Bombar et al., 2016). While studies have been reporting on the widespread distribution of UCYN-A and heterotrophic diazotrophs, their contribution to in situ activity remains until now poorly quantified.

4.3 Key environmental drivers of N₂ fixation

Environmental conditions that promote autotrophic and heterotrophic N₂ 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 N₂ 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 N₂

405 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 N₂ fixation activity at the time of our study. Lemaitre et al. (2018) report that the upper 100–120 m waters of the Iberian Basin (stations Geo-1 and Geo-13) and the West European Basin (Geo-21) carried significant
410 particulate organic carbon loads (POC of 166, 171 and 411 mmol C m⁻², respectively) with a dominant fraction of small size POC (1–53 μm; 75%, 92% and 64% of 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 observed at stations Geo-13 and Geo-21, evidencing an efficient shallow remineralisation (only 3 to 4% of euphotic layer integrated PP reaching the depth of export at these stations; Lemaitre et al., 2018). This
415 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.,
420 2013) do not exhibit a clear PO₄³⁻ excess in the region (P* ranging between –0.1 and 0.1 μmol L⁻¹; Fig. 1 and Tables S1 and S2). Nevertheless, Spearman rank correlations indicate that volumetric N₂ fixation rates were significantly correlated with the BG2014/14 shipboard P* values (n = 24, p < 0.01), with stations Bel-11 and Bel-13 weighing heavily in this correlation. Without the data from these two sites the correlation between in situ P* and N₂ fixation rates is no longer significant (n = 16, p = 0.163), with P* becoming highly correlated with PP and Chl *a* (n = 16, p =
425 0.0257 and 0.016, respectively). This suggests that P* effect on N₂ 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 Bel-9). The impact of weak P* values in oligotrophic waters depleted in DIN and PO₄³⁻ but replete in dFe might in fact reflect the direct use 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
430 extracellular alkaline phosphatase). Therefore, a likely enhanced DOP release towards the end of the spring bloom may have contributed to sustaining N₂ fixation in the studied region. Such DOP utilization has indeed been reported for various marine organisms, particularly diazotrophic cyanobacteria (Dyhrman et al., 2006; Dyhrman & Haley, 2006) and bacterial communities (Luo et al., 2009).

Supply routes of dFe to the surface waters of the investigated area relied on lateral advection from the continental
435 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). In the following we discuss that 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-
440 based dust deposition (dry + 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 N₂ fixation rates being actually inversely correlated to the satellite-based average dust input (p < 0.01, Table S3). In contrast, satellite-based dust deposition (dry + wet) averaged over the month of April 2014 (i.e. preceding the timing of sampling) indicate high values over the subtropical waters located
445 south of the studied region (Fig. S3a;). The θ/S diagrams at stations Bel-11 and Bel-13 (and to a lesser extent at Geo-13; Fig. 2) illustrate the presence of very warm and saline waters and satellite SST images suggest these were

advection from the subtropics (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 N₂ fixation activity recorded at stations Bel-11 and Bel-13. This resulted in N₂ 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 N₂ fixation was below detection limit (stations Bel-3 and Bel-5), dust deposition in April 2014 was also the lowest, suggesting that N₂ 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 N₂ 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 N₂ fixation. A principal component analysis (PCA; Tables 2 and S2) 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. 6) and an inverse relation with PP and associated variables (Chl *a*, NH₄⁺, NO₃⁻ + NO₂⁻), 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. 6. Axis 2 is defined by the positive relation with surface salinity and P* (Fig. 6) 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. 6) is interpreted to reflect fluvial inputs (Tonnard et al., 2018). Finally, this statistical analysis indicates that N₂ 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 N₂ fixation activities ($81\text{--}1533 \text{ } \mu\text{mol N m}^{-2} \text{ d}^{-1}$) 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 $140 \text{ } \mu\text{mol N m}^{-2} \text{ d}^{-1}$). In contrast we did not detect any N₂ fixation activity in the central Bay of Biscay. At sites where significant N₂ fixation activity was detected, rates were similar to or up to an order of magnitude larger compared to values for the eastern tropical and subtropical North Atlantic, regions commonly believed to represent the main harbour of oceanic N₂ fixation for the eastern Atlantic. Assuming that the carbon vs nitrogen requirements by these N₂ fixers obey the Redfield stoichiometry, N₂ fixation was found able to contribute 1–3% of the euphotic layer daily PP and even up to 23–25% at the sites where N₂ fixation activity was highest. The Prymnesiophyte-symbiont *Candidatus Atelocyanobacterium thalassa* (UCYN-A) contributed most to the *nifH* sequences recovered at the two sites where N₂ fixation activity was highest, while the remaining sequences belonged exclusively to heterotrophic bacteria. We support that the unexpectedly high N₂ 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 N₂ fixation activity and phytoplankton bloom under iron-replete conditions in the studied region and similar areas, as these would require to be considered in future assessment of global N₂ fixation.

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

The Supplement related to this article is available.

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

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835

Tables

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

Province	Station	Latitude (° N)	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
ENACW _{sp}	Bel-3	46.5	0	-	0	-
	Bel-5	45.3	0	-	0	-
	Bel-7	44.6	2	0.4	1	0.4
	Geo-21	46.5	1	0.02	1	0.0
ENACW _{st}	Bel-9	42.4	1	0.1	1	0.1
	Bel-11	40.7	28	1.9	25	1.8
	Bel-13	38.8	25	1.3	23	1.2
	Geo-1	40.3	3	0.2	3	0.1
	Geo-2	40.3	3	0.1	3	0.1
	Geo-13	41.4	3	0.1	3	0.1

840

Figure legends

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 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 an orange dashed line. Black dashed and solid contour lines illustrate 500 m and 1500 m isobaths, respectively. (Schlitzer, R., Ocean Data View).

Figure 2: θ/S diagrams obtained using CTD profiles down to 1500 m depth during (a) the Belgica BG2014/14 cruise (stations Bel-3, 5, 7, 11 and 13), (b) the GEOVIDE cruise (stations Geo-1, 2, 13 and 21) and (c) both expeditions combined. Diamonds indicate the characteristics of the major water masses encountered as reported in Fiúza (1984) and García-Ibáñez et al. (2015): Eastern North Atlantic Central Waters (ENACW) of subpolar (ENACWsp) and subtropical (ENACWst) origin, Mediterranean Water (MW) and Labrador Sea Water (LSW). (Schlitzer, R., Ocean Data View).

Figure 3: Spatial distribution of Chl *a* (a, d), NH_4^+ (b, e) and $\text{NO}_3^- + \text{NO}_2^-$ (c, f) concentrations along the Belgica BG2014/14 (a to c) and GEOVIDE (d to 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).

Figure 4: Spatial distribution (\pm SD) of depth-integrated primary production (duplicates are in light and dark green with the corresponding bar values on top in $\text{mmol C m}^{-2} \text{d}^{-1}$) determined during the (a) Belgica BG2014/14 and (b) GEOVIDE cruises. Error bars represent the propagated measurement uncertainty of all parameters used to compute volumetric uptake rates. Time series of area-averaged chlorophyll *a* concentration (mg m^{-3}) for the period between December 2013 and December 2014 for the $0.5^\circ \times 0.5^\circ$ grid surrounding each sampled station during the (c) Belgica BG2014/14 and (d) GEOVIDE cruises, registered by Aqua MODIS satellite (Giovanni online satellite data system). Dashed box illustrated the sampling period for both cruises (May 2014).

Figure 5: Spatial distribution (\pm SD) of depth-integrated N_2 fixation rates (duplicates are in light and dark blue with the corresponding bar values on top in $\mu\text{mol N m}^{-2} \text{d}^{-1}$) determined during the (a) Belgica BG2014/14 and (b) GEOVIDE cruises. Error bars represent the propagated measurement uncertainty of all parameters used to compute volumetric uptake rates. Diversity of *nifH* sequences during (c) the Belgica BG2014/14 cruise, successfully recovered only at stations Bel-11 and Bel-13 (5 m), and during (d) the GEOVIDE cruise for stations Geo-2 (100 m), Geo-13 (35 m) and Geo-21. The total number of sequences recovered from each station sample is indicated on top of the bars, and the exact percentage represented by each group is shown inside the bars.

Figure 6: Euclidean distance biplot illustrating the axis loadings corresponding to the two components as obtained from the result of PCA based on Spearman rank correlation with depth-integrated rates of N_2 fixation and primary production (PP), phosphate excess (average P^* at 20 m depth surrounding each sampled site from the April to June; World Ocean Atlas 2013 climatology from 1955 to 2012) (Garcia et al., 2013), average dust deposition (dry + wet)

derived during April 2014 satellite data (Giovanni online data system, NASA Goddard Earth Sciences Data and Information Services Center) and ambient variables (temperature, salinity, and nutrient data). Coloured dots represent
885 the projection of each station corresponding to their biogeochemical characteristics. Axis 1 is found highly inversely correlated to PP, Chl *a*, NH_4^+ and $\text{NO}_3^- + \text{NO}_2^-$ concentrations, while highly positively related to temperature and N_2 fixation rates, with axis loading of -0.812 , -0.768 , -0.936 , -0.783 , 0.942 and 0.506 , respectively (see table S5). Axis 2 is highly positively correlated to climatological P^* , salinity and N_2 fixation rates (axis loading of 0.584 , 0.943 and 0.602 , respectively).

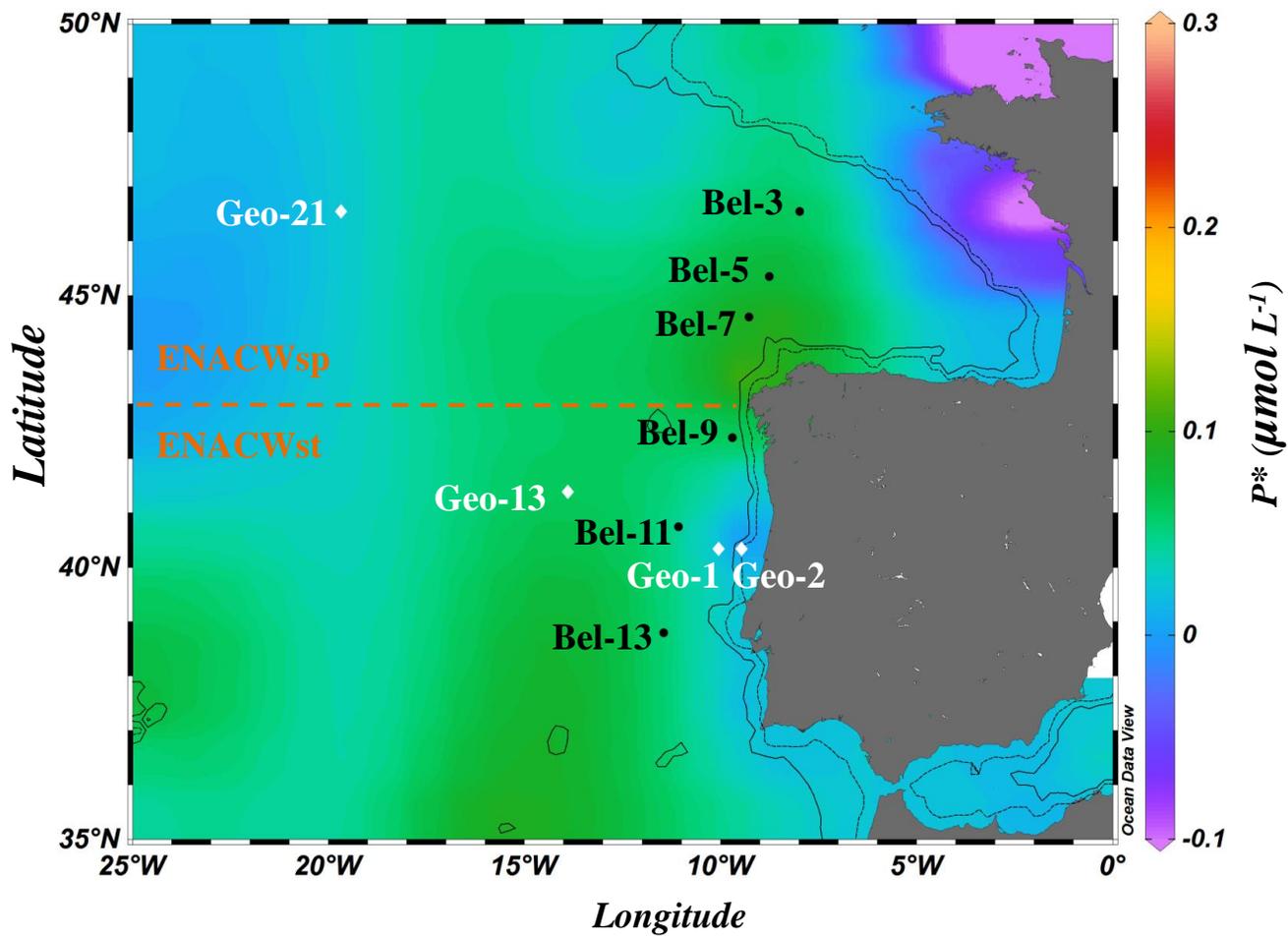


Figure 1

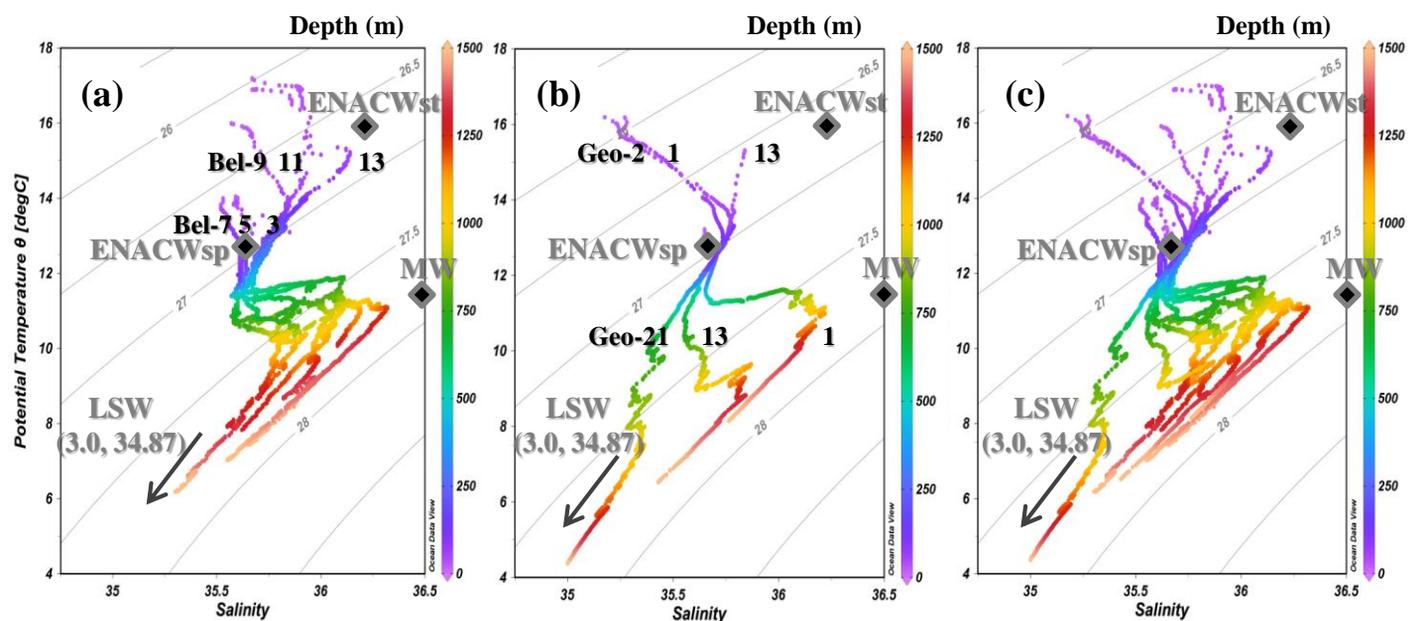
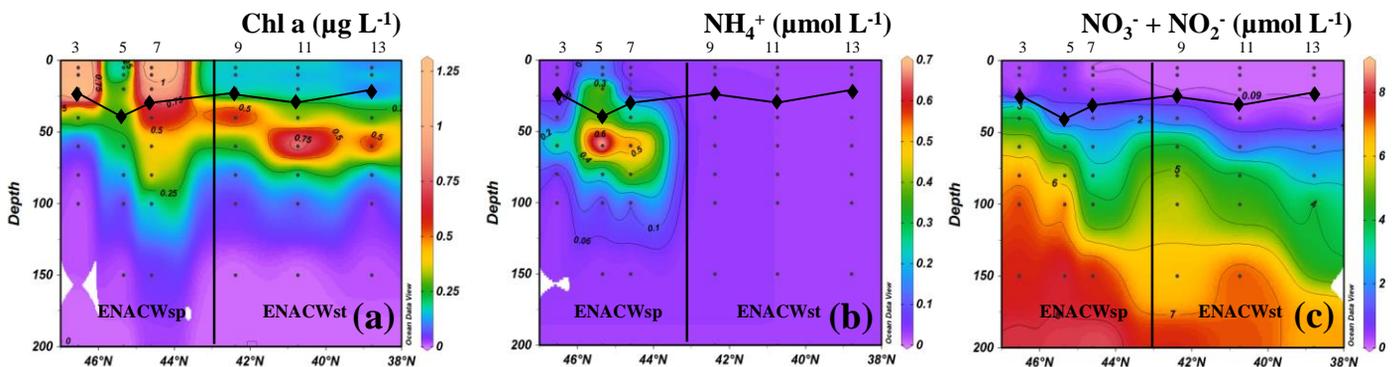


Figure 2

Belgica BG2014/14



GEOVIDE

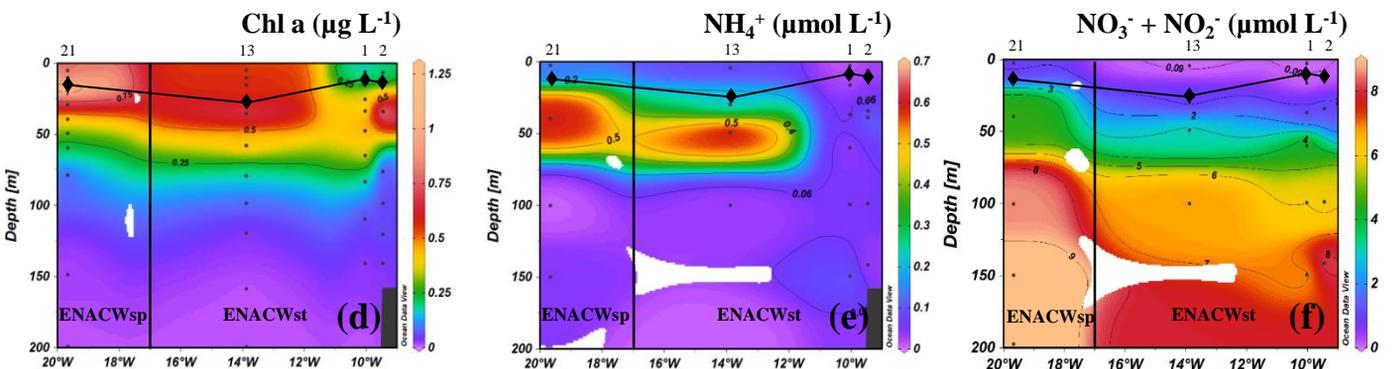


Figure 3

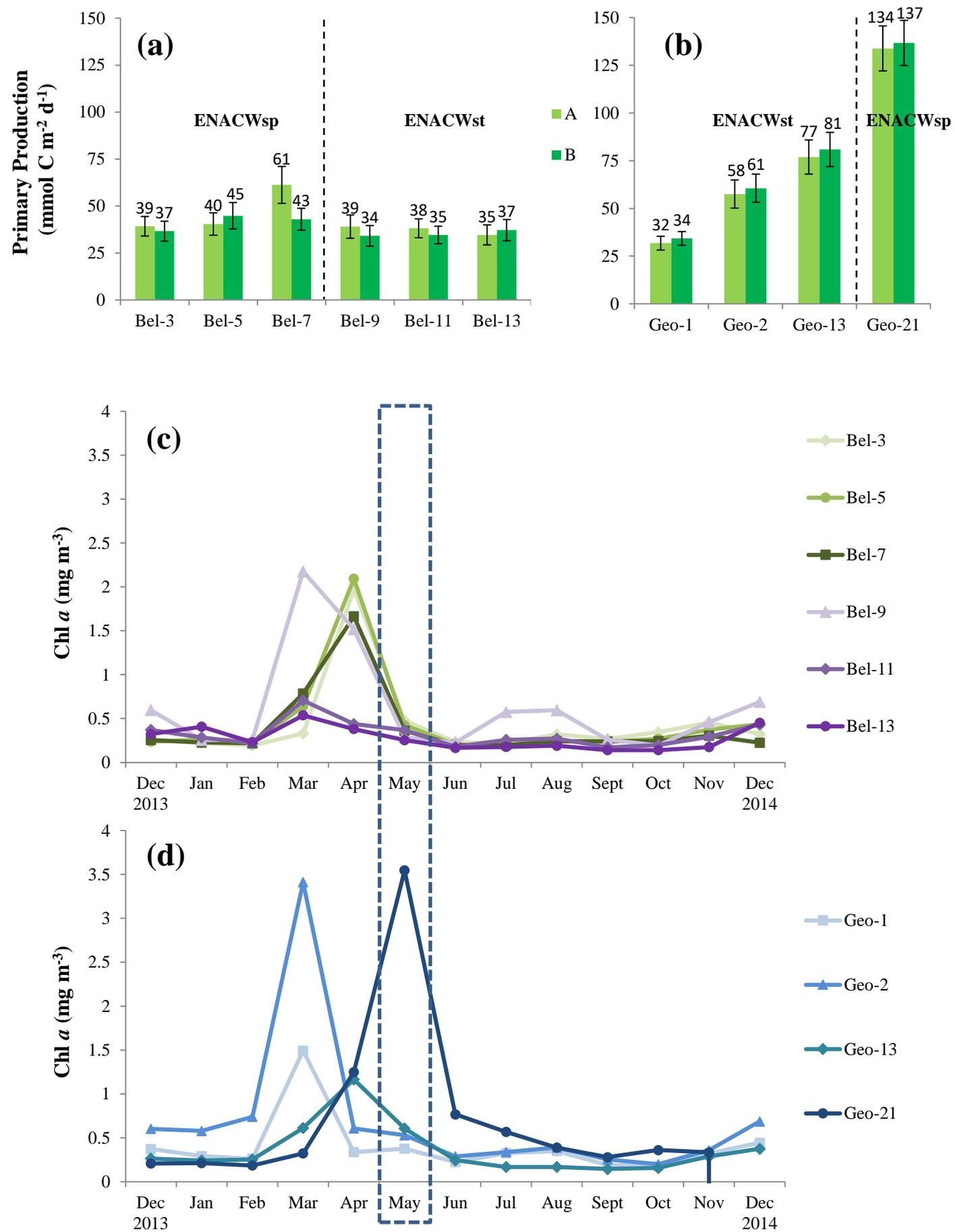


Figure 4

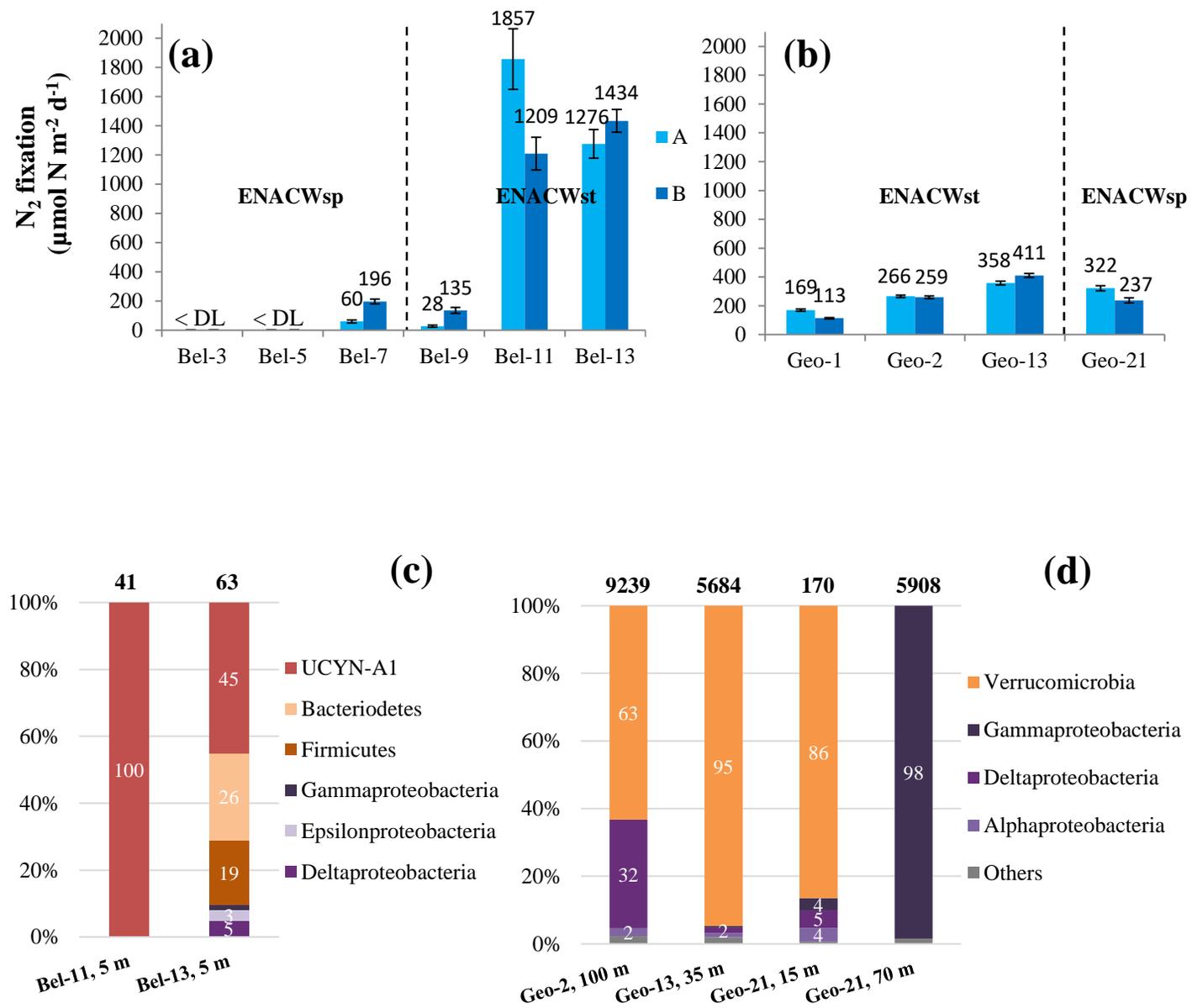


Figure 5

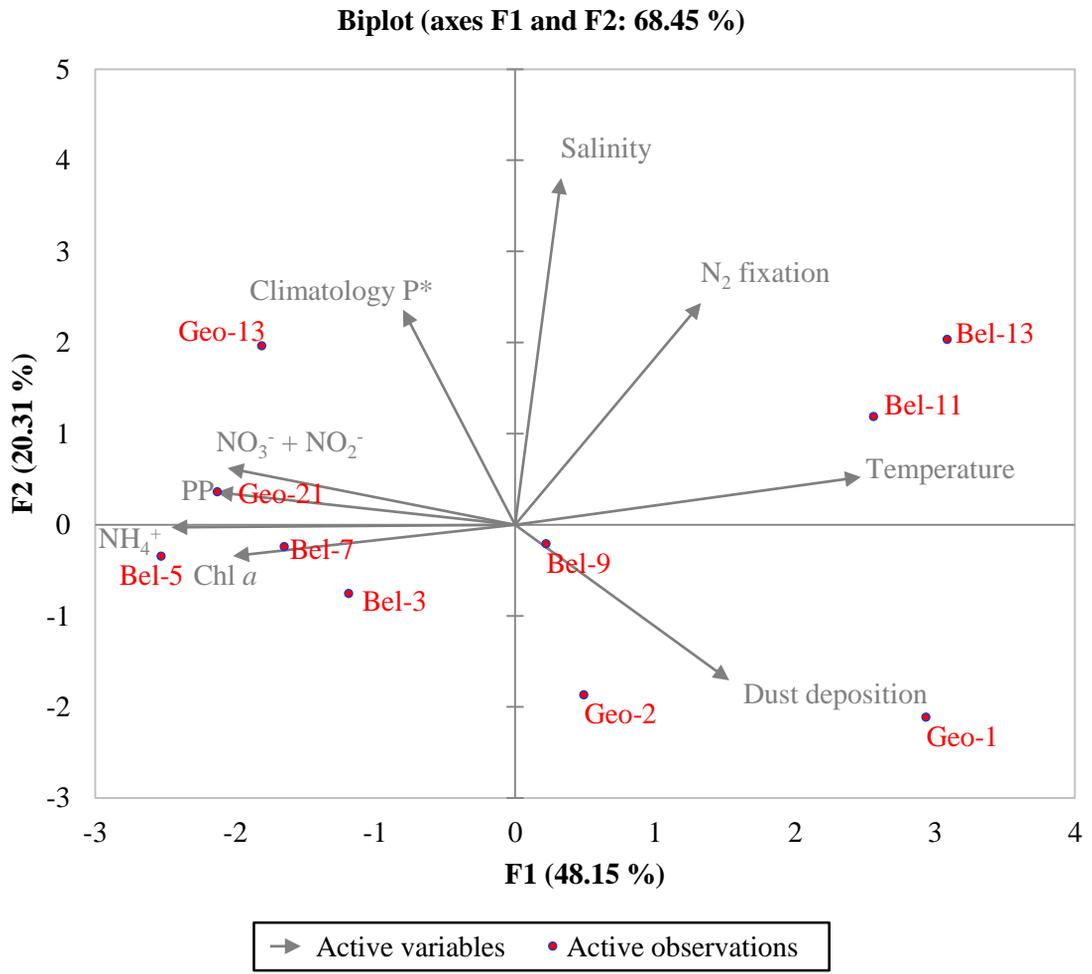


Figure 6

Supplementary Material

Evidence of high N₂ fixation rates in productive waters of the temperate Northeast Atlantic

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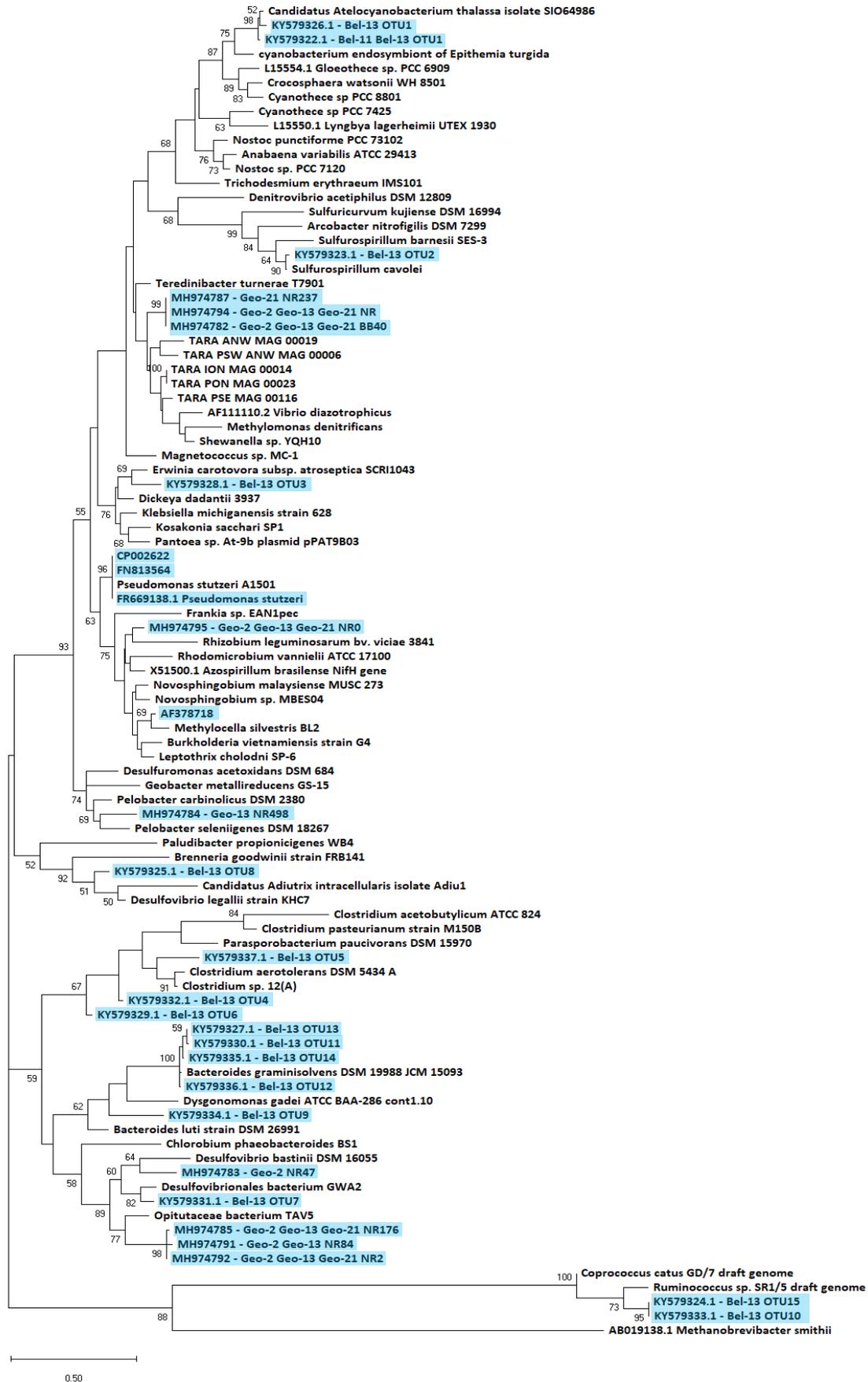
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Contents of this file

Figures S1 to S3

Tables S1 to S4

Supplementary Material



Supplementary Figure S1. 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 Neighbor-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 (5 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 indicated with a blue rectangle. 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.

Supplementary Material

Supplementary Table S1. Summary of the dataset used to run the principal component analyses relating the volumetric rates of N₂ fixation and primary production to environmental variables

Station	Date	Depth [m]	Lat. [°N]	Long. [°E]	N ₂ fixation [nmol N L ⁻¹ d ⁻¹]	Primary production [μmol C m ⁻³ d ⁻¹]	Temperature [°C]	Salinity [psu]	NH ₄ ⁺ [μM]	NO ₃ ⁻ +NO ₂ ⁻ [μM]	P* [μM]	Chl <i>a</i> [μg L ⁻¹]
Bel-3	24-May-2014	5	46.5	-8.0	< DL	1180.8	13.86	35.58	< DL	0.27	0.04	1.42
Bel-3	24-May-2014	10	46.5	-8.0	< DL	776.1	13.87	35.58	< DL	0.36	0.04	1.25
Bel-3	24-May-2014	25	46.5	-8.0	< DL	995.9	13.78	35.58	< DL	1.14	0.06	1.10
Bel-3	24-May-2014	50	46.5	-8.0	< DL	109.7	12.44	35.62	0.35	4.62	-0.03	0.16
Bel-5	25-May-2014	5	45.3	-8.8	< DL	429.1	13.91	35.59	0.19	0.66	0.02	0.16
Bel-5	25-May-2014	30	45.3	-8.8	< DL	360.6	13.91	35.59	0.59	0.91	0.00	0.12
Bel-5	25-May-2014	70	45.3	-8.8	< DL	433.9	13.24	35.61	0.59	4.71	-0.04	0.21
Bel-5	25-May-2014	130	45.3	-8.8	< DL	9.7	11.92	35.62	< DL	7.74	-0.14	0.00
Bel-7	26-May-2014	5	44.6	-9.3	1.1	849.1	13.94	35.51	< DL	< DL	0.05	1.19
Bel-7	26-May-2014	16	44.6	-9.3	1.0	707.3	13.94	35.52	< DL	0.24	0.05	1.14
Bel-7	26-May-2014	30	44.6	-9.3	2.0	1018.3	13.86	35.52	0.09	1.01	0.00	0.98
Bel-7	26-May-2014	80	44.6	-9.3	1.6	70.2	13.32	35.55	0.34	2.84	0.02	0.16
Geo-21	31-May-2014	10	46.5	-19.7	8.2	2824.8	14.57	35.68	0.18	1.52		0.88
Geo-21	31-May-2014	18	46.5	-19.7	< DL	3443.7	13.70	35.69	0.39	2.21		1.21
Geo-21	31-May-2014	25	46.5	-19.7	4.9	3500.1	13.47	35.68	0.50	2.82		0.73
Geo-21	31-May-2014	40	46.5	-19.7	1.4	1155.1	12.84	35.65	0.68	4.13		0.39
Geo-21	31-May-2014	60	46.5	-19.7	2.0	393.8	12.84	35.69	0.41	5.32		0.19
Geo-21	31-May-2014	91	46.5	-19.7	2.3	73.8	12.51	35.70	< DL	7.19		

Supplementary Table S1 continued.

Station	Date	Depth [m]	Lat. [°N]	Long. [°E]	N ₂ fixation [nmol N L ⁻¹ d ⁻¹]	Primary production [μmol C m ⁻³ d ⁻¹]	Temperature [°C]	Salinity [psu]	NH ₄ ⁺ [μM]	NO ₃ ⁻ +NO ₂ ⁻ [μM]	P* [μM]	Chl <i>a</i> [μg L ⁻¹]
Bel-9	27-May-2014	5	42.4	-9.7	3.9	335.9	16.04	35.55	< DL	< DL	0.05	0.16
Bel-9	27-May-2014	25	42.4	-9.7	0.7	207.3	15.96	35.56	< DL	0.38	0.04	0.18
Bel-9	27-May-2014	45	42.4	-9.7	0.9	571.2	14.33	35.83	< DL	2.07	-0.01	0.55
Bel-9	27-May-2014	120	42.4	-9.7	< DL	6.9	13.24	35.78	< DL	5.99	-0.08	0.01
Bel-11	28-May-2014	5	40.7	-11.1	65.4	565.3	16.95	35.56	< DL	< DL	0.05	0.15
Bel-11	28-May-2014	35	40.7	-11.1	7.0	630.7	15.18	35.84	< DL	< DL	0.05	0.28
Bel-11	28-May-2014	45	40.7	-11.1	< DL	292.3	15.26	35.83	< DL	0.61	0.02	0.12
Bel-11	28-May-2014	80	40.7	-11.1	4.9	334.6	14.01	35.90	< DL	4.35	-0.08	0.23
Bel-13	29-May-2014	5	38.8	-11.4	45.0	599.4	17.23	35.68	< DL	< DL	0.05	0.22
Bel-13	29-May-2014	30	38.8	-11.4	10.5	323.2	16.46	35.89	< DL	< DL	0.09	0.09
Bel-13	29-May-2014	45	38.8	-11.4	12.6	692.0	15.38	35.97	< DL	0.52	0.12	0.20
Bel-13	29-May-2014	80	38.8	-11.4	2.4	92.6	14.84	36.10	< DL	2.39	0.07	0.08
Geo-1	19-May-2014	6	40.3	-10.0	4.8	621.8	16.70	35.11	0.33	< DL		0.16
Geo-1	19-May-2014	11	40.3	-10.0	7.1	696.9	16.53	35.18	< DL	< DL		0.19
Geo-1	19-May-2014	16	40.3	-10.0	4.8	667.8	16.09	35.26	< DL	< DL		
Geo-1	19-May-2014	25	40.3	-10.0	2.5	579.1	15.33	35.36	< DL	0.42		0.33
Geo-1	19-May-2014	34	40.3	-10.0	1.2	842.3	15.14	35.46	< DL	0.76		0.35
Geo-1	19-May-2014	48	40.3	-10.0	1.1	676.9	14.35	35.62	< DL	2.44		0.46
Geo-2	20-May-2014	11	40.3	-9.5	4.7	474.3	16.82	34.99	< DL	< DL		0.21
Geo-2	20-May-2014	31	40.3	-9.5	2.8	1170.5	14.67	35.54	< DL	0.93		0.47
Geo-2	20-May-2014	39	40.3	-9.5	2.7	1149.5	13.97	35.70	0.09	1.38		0.98
Geo-2	20-May-2014	85	40.3	-9.5	2.3	72.5	13.32	35.77	< DL	4.39		0.02

Supplementary Table S1 final.

Station	Date	Depth [m]	Lat. [°N]	Long. [°E]	N ₂ fixation [nmol N L ⁻¹ d ⁻¹]	Primary production [μmol C m ⁻³ d ⁻¹]	Temperature [°C]	Salinity [psu]	NH ₄ ⁺ [μM]	NO ₃ ⁻ +NO ₂ ⁻ [μM]	P* [μM]	Chl <i>a</i> [μg L ⁻¹]
Geo-13	24-May-2014	15	41.4	-13.9	5.5	1670.7	15.47	35.84	0.21	0.34		0.56
Geo-13	24-May-2014	30	41.4	-13.9	1.0	403.0	14.73	35.81	0.34	0.68		0.68
Geo-13	24-May-2014	43	41.4	-13.9	2.4	910.9	13.66	35.77	0.73	2.21		
Geo-13	24-May-2014	58	41.4	-13.9	2.2	790.5	13.36	35.76	0.68	3.47		0.45
Geo-13	24-May-2014	75	41.4	-13.9	3.9	338.1	13.14	35.76	0.07	4.54		0.17
Geo-13	24-May-2014	116	41.4	-13.9	3.1	22.8	12.91	35.75	< DL	6.27		0.01

Supplementary Material

Supplementary Table S2. Summary of the dataset used to run the principal component analyses relating the depth-integrated rates of N₂ fixation and primary production to environmental variables.

Station	MLD	Lat. [°N]	Long. [°E]	N ₂ fixation [μmol N m ⁻² d ⁻¹]	Primary Production [mmol C m ⁻² d ⁻¹]	Euphotic layer averaged Temperature [°C]	Euphotic layer averaged Salinity [psu]	Euphotic layer integrated Chl <i>a</i> [mg m ⁻²]	Euphotic layer integrated NH ₄ ⁺ [μmol m ⁻²]	Euphotic layer integrated NO ₃ ⁻ +NO ₂ ⁻ [μmol m ⁻²]	Climatology P* at 20 m depth [μmol m ⁻²]	Satellite- based dust deposition [Apr. 2014] [μg m ⁻² d ⁻¹]	Satellite- based dust deposition [May 2014] [μg m ⁻² d ⁻¹]
Bel-3	27	46.5	-8.0	0	37.9	13.5	35.6	47.1	6.7	86.2	0.06	1263	2539
Bel-5	41	45.3	-8.8	0	42.6	13.2	35.6	17.1	54.0	509.0	0.06	1647	1914
Bel-7	33	44.6	-9.3	128	52.1	13.8	35.5	62.3	12.9	107.1	0.07	2147	1443
Geo-21	17	46.5	-19.7	279	135.3	13.3	35.7	38.1	34.2	452.5	0.04	2147	1443
Bel-9	26	42.4	-9.7	81	36.6	14.9	35.7	32.6	7.7	332.2	0.06	3650	1088
Bel-11	33	40.7	-11.1	1533	36.4	15.3	35.8	15.2	5.1	93.5	0.05	2799	618
Bel-13	25	38.8	-11.4	1355	35.9	16.0	35.9	12.1	5.1	58.3	0.06	2147	618
Geo-1	12	40.3	-10.0	141	33.1	15.7	35.3	14.2	5.3	68.4	0.03	3650	618
Geo-2	14	40.3	-9.5	262	59.1	14.7	35.5	37.8	6.0	219.4	0.01	4758	820
Geo-13	29	41.4	-13.9	384	78.9	13.9	35.8	42.4	34.0	274.2	0.07	1647	820

Supplementary Material

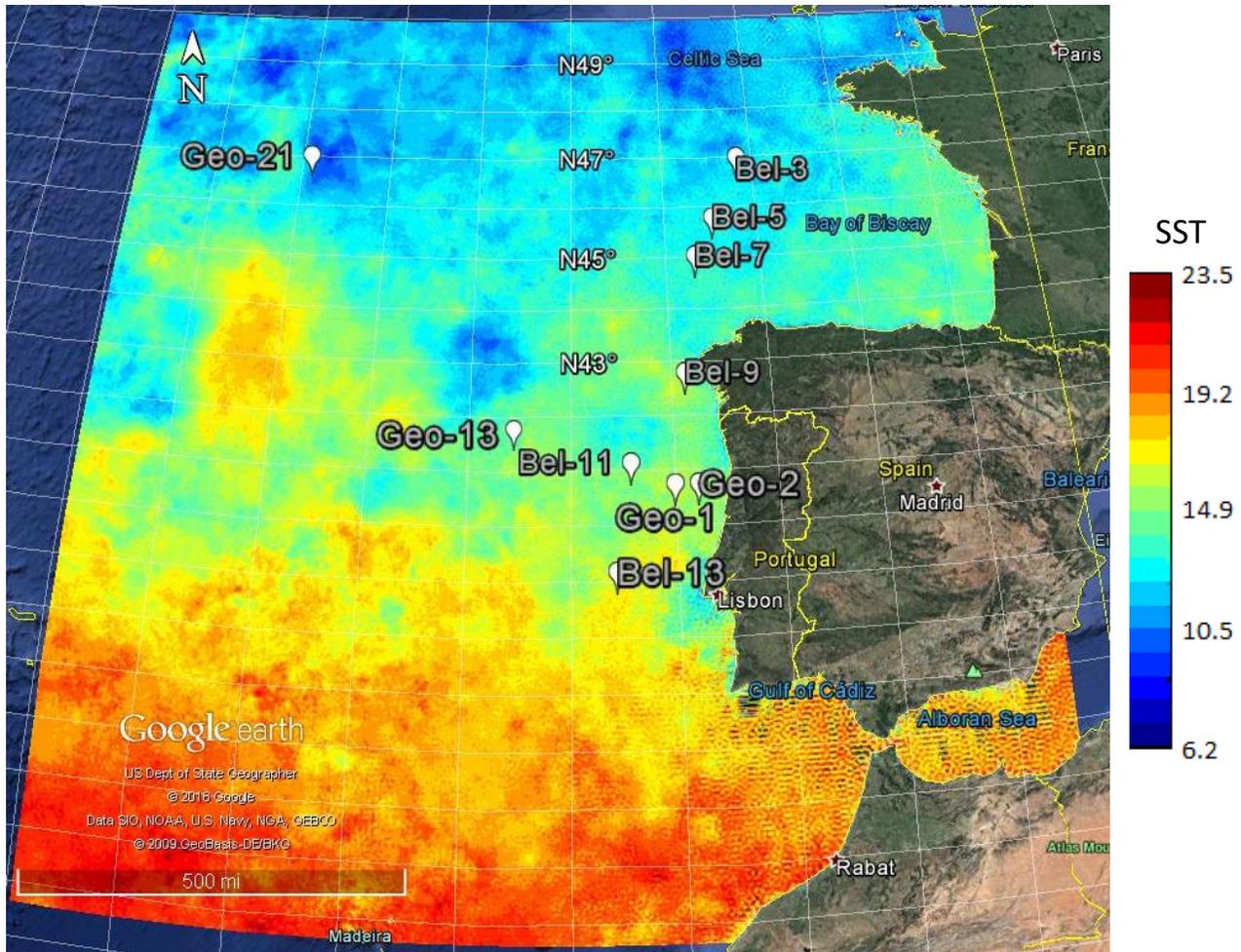
Supplementary Table S3. Spearman correlation matrix opposing depth-integrated rates of N₂ fixation and primary production (PP), from BG2014/14 and GEOVIDE cruises together, to euphotic layer averaged or integrated environmental variables. The correlation factor (r) and its significance given by the p-value (p) at p < 0.001, p < 0.01 and p < 0.05 presented with ***, ** and *, respectively, and the number of observations (n) are shown for each combination tested. Also, dFe correlations were made only for GEOVIDE sampling stations.

		Average salinity	Integrated [NH ₄ ⁺] [μmol m ⁻²]	Integrated [NO ₃ ⁻ + NO ₂ ⁻] [μmol m ⁻²]	Average in situ P* [μM]	Surface climatology P* [μM]	Integrated dFe [nmol m ⁻²]	Satellite-based dust deposition April 2014 [μg m ⁻² s ⁻¹]	Satellite-based dust deposition May 2014 [μg m ⁻² s ⁻¹]	PP [mmol C m ⁻² d ⁻¹]	N ₂ fixation [μmol N m ⁻² d ⁻¹]
Average temperature [°C]	r	0.188	-0.869***	-0.721*	0.0788	-0.0732	0.8	0.587	-0.901***	-0.721*	0.553
	<i>p</i>	0.583	0.0000002	0.0157	0.811	0.811	0.333	0.0665	0.0000002	0.0157	0.0892
	n	10	10	10	10	10	4	10	10	10	10
Average salinity			-0.109	0.152	0.00606	0.366	-0.6	-0.29	-0.265	-0.0182	0.62*
			0.733	0.656	0.973	0.275	0.417	0.404	0.446	0.946	0.048
			10	10	10	10	4	10	10	10	10
Integrated [NH ₄ ⁺] [μmol m ⁻²]				0.857***	-0.0304	0.26	-0.8	-0.471	0.737*	0.729*	-0.482
				0.0000002	0.919	0.446	0.333	0.16	0.0131	0.0131	0.148
				10	10	10	4	10	10	10	10
Integrated [NO ₃ ⁻ + NO ₂ ⁻] [μmol m ⁻²]					-0.0182	0.116	-0.8	-0.228	0.512	0.758**	-0.195
					0.946	0.733	0.333	0.512	0.116	0.0087	0.559
					10	10	4	10	10	10	10
Average in situ P* [μM]						-0.274	-0.4	0.161	-0.253	0.479	0.511
						0.425	0.75	0.631	0.468	0.148	0.116
						10	4	10	10	10	10
Surface climatology P* [μM]							-0.8	-0.491	0.205	0.0732	-0.0856
							0.333	0.137	0.559	0.811	0.785
							4	10	10	10	10
Integrated dFe [nmol m ⁻²]								0.8	-0.632	-0.8	-0.6
								0.333	0.333	0.333	0.417
								4	4	4	4
Satellite-based dust deposition April 2014 [μg m ⁻² s ⁻¹]									-0.588	-0.265	0.257
									0.0665	0.446	0.446
									10	10	10
Satellite-based dust deposition May 2014 [μg m ⁻² s ⁻¹]										0.512	-0.774**
										0.116	0.00686
										10	10
PP [mmol C m ⁻² d ⁻¹]											-0.0365
											0.892
											10

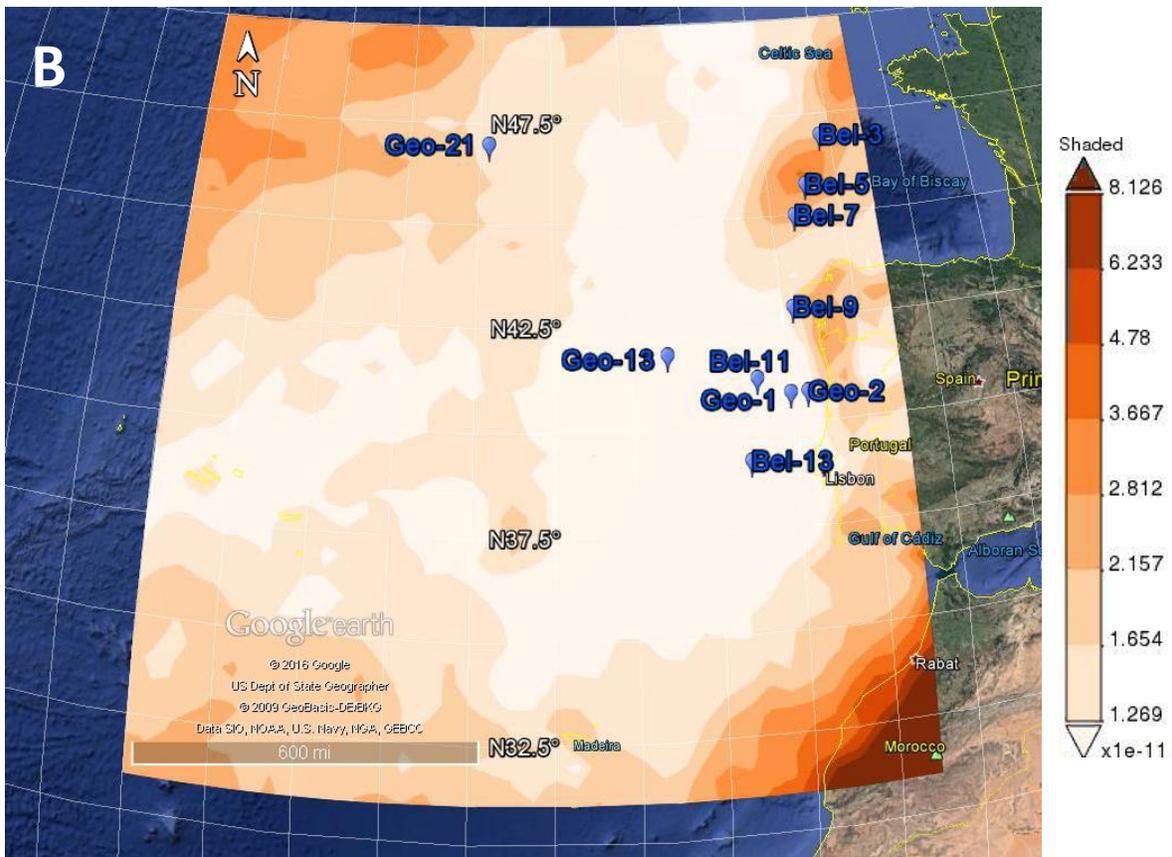
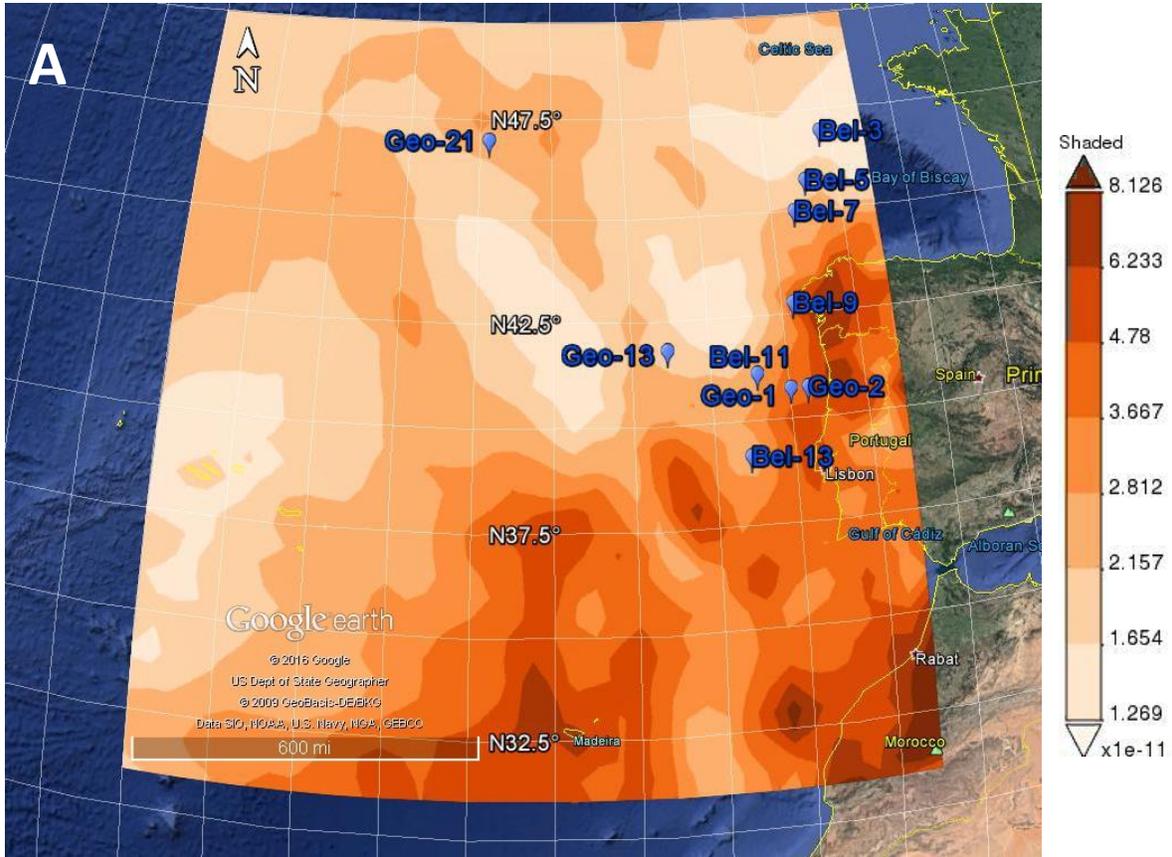
Supplementary Material

Supplementary Table S4. Spearman correlation matrix opposing volumetric rates of N₂ fixation and primary production (PP) to depth-specific environmental variables for the combined Belgica 2014/14 and GEOVIDE cruises. The correlation factor (*r*) and its significance given by the *p*-value (*p*) at $p < 0.001$, $p < 0.01$ and $p < 0.05$ presented with ***, ** and *, respectively, and the number of observations (*n*) are shown for each combination tested. Note that when nutrient concentrations were < DL we used the DL value to run the correlation test. Also, P* correlations were only made for the Belgica 2014/14 studied sites and dFe correlations only for GEOVIDE sampling stations.

		Salinity	[NH ₄ ⁺] [μM]	[NO ₃ ⁻ + NO ₂ ⁻] [μM]	In situ P* [μM]	DFe [nM] (Tonnard et al., 2018)	Chl <i>a</i> [μg L ⁻¹]	PP [μmol C m ⁻³ d ⁻¹]	N ₂ fixation [nmol N L ⁻¹ d ⁻¹]
Temperature [°C]	r	-0.191	-0.418**	-0.854***	0.628**	0.291	0.00461	0.158	0.54***
	<i>p</i>	0.202	0.00398	0.0000002	0.00104	0.267	0.976	0.292	0.00012
	<i>n</i>	46	46	46	24	16	43	46	46
Salinity			0.0922	0.39**	0.0288	0.0883	-0.195	-0.214	0.106
			0.54	0.00765	0.892	0.738	0.208	0.152	0.482
			46	46	24	16	43	46	46
[NH ₄ ⁺] [μM]				0.344*	-0.441*	-0.462	0.128	0.255	-0.154
				0.0194	0.031	0.0695	0.411	0.0869	0.307
				46	24	16	43	46	46
[NO ₃ ⁻ + NO ₂ ⁻] [μM]					-0.716***	-0.253	-0.216	-0.314*	-0.362*
					0.0000325	0.336	0.162	0.0338	0.0136
					24	16	43	46	46
In situ P* [μM]						undefined	0.149	0.369	0.557**
						<i>undefined</i>	0.481	0.0746	0.00481
						0	24	24	24
DFe [nM] (Tonnard et al., 2018)							-0.57	-0.416	0.175
							0.0322	0.104	0.505
							14	16	16
Chl <i>a</i> [μg L ⁻¹]								0.85***	-0.0874
								0.0000002	0.575
								43	43
PP [μmol C m ⁻³ d ⁻¹]									0.143
									0.342
									46



Supplementary Figure S2. High resolution trihourly averaged sea surface temperature (SST) on May 29th 2014 (sampling date of station Bel-13, 10 days following the sampling of station Geo-1), derived from the European Sea (sea surface subskin temperature) of Copernicus Marine Environment Monitoring Service (CMEMS, European Commission). White markers indicate the location of the stations sampled during our study (image provided by Google Earth Pro).



Supplementary Material

Supplementary Figure S3. April (A) and May (B) 2014 monthly averaged dry + wet dust deposition ($\text{kg m}^{-2} \text{s}^{-1}$) derived from Giovanni online satellite data system (NASA Goddard Earth Sciences Data and Information Services Center). White markers indicate the location of the stations sampled during our study (image provided by Google Earth Pro).

Supplementary Table S5. Principal component matrix illustrating the components (or axis) loadings, in other words the correlation of each variable to a determined axis as obtained with the XLSTAT software. The percentage of variability of the system explained by each of the two axes is indicated, for a total explained variance of 68%.

	Axis 1	Axis 2
% Variability explained:	48%	20%
Variables		
Euphotic layer integrated primary production	-0.812	0.088
Euphotic layer averaged temperature	0.942	0.130
Euphotic layer integrated Chl <i>a</i>	-0.768	-0.085
Euphotic layer integrated $[\text{NH}_4^+]$	-0.936	-0.007
Euphotic layer integrated $[\text{NO}_3^- + \text{NO}_2^-]$	-0.783	0.154
Climatology surface P* (20 m)	-0.305	0.584
Euphotic layer averaged salinity	0.125	0.943
Satellite dry + wet dust deposition (April 2014)	0.583	-0.423
Euphotic layer integrated N₂ fixation	0.506	0.602

Fonseca-Batista et al. manuscript revision

Biogeosciences Editor's comments

This study was previously submitted to Frontiers in Microbiology (FiTM) and was rejected after critical reviews by two referees. Both reviewers are positive about the publication of this study in FiTM, although the comments are pretty critical, particularly reviewer#2. So the reason of rejection appears to be that the authors are not able to adequately address the concerns raised by the reviewers. Both reviewers are nice, despite of being somehow critical.

I have carefully read the comments and replies to the comments, and reviewed the ms myself. The manuscript could be published only after major revision suggested by both reviewers.

We would like to thank the Editor for taking the time to review this manuscript himself. We have carefully considered the comments and have revised the manuscript accordingly. We addressed all the comments in a point-by-point manner.

The key concerns are from reviewer#2, and I fully side with him/her. “Overall the text is too speculative- the discussion, although well written, is much too long with 10 pages, and especially the last 5 pages are full of speculation and partly wander too far from discussing actually measured data. It is impressive from how many different angles the authors try to shed light on their data, but when large parts of text end in hypothetical conclusions, assumptions, possibilities, it somehow makes the story implode back onto its core data, and it makes reading the long discussion a bit frustrating. I feel that the few interesting points could be summarized and put into necessary context in a much shorter way. I wonder whether the manuscript could be partly rewritten in order to focus more on the rates and less on the weak *nifH* data”.

We understand the Editor's concerns related to the comments made by the Reviewer #2 during processing by Frontiers in Marine Science (FiMS). However, we would like to point out that significant modifications had been made in the manuscript prior to the submission to Biogeosciences, and further adjustments were now implemented to focus the text on our data and less on hypothetical environmental drivers:

- (1) the discussion section is now 5 pages (compared to the initial 10 pages mentioned above). The most criticized section related to the environmental drivers of N₂ fixation now only has 2 pages (instead of 5).**
- (2) the main hypotheses put forward to explain the distribution and magnitude of N₂ fixation rates observed in this study were better summarized, made more concise and backed up with additional data from the GEOVIDE cruise that were not included in the last version submitted to FiMS. These supporting data include particulate organic carbon concentration in the surface waters and export efficiency (reflecting the potential for shallow recycling) (Lemaitre et al., under review in this same BG special issue for the GEOVIDE project), dissolved iron concentrations (Tonnard et al., under review in the GEOVIDE special issue), and *nifH* data on the diazotroph community in the Iberian Basin and West European Basin.**

The major revisions should be made including:

- (1) Stay focused on what you want to say. The authors need to focus on N₂ fixation and productive water in this study, and get all relevant placed in the text first.
 (2) The figures and tables need to be re-structured.

In summary, the major re-structuring is required as following.

- (1) Table 1 might be useful, but table 2 can be placed in the supplementary text.

A major restructuring of the figures and tables was implemented as suggested by the Editor:

- **both pair of Figures (Fig. 4-5 and Fig. 7-8) have now been merged.**
- **Fig. 6 was removed from the article, the phytoplankton pigments data is now only cited as part of another manuscript in preparation, as follows: Tonnard et al., in preparation.**
- **Table 2 was transferred to supplementary material.**

Based on the BG Editor's latest comments, texts were adapted, particularly in the abstract, introduction, methods and results (to add DNA analysis and results from GEOVIDE cruise) and finally in the discussion to focus more on N₂ fixation rates, their significance at the regional and basin scales, and their contribution to primary production.

The key points includes:

- (1) N₂ fixation rate should be the figure 2, and primary production figure 3. Other figures are then arranged in an order to explain the N₂ fixation and primary production
 (2) In addition, Fig.7 and Fig.8 can be merged. Figure 4 and Figure 5 might be merged as well.
 (3) Figure 6 may not be appropriate in this study because it will be published somewhere else.

Figures and Tables were re-arranged as suggested (see above). Concerning the order of figure presentation, we preferred discussing the sections on θ/S diagrams and nutrients and chlorophyll prior to the rates measurements. We believe that introducing the physico-chemical features of the studied region not only allows one to easily grasp the environmental context of the study, but also to highlight specific regions with characteristic traits of interest which will not need to be detailed when discussing N₂ fixation rates and Primary Production.

Other key concerns for example are the following

- (4) In the abstract, the table 1 was not mentioned which does not echo with the title "productive water"

We agree, the abstract was adapted to focus more on N₂ fixation rates, their contribution to primary production, but also to reduce the attention brought to specific hypothetical environmental drivers.

- (5) Line 24 delete the phrase of (38.8–46.5° N; 8.0–19.7° W) in May 2014

The latitudinal and longitudinal range of the studied region was deleted, we just kept "May 2014" to indicate the time of sampling which is relevant based on the importance of seasonality in this work, a condition now better highlighted in the abstract (line 23)

- (6) L26-28. This statement is too descriptive

The sentence was modified, to bring the reader's attention to the facts that (1) such high N₂ fixation rates have not yet been observed earlier along the whole eastern Atlantic boundary, (2) elevated rates like these have only been reported at the western Atlantic boundary in very specific nutrient-rich environments such as: coastal, shelf and mesohaline waters. The sentence now reads as follows (lines 29-32):

“In the Atlantic Ocean, N_2 fixation rates exceeding $1000 \mu\text{mol N m}^{-2} \text{d}^{-1}$ have previously only been reported in the temperate and tropical western North Atlantic waters having coastal, shelf or mesohaline characteristics, as opposed to the mostly open ocean conditions studied here.”

(7) L28. Delete the phrase In agreement with previous studies.

The sentence was deleted and the text was adapted as follows (lines 32-35 and 37-40):

*“At the two sites where N_2 fixation activity was the highest; *nifH* sequences assigned to the prymnesiophyte-symbiont *Candidatus Atelocyanobacterium thalassa* (UCYN-A) dominated the *nifH* sequence pool recovered from DNA samples, while the remaining sequences, as for all the ones recovered from the other sites, belonged exclusively to non-cyanobacterial phylotypes.”*

...

*“Earlier studies in the Iberian region were conducted largely outside the bloom period, unlike the present work which was carried out in spring, yet in all cases the assessment of *nifH* gene diversity, suggests a predominance of UCYN-A and non-cyanobacterial diazotrophs.”*

(8) L35-38. Please provide solid evidence, instead of proposing something.

The hypotheses are now introduced along with supporting information presented in the corresponding discussion section, regarding the abundance of particulate organic carbon in surface waters (based on Lemaitre et al., under review in the same GEOVIDE BG Special Issue), and on the in situ excess phosphorus data. The text was adapted as follows (lines 35-43):

*“Previous studies in the Iberian Basin have systematically reported lower N_2 fixation rates (from < 0.1 to $140 \mu\text{mol N m}^{-2} \text{d}^{-1}$), as compared to those found in the present study, and this regardless of whether the bubble-addition method or the dissolution method were applied. Earlier studies in the Iberian region were conducted largely outside the bloom period, unlike the present work which was carried out in spring, yet in all cases the assessment of *nifH* gene diversity, suggests a predominance of UCYN-A and non-cyanobacterial diazotrophs. We support that the unexpectedly high N_2 fixation activities recorded at the time of our study were promoted by the availability of phytoplankton-derived organic matter produced during the spring bloom, as evidenced by the significant surface particulate organic carbon concentrations, and by the presence of excess phosphorus signature in surface waters, particularly at the sites with extreme activities.”*

(9) L39-40. These data appear to be placed in the supplementary materials. Therefore, it is no appropriate to be discussed in the abstract.

We agree, that sentence was deleted.

Biogeosciences Reviewer #1's comments

Review of “Evidence of high N₂ fixation rates in productive waters of the temperate Northeast Atlantic” by Fonseca-Batista et al.

The editor should understand that neither N fixation, N-fixing gene abundances, nor bioecology are within my realm of expertise. Despite that, I have sailed on many research cruises with N-fixation scientists, and even collected nifH samples for them on my own cruises. So I should be classified as a knowledgeable non-expert: I appreciate the research area but cannot analyze or critique details.

From that perspective, the paper is a fine contribution, calling attention to high N fixation rates and relatively high N-fixing gene copies in the temperate eastern Atlantic in the springtime following the spring bloom. This has not been observed before, partially due to methodological issues and partly due to the lack of observations in this season. Obviously it may change our view of how to assess global N fixation rates and how to model them.

We would like to thank the Reviewer #1 for reviewing this manuscript even though the topic it covers are not exactly within his/her area of expertise. We have considered the comments, and we appreciate the Reviewer's recognition of our manuscript.

I could only find a couple of small issues with the text (noted below), otherwise I believe it can be published with only minor revisions, hopefully with more guidance from a reviewer expert in this field.

(1) p. 1, line 31: For the sake of clarity, I recommend modifying the text to “At the sites where N₂ fixation activity was the highest, we recovered sequences affiliated to UCYN-A1 (obligate symbiont of eukaryotic prymnesiophyte algae).”

The sentence was modified and now reads as follows (lines 32-35):

“At the two sites where N₂ fixation activity was the highest; nifH sequences assigned to the prymnesiophyte-symbiont Candidatus Atelocyanobacterium thalassa (UCYN-A) dominated the nifH sequence pool recovered from DNA samples, while the remaining sequences, as for all the ones recovered from the other sites, belonged exclusively to non-cyanobacterial phylotypes.”

(2) p. 5, line 161: the Ambar and Fiúza, 1994 paper is not in the list of references.

The missing reference was added to the reference list:

“Ambar, I., Fiúza, A.F.G. (1994). Some features of the Portugal Current System: a poleward slope undercurrent, an upwelling-related summer southward flow and an autumn-winter poleward coastal surface current. In: Proceedings of the Second International Conference on Air-Sea Interaction and on Meteorology and Oceanography of the Coastal Zone. Katsaros, K.B., Fiúza, A.F.G., Ambar, I., American Meteorological Society, pp. 286-287.”