Dear Mr. Debany Fonseca-Batista,

I have again reviewed your ms on my own and unfortunately it cannot be published at its present form.

As you may still remember the key problem of your manuscript is the lack of logics, although the data have potential of publication.

PLEASE STAY FOCUSE on what you wanna convey as stated in the title: “Evidence of high N2 fixation rates in productive waters of the temperate Northeast Atlantic”. Based on this title, the authors would expect to see the direct evidence for (1) N2 fixation rate; (2) productive water, i.e., 13C inorganic uptake; AND the reason behind it including (3) water nutrient and pigement; (4) taxonomic identity of N fixer; (4) environmental force determining the N2 fixation and inorganic 13C uptake.

Therefore, in the abstract, it is important to show your key findings in a logic way. i.e., you have observed N2 fixation at 8 stations out of 10 sampled; you also need to show the flux and importance of inorganic 13C uptake. Then you might explain what environmental variables play a key role in regulating N2 fixation and 13CO3 uptake, in addition to the taxonomic identity of N2 fixation.
Meanwhile, you could explain why the extraordinarily high rate of N2 fixation occurred.

Dear Editor, we thank you for the time and work you have been putting in order to improve the reading of our manuscript. In order to take into account the above general comments you have brought to our attention, we first decided to adapt the title of the manuscript, which now reads as “Evidence of high N_2 fixation rates in the temperate Northeast Atlantic”. Secondly, we incremented the abstract with more details about our findings in terms of primary production at the time of our study, while leaving out the information about previous studies in the same region (for N_2 fixation rate measurements and diazotrophs community assessment).

Your current abstract cannot be published, and please first revise the Result section (figures and tables), and then materials and methods, then abstract.

With regards to the Material and Methods and Results sections, we have modified the text in order to comply with the Editor’s general views. Details about these adaptations are given below.

Once again, I would like to emphasize that your ms has potential of publication, but it should be organized in a straightforward manner regarding the key findings you want to show.
If you do not want spend more time on it, it is also acceptable that you can notify the editorial office to withdraw this BG submission, and then seek for publication of this study somewhere else.

Best wishes
Zhongjun Jia

Comments to bg-2018-220

Abstract

(1) L26. Please delete the following phrase. (65 and 45 nmol N L-1 d-1 at surface level, respectively).

The sentence was deleted

(2) L27. Please delete the following sentence. “Although diazotrophic activity was not detected at two northern stations in the central Bay of Biscay”. It is not necessary to emphasize these negative two sites. The authors can simply focus on the 8 sites where N2 fixation occurred during the sampling period.

The sentence was deleted

(3) L28. Might contribute to 1-3% f euphotic layer daily PP

The sentence was adapted as follows (lines 30 to 32):

“When converted to carbon uptake using Redfield stoichiometry, N_2 fixation rates could have supported 1 to 3% of euphotic layer daily PP at most sites, except at the two most active sites where this contribution to daily PP reached as high as 25%”.

(4) L29-32. Pls delete the following sentence. In the Atlantic Ocean, N2 fixation 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.

The sentence was deleted

(5) L35-37. Please delete the description of the early study, while emphasize your own results.

The sentence was deleted

(6) L37-40. Please rephrase the following statement, and delete the description of early study. 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.

The sentence was deleted

Materials and Methods. It should be re-structured as following.

(1) 2.1. Site description and water sample collection

(2) 2.2. N2 fixation Measurement

(3) 2.3. inorganic uptake determination
(4) 2.3. Physiochemical and biological properties of oceanic water
(5) 2.4. DNA extraction and illumine sequencing of nifH genes
(6) 2.5. Statistical analysis

The Material and Methods section was adapted in order to fit as closely as possible to the recommendation of the Editor. However, in order to provide the reader with a more linear presentation, as commonly done in oceanographic publications, the methods for nutrient measurements were described just after the site description and sample collection sub-section. Stable isotope incubation experiments (^{15}N and ^{13}C) were described within a single sub-section, this particularly because each incubation bottle was spiked with both tracers. This way of presentation is the most commonly used in the literature. The Material and Methods section is now organized as follows:

2.1 Site description and sample collection
2.2 Nutrient measurements
2.3 $^{15}N_2$ fixation and ^{13}C-HCO_3^- uptake rates
2.4 DNA sampling and *nifH* diversity analysis
2.5 Statistical analysis

In addition
(7) The present writing could be improved for reading with great ease. The authors need to specifically describe how the water were sampled for determination of various properties. For example, L107, where the 12 or 14 Niskin bottles were placed (at depth) for seawater sampling?

The text within the sub-section "Site description and sample collection" was adapted as follows (lines 111 to 118):

"Temperature, salinity and photosynthetically active radiation (PAR) profiles down to 1500 m depth were obtained using a conductivity-temperature-depth sensor (SBE 09 and SBE 911+, during the BG2014/14 and GEOVIDE cruises, respectively) fitted to rosette frames. For all biogeochemical measurements seawater samples were collected from Niskin bottles attached to the rosette and triggered at specific depths in the upper 200 m. In particular, for stable isotope incubation experiments seawater was collected in 4.5 L acid-cleaned polycarbonate (PC) bottles from four depths corresponding to 54%, 13%, 3% and 0.2% of surface PAR at stations Bel-3, 5, 7, 9, 11, and Geo-2. At stations Geo-1, 13 and 21, two additional depths corresponding to 25% and 1% of surface PAR were also sampled for the same purpose".

(8) The methodological description for environmental condition assessment (such as nitrate measurment) should be placed after the description of sample collection

The methodology for nutrient measurements is described in the sub-section following the site description and sample collection.

(9) L121-124. This part could be placed in the section 2.1 as sample collection for N2 fixation and primary production

The sentence was moved to the sample collection sub-section, as cited above (lines 115 to 118).

(10) L130. 13C-HCO2 spiking could be placed in a separate paragraph.

Nitrogen and carbon stable isotope incubations were carried out simultaneously in the same incubation bottles, this is now clarified in the text as follows (lines 133 to 135):
"N_2 fixation and primary production (PP) were determined simultaneously from the same incubation sample in duplicate using the ^{15}N-N_2 dissolution method (Großkopf et al., 2012) and ^{13}C-$NaHCO_3$ tracer addition (Hama et al., 1983) techniques, respectively".

This is why we find it appropriate to keep the description of the whole incubation experiment in a single sub-section.

Results. It should be re-structured as following.
(7) 3.1. Inorganic 13C assimilation and N2 fixation. NEW figure 2, it can be made by combining Fig. 4ab and Fig. 5ab in the original ms as NEW Figure 2abcd. In this section, please describe the 13C changes of organic matter, instead of using the term primary production. Or you may start the paragraph by saying that the primary production was assessed by the increase of 13C in organic carbon
(8) 3.2. Water nutrients and pigment distribution. NEW Figure 3. It can be made by combining Figure 3 and Figure 4c and Figure 4d in the original ms.
(9) 3.3. Taxonomic identities of N2 fixers. NEW Figure 4. It is the supplementary Figure S1.
(10)3.4. Environmental determinants of N2 fixation in productive water. NEW Figure 5. i.e. the Figure 6 in the original manuscript.

We thank the Editor for the suggestions to reorganize the Results section. We have undertaken most of the changes pointed out by the Editor.

- **Changes in the figure arrangements:**

The new Fig. 3 combines the primary production rates and the N_2 fixation rates.
The phylogenetic tree that was previously in the supplemental information is now Fig. 5.
The water mass diagrams (prior Fig. 2) have been moved to the supplemental information.

- **Changes to the text:**

- **To comply with the approach commonly used in the oceanographic literature, the Result section first describes the environmental context of our study by presenting the nutrient levels, and related biomass (chl *a*). As such, we kept nutrient and chlorophyll sections as in Fig. 2.**
- **Given the new structure of the result presentation, we have not merged the nutrient and chlorophyll sections with the satellite-based time series data, since the latter are discussed in the primary production section. The satellite-based chl *a* data is used in the manuscript to support our**

primary production rate measurements, and to relate them to the stage of the spring bloom found at the different sites studied.

- **The term "primary production" is now clearly define at the beginning of the Results' sub-section 3.2 (lines 227 to 230) which is now entitled "Primary production and satellite-based Chl *a* observations. In addition we have now added a paragraph in the methods describing the calculations, which are also clearly outlined in the reference cited in this section: Hama et al. (1983) (lines 150 to 154).**

In the methods (lines 150 to 154):
"N_2 fixation and carbon uptake volumetric rates were computed as shown in Equation 1:

$$N_2 \text{ or } HCO_3^- \text{ uptake rate } (\text{nmol } L^{-1} d^{-1} \text{or } \mu\text{mol } m^{-3} d^{-1}) = \frac{A_{PN \text{ or } POC}^{final} - A_{PN \text{ or } POC}^{t=0}}{A_{N_2 \text{ or } DIC} - A_{PN \text{ or } POC}^{t=0}} \times \frac{[PN \text{ or } POC]}{\Delta t} \quad \textbf{(1)}$$

where A represents the ^{15}N or ^{13}C atom% excess of PN or POC at the beginning (t =0) and end (final) of the incubation, or of the dissolved inorganic pool (N_2 or dissolved inorganic carbon, DIC); and Δt the incubation period".

Hama, T., Miyazaki, T., Ogawa, Y., Iwakuma, T., Takahashi, M., Otsuki, A., & Ichimura, S. (1983). Measurement of photosynthetic production of a marine phytoplankton population using a stable 13C isotope. Marine Biology, 73, 31–36.

In the Results (lines 227 to 230):
"Primary production (PP), estimated through the incorporation of enriched bicarbonate (^{13}C-$NaHCO_3$) into the particulate organic carbon (POC) pool, illustrated volumetric rates ranging from 7 to 3500 µmol C m^{-3} d^{-1} (see Supporting Information Table S1) and euphotic layer integrated rates ranging from 32 to 137 mmol C m^{-2} d^{-1} (Fig. 3a, b, and Supporting Information Table S2)".

- **N_2 fixation rates were presented in the last sub-section of the Results, along with the taxonomic affiliation of the diazotrophs to support our observations, including the phylogenetic tree (new Fig. 6).**

- **Finally, a sub-section presenting the major findings related to statistical analyses (Spearman rank correlation) has been added at the end of the Results section, as suggested by the Editor (lines 280 to 287).**

The figure 2 in the current ms about water mass could be placed in the supplementary section.

The potential temperature versus salinity diagrams (previously Fig. 2) were moved to the Supplementary Material as Fig. S1, as proposed.

Evidence of high N_2 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 N_2 fixation activities at 8 of the 10 stations sampled, ranging overall between 81 and 384 µmol N m^{-2} d^{-1} (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)~~. Primary production was relatively lower along the Iberian Margin with 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}. Our observations in combination with area-averaged Chl *a* satellite data, contemporaneous with our study period, revealed that post-bloom conditions prevailed at most sites, while at the northwesternmost station the bloom was still ongoing. ~~Although diazotrophic activity was not detected at two northern stations in the central Bay of Biscay, w~~When converted to carbon uptake using Redfield stoichiometry, we find that N_2 fixation ~~at the eight other sites~~ rates could have ~~generally contributed~~ supported~~to~~ 1 to ~~–~~ 3% of euphotic layer daily PP at most sites~~, and up to~~ except at the two most active sites where this contribution to daily PP could reached as high as 25%~~ and 23%, respectively at the two most active sites~~. ~~In the Atlantic Ocean, N_2 fixation 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 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. At all the other sites where *nifH* sequences were recovered, these belonged exclusively to non-cyanobacterial phylotypes. ~~Previous studies in the Iberian Basin have systematically reported lower N_2 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-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 also sustained 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 N_2 fixation in productive waters of the temperate North Atlantic to better constrain nitrogen input to the Atlantic Ocean inventory.

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 N_2-fixing micro-organisms (diazotrophs) of dissolved N_2 gas into bioavailable nitrogen also contributes to new production in the euphotic layer and as such, to the subsequent sequestration of atmospheric carbon dioxide into the deep ocean (Gruber, 2008). Estimating the overall contribution of N_2 fixation to carbon sequestration in the ocean requires an assessment of the global marine N_2 fixation.

Until recently most studies of N_2 fixation have focused on the tropical and subtropical regions of the global ocean, with few attempts to measure N_2 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 for 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 reported in oligotrophic tropical and subtropical oceanic waters, thought to represent the optimal environment for its growth and N_2-fixing activity (Dore et al., 2002; Breitbarth et al., 2007; Montoya et al., 2007; Needoba et al., 2007; Moore et al., 2009; Fernández et al., 2010; Snow et al., 2015). In low latitude regions, warm, stratified surface waters depleted in dissolved inorganic nitrogen (DIN), are assumed to give a competitive advantage to diazotrophs over other phytoplankton since only they can draw N from the unlimited dissolved N_2 pool for their biosynthesis. As such, past estimates of global annual N_2 fixation were mainly based on information gathered from tropical and subtropical regions, while higher latitude areas have been poorly explored for diazotrophic activity (Luo et al., 2012).

Studies using genetic approaches targeting the *nifH* gene encoding the nitrogenase enzyme, essential for diazotrophy, have shown the presence of diverse diazotrophs throughout the world's oceans, extending their ecological niche (Farnelid et al., 2011; Cabello et al., 2015; Langlois et al., 2015). Small diazotrophs such as unicellular diazotrophic cyanobacteria (UCYN classified in groups A, B and C) and non-cyanobacterial diazotrophs, mostly heterotrophic bacteria (e.g. Alpha- and Gammaproteobacteria), have been observed over a wide range of 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 $^{15}N_2$ bubble-addition technique (Mohr et al., 2010) and the presence of an abundant diazotrophic community in high latitude regions actively fixing N_2 (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 diversity at higher latitudes.

In the Northeast Atlantic, the large input of iron-rich Saharan dust alleviating dissolved iron (dFe) limitation of the nitrogenase activity (Fe being a co-factor of the N_2-fixing enzyme) (Raven, 1988; Howard & Rees, 1996; Mills et al., 2004; Snow et al., 2015) and the upwelling of subsurface waters with low DIN (dissolved inorganic nitrogen) to phosphate ratios, make this region highly favorable for N_2 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). Here, we present N_2 fixation rate measurements and the taxonomic affiliation of the diazotrophic community from two consecutive missions carried out in the Northeast sector of the Atlantic Ocean in May 2014, during and after the spring bloom.

2 Material and Methods

2.1 Site description and sample collection

Field experiments were conducted during two nearly simultaneous cruises in May 2014. The Belgica BG2014/14 cruise (21–30 May 2014, R/V *Belgica*), investigated the Bay of Biscay and the western Iberian Margin. In parallel, the GEOVIDE expedition in the framework of the international GEOTRACES 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 four stations within the Iberian Basin investigated for N_2 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).

~~Sites sampled in~~ All sampling sites were located within the Iberian Basin Portugal Current System (PCS) (Ambar and Fiúza, 1994) which is influenced by highly fluctuating wind stresses (Frouin et al., 1990). The predominant upper layer water mass in this basin is the Eastern North Atlantic Central Water (ENACW), a winter mode water which according to Fiúza (1984) consists of two components (see θ/S diagrams in Supporting Information Fig. S1): (i) the lighter, relatively warm (> 14°C) and salty (> 35.6) ENACWst 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 ENACWsp, underlying the ENACWst, 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) ENACWsp stations north of 43° N (Bel-3, Bel-5, Bel-7, and Geo-21) only affected by the ENACWsp (Fig. S1a, b) and (ii) ENACWst stations, south of 43° N, characterized by the upper layer being influenced by ENACWst and the subsurface layer by ENACWsp (Fig. S1a, b). Most of these ENACWst stations were open ocean sites (Bel-9, Bel-11, Bel-13, and Geo-13) while two stations were in proximity of the Iberian shelf (Geo-1 and Geo-2) (Tonnard et al., 2018).

Temperature, salinity and photosynthetically active radiation (PAR) profiles down to 1500 m depth were ~~determined~~obtained using a conductivity-temperature-depth sensor (SBE 09 and SBE 911+, during the BG2014/14

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

2.21 ~~Environmental conditions~~Nutrient measurements

~~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.~~ Surface w~~W~~ater ~~column~~ concentrations of ammonium (NH_4^+) during both cruises were measured on board as well as nitrate + nitrite (NO_3^- + NO_2^-) concentrations during the GEOVIDE expedition. During the BG2014/14 cruise, samples for NO_3^- + NO_2^- and phosphate (PO_4^{3-}) measurements were filtered (0.2 µm) and stored at –20°C until analysis at the home-based laboratory. PO_4^{3-} data are not available for the GEOVIDE cruise.

Nutrient concentrations were determined using the conventional fluorometric (for NH_4^+) (Holmes et al., 1999) and colorimetric methods (for the other nutrients) (Grasshoff et al., 1983) with detection limits (DL) of 64 nmol L^{-1} (NH_4^+), 90 nmol L^{-1} (NO_3^- + NO_2^-) and 60 nmol L^{-1} (PO_4^{3-}). 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~~3 $^{15}N_2$ fixation and ^{13}C-HCO_3^- uptake rates

N_2 fixation and primary production (PP) were determined simultaneously from the same incubation sample in duplicate using the ^{15}N-N_2 dissolution method (Großkopf et al., 2012) and ^{13}C-$NaHCO_3$ tracer addition (Hama et al., 1983) techniques, respectively. ~~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 $^{15}N_2$ dissolution method can be found in Fonseca-Batista et al. (2017). Briefly, $^{15}N_2$-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 $^{15}N_2$ gas (98 ^{15}N atom%, Eurisotop, lot number 23/051301) and left to equilibrate. This $^{15}N_2$ 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 $^{15}N_2$-enriched seawater, spiked with 3 mL of a $NaH^{13}CO3$ 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 shown in Equation 1:

$$N_2 \text{ or } HCO_3^- \text{ uptake } rate \text{ (nmol } L^{-1}d^{-1} \text{or } \mu\text{mol } m^{-3}d^{-1}) = \frac{A_{PN\ or\ POC}^{final} - A_{PN\ or\ POC}^{t=0}}{A_{N_2\ or\ DIC} - A_{PN\ or\ POC}^{t=0}} \times \frac{[PN\ or\ POC]}{\Delta t} \quad (1)$$

where A represents the ^{15}N or ^{13}C atom% excess of PN or POC at the beginning (t =0) and end (final) of the incubation, or of the dissolved inorganic pool (N_2 or dissolved inorganic carbon, DIC); and Δt the incubation period. 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.~~4~~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 ng μL^{-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 Proteinase K 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 nifH1/2 primers (Langlois et al., 2005; Ratten, 2017) attached to Illumina adaptors and barcodes for multiplexing in the Illumina MiSeq instrument. Next generation sequencing was carried out at the Integrated Microbiome Resource (IMR) of the Centre for Comparative 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~~6). 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.

2.5 Statistical analysis

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

3 Results

3.1 Ambient environmental settings

Surface waters of all the ENACWst stations showed a relatively strong stratification resulting from the progressive spring heating, with sea surface temperature (SST) ranging from 15.3 (Geo-13) to 17.2°C (Bel-13). Nutrients were depleted at the surface (NO_3^- + NO_2^- < 0.09 µM in the upper 20 m; Fig. ~~3~~2c, f) and surface Chl *a* concentrations were low (< 0.25 µg L^{-1}; Fig. ~~3~~2a, d) but showed a subsurface maximum (between 0.5 and 0.75 µg 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 (NO_3^- + NO_2^- = 0.7 µM in the lower mixed layer depth, MLD) and higher Chl *a* (> 0.5 µg 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 NO_3^- + NO_2^- ranging from 0.3 to 0.8 µM) and had a higher phytoplankton biomass (Chl *a* between 0.7 to 1.2 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° W), which appeared to be located within an anticyclonic mesoscale eddy, as evidenced by the downwelling structure detected in the Chl *a* and NO_3^- + NO_2^- profiles (Fig. ~~3~~2a, c) at this location (as well as T and S sections, data not shown).

3.2 Primary production and satellite-based chl *a* observations

Primary production (PP), estimated through the incorporation of enriched bicarbonate (^{13}C-$NaHCO_3$) into the particulate organic carbon (POC) pool, illustrated ~~V~~volumetric rates ~~of carbon uptake ranged~~ ranging from 7 to 3500 µmol C m^{-3} d^{-1} (see Supporting Information Table S1) and euphotic layer integrated rates ~~varied~~ ranging from 32 to 137 mmol C m^{-2} d^{-1} (Fig. 3~~4~~a, 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 mmol C m^{-2} d^{-1}, except at station Bel-7 where it was slightly higher (52 mmol C m^{-2} d^{-1}; Fig. 3~~4~~a, 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 mmol C m^{-2} d^{-1}, respectively; Fig. ~~4~~3b) as well as closer to the shelf (reaching 59 mmol C m^{-2} d^{-1} at Geo-2). These results are in the range of past measurements in this region for the same period of the year, ranging from 19 to 103 mmol C m^{-2} d^{-1} (Marañón et al., 2000; Fernández et al., 2005; Poulton et al., 2006; Fonseca-Batista et al., 2017). Area-averaged Chl *a* derived from satellite imagery for a time-period overlapping with our ~~Our observations also coincide with the area-averaged Chl *a* time series obtained from satellite data~~(Giovanni online data system; Fig. 4a, b~~c, d~~) ~~which~~ revealed that post-bloom conditions prevailed at most sites (Bel-3 to Bel-13 and Geo-1 to Geo-13) while ~~the~~ bloom conditions were~~as~~ still ~~ongoing~~ found 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).~~

3.3 N_2 fixation and dominant diazotrophs at the sampling sites

Volumetric N_2 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 nmol N L^{-1} d^{-1} (see ~~Supporting Information~~ Table S1), with areal rates ranging between 81 and 1533 µmol N m^{-2} d^{-1} (Fig. 3c, d~~5a, b~~, and ~~Supporting Information~~ Table S2).

We observed intense N_2 fixation activities at the two sites (Bel-11 and Bel-13) most affected by ENACW waters of subtropical origin (Fig. S1~~Fig. 2~~). At stations Bel-11 and Bel-13, volumetric rates of N_2 fixation ranged from 2.4 to 65.4 nmol N L^{-1} d^{-1}, with highest rates found at surface level (65.4 and 45.0 nmol N L^{-1} d^{-1}, respectively), while areal rates averaged 1533 and 1355 µmol N m^{-2} d^{-1}, respectively. N_2 fixation was detected at all four GEOVIDE stations. Shelf-influenced (Geo-1 and Geo-2) and open ocean (Geo-13) ENACWst sites, geographically close to Bel-11 and Bel-13, also displayed high N_2 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. 3c, d~~5a, b~~, and ~~Supporting Information~~ Table S2). Significant N_2 fixation rates were also measured at stations that overall exhibited the highest primary production rates, including Bel-7, Geo-13 and Geo-21 (Fig. 3). We computed the relative contribution of N_2 fixation to PP by converting N_2 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). N_2 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 N_2 fixation rates. Cloning of the *nifH* amplicons found in surface waters (54% PAR level where volumetric rates of N_2 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. 5a~~c~~ and ~~S1~~6) (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. 5a~~c~~ and ~~S1~~6). 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. 5a~~c~~ and ~~S1~~6). For the GEOVIDE cruise, *nifH* screening returned positive *nifH* presence at stations Geo-2, Geo-13 and Geo-21. Next generation sequencing of these amplicons yielded in total 21001 reads, with a range of 170 to 9239 *nifH* amplicons per sample, belonging exclusively to non-cyanobacterial diazotrophs, with the major affiliation to Verrucomicrobia, and Gamma-, Delta- and Alpha-proteobacteria, representing 54, 28, 15 and 1% of total *nifH* amplicons, respectively (Fig. 5b~~d~~ and ~~S1~~6). 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.

3.4 Relationship between N_2 fixation rates and environmental variables

N_2 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 (NO_3^- + NO_2^- from < 0.1 to 7.6 µM). Water column integrated N_2 fixation tended to increase with the 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 N_2 fixation tended to increase with temperature (n = 46, $p < 0.01$, Table S4) and excess phosphorus concentration (only available Belgica studied sites, n = 24, $p < 0.01$) while being negatively correlated to nitrate plus nitrite concentration (n = 46, $p < 0.01$).

4 Discussion

During two quasi simultaneous expeditions to the Iberian Basin and the Bay of Biscay in May 2014 (38.8–46.5° N), we observed N_2 fixation activity in surface waters of most stations (except at the two northernmost sites in the Bay of Biscay). Our results are in support of other recent studies, that have observed diazotrophic communities and significant N_2 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 N_2 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.

4.1 Significance of N_2 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 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. 3c, d~~5a, b~~, and Tables S1 and S2). Although N_2 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, N_2 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, N_2 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 our computation relies on the assumption that only photoautotrophic diazotrophs contribute to bulk N_2 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 N_2 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 N_2 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 µmol N m^{-2} d^{-1}) (Großkopf et al., 2012; Subramaniam et al., 2013; Fonseca-Batista et al., 2017). Besides these two higly active sites, N_2 fixation rates at the other studied locations (ranging between 81–384 µmol N m^{-2} d^{-1}) were still in the upper range of values reported for the whole eastern Atlantic region. 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 ~1000 µmol N m^{-2} d^{-1} as observed here, have only been reported for temperate coastal waters of the Northwest Atlantic (up to 838 µmol N m^{-2} 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 µmol N m^{-2} 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 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 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 (M. Tonnard, personal communication, January 2018). 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 symbionts, followed by heterotrophic bacteria, while *Trichodesmium* filaments were low or undetectable (Needoba et al., 2007; Rees et al., 2009; Mulholland et al., 2012; Agawin et al., 2014; Shiozaki et al., 2015; Moreira-Coello et al., 2017).

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

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

Besides UCYN-A, all the remaining *nifH* sequences recovered from both cruises, although obtained through different approaches, belonged to non-cyanobacterial diazotrophs. The phylogenetic tree (Fig. 6) showed that the non-cyanobacterial diazotrophs clustered with (1) Verrucomicrobia, a phylum yet poorly known that includes aerobic to microaerophile methanotrophs groups, found in a variety of environments (Khadem et al., 2010; Wertz et al., 2012), (2) anaerobic bacteria, obligate or facultative, mostly affiliated to Cluster III phylotypes of functional nitrogenase (e.g., Bacteriodetes, Firmicutes, Proteobacteria) and finally (3) phylotypes from Clusters I, II, and IV (e.g., Proteobacteria and Firmicutes). Among the Cluster III phylotypes, Bacteroidetes are commonly encountered in the marine environment, and are known as specialized degraders of organic matter that preferably grow attached to particles or algal cells (Fernández-Gómez et al., 2013). N_2 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~~6). Anaerobic Cluster III phylotypes have been previously recovered from different ocean basins (Church et al., 2005; Langlois et al., 2005, 2008; Man-Aharonovich et al., 2007; Rees et al., 2009; Halm et al., 2012; Mulholland et al., 2012). These diazotrophs were suggested to benefit from anoxic microzones found within marine snow particles or zooplankton guts to fix N_2 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 dominance of Gammaproteobacteria phylotypes belonging to a recently identified clade of marine diazotrophs within the Oceanospirillales (Delmont et al., 2018).

These observations tend to strengthen the idea 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 N_2 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 N_2 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; Rahav et al., 2013, 2016; Loescher et al., 2014).

Findings from the GEOVIDE cruise tend to support the hypothesis of a stimulating effect of organic matter availability on N_2 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 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 availability of organic matter in the upper layers likely contributed to supplying remineralized P (organic P being generally more labile than other organic nutrients; Vidal et al., 1999, 2003) and to enhancing the residence time of dFe originating from atmospheric deposition due to the formation of organic ligands (Jickells, 1999; de Baar and de Jong, 2001; Sarthou et al., 2003).

P* values from the BG2014/14 cruise (Table S1) and the climatological P* data for the Iberian Basin (Garcia et al., 2013) do not exhibit a clear PO_4^{3-} 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 N_2 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 (data not shown) the correlation between in situ P* and N_2 fixation rates is no longer significant (n= 16, $p = 0.163$), with P* becoming highly correlated with PP and Chl *a* (n = 16, $p = 0.0257$ and 0.016, respectively). This suggests that P* effect on N_2 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 PO_4^{3-} 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 extracellular alkaline phosphatase). Therefore, a likely 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 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 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-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 N_2 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 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. S1~~Fig. 2~~) illustrate the presence of very warm and saline waters and satellite SST images suggest these were advected 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 N_2 fixation 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.

For the central Bay of Biscay, where N_2 fixation was below detection limit (stations Bel-3 and Bel-5), dust deposition in April 2014 was also the lowest, suggesting that N_2 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 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.

These statements are further supported by the outcome of a multivariate statistical analysis providing a comprehensive view of the environmental features influencing N_2 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~~7) and an inverse relation with PP and associated variables (Chl *a*, NH_4^+, NO_3^- + NO_2^-), 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~~7. Axis 2 is defined by the positive relation with surface salinity and P* (Fig. ~~6~~7) and reflects the advection of surface waters of subtropical origin, for stations Bel-11, Bel-13 and Geo-13. For stations Geo-1 and Geo-2, the inverse relation with surface salinity (Fig. ~~6~~7) is interpreted to reflect fluvial inputs (Tonnard et al., 2018). Finally, this statistical analysis indicates that N_2 fixation activity was likely influenced by the two PCA components, tentatively identified as productivity (axis 1) and surface water advection (axis 2) from the shelf and the subtropical region.

5 Conclusions

The present work highlights the occurrence of elevated N_2 fixation activities (81–1533 µmol N m^{-2} 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 µmol N m^{-2} d^{-1}). In contrast we did not detect any N_2 fixation activity in the central Bay of Biscay. At sites where significant N_2 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 N_2 fixation for the eastern Atlantic. Assuming that the carbon vs nitrogen requirements by these N_2 fixers obey the Redfield stoichiometry, N_2 fixation was found able to contribute 1–3% of the euphotic layer daily PP and even up to 23–25% at the sites where N_2 fixation activity was highest. The Prymnesiophyte-symbiont *Candidatus* Atelocyanobacterium thalassa (UCYN-A) contributed most to the *nifH* sequences recovered at the two sites where N_2 fixation activity was highest, while the remaining sequences belonged exclusively to heterotrophic bacteria. We support that the unexpectedly high N_2 fixation activity recorded at the time of our study was sustained by (i) organic matter availability in these open waters, resulting from the prevailing vernal bloom to post-bloom conditions, in combination with (ii) excess phosphorus signatures which appeared to be tightly related to diazotrophic activity particularly at the two most active sites. Yet these observations and hypotheses rely on the availability of dFe with evidence for input from shelf waters and pulsed atmospheric dust deposition being a significant source of iron. Further studies are required to investigate this possible link between N_2 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 N_2 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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841 **Tables**

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844 **Table 1:** Relative contribution (%) of N_2 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 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 ($P^* = [PO_4^{3-}] - [NO_3^-] / 16$) 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).

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).

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}); N_2 fixation **(c, d)** (duplicates are in light and dark blue bars with the corresponding values in µmol N m^{-2} d^{-1}) determined during the Belgica BG2014/14 **(a, c)** and GEOVIDE **(b, d)** cruises. Error bars represent the propagated measurement uncertainty of all parameters used to compute volumetric uptake rates.

~~**Figure 4:** Spatial distribution (± SD) of depth integrated primary production (duplicates are in light and dark green with the corresponding bar values on top in mmol C m^{-2} 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}) registered by Aqua MODIS satellite (Giovanni online satellite data system) between December 2013 and December 2014 for the 0.5° x 0.5° grid surrounding the different stations during the **(a~~c~~)** Belgica BG2014/14 and **(~~d~~b)** GEOVIDE cruises. The dashed box highlights the sampling period for both cruises (May 2014).

~~**Figure 5:** Spatial distribution (± SD) of depth integrated N_2 fixation rates (duplicates are in light and dark blue with the corresponding bar values on top in µmol N m^{-2} 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 **(a~~c~~)** the Belgica BG2014/14 cruise (successfully recovered only at stations Bel-11 and Bel-13, 5 m) and **(b~~d~~)** the GEOVIDE cruise (stations Geo-2, 100 m; Geo-13, 35 m and Geo-21, 15 and 70 m. The total numbers of recovered sequences are indicated on top of the bars, and the exact percentage represented by each group is shown inside the bars.

Figure 6: Phylogenetic tree of *nifH* predicted amino acid sequences generated using the Maximum Likelihood method of the Kimura 2-parameter model (Kimura, 1980) via the Molecular Evolutionary Genetics Analysis software (MEGA 7.0) (Kumar et al., 2016). Initial tree(s) for the heuristic search were obtained automatically by applying 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 ≥ 95% similarity at the nucleotide level with representative clones) are highlighted in blue. For the *nifH* sequences recovered from the GEOVIDE cruise, only those contributing to the cumulative 98% of recovered sequences were included in this tree. Bootstrap support values (≥ 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.

Figure 7: Euclidean distance biplot illustrating the axis loadings for the two main PCA components based on the Spearman rank correlation matrix shown in Table S3. Variables taken into account include depth-integrated rates of N_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., 2013); satellite average dust deposition (dry + wet) derived during April 2014 (Giovanni online data system, NASA Goddard Earth Sciences Data and Information Services Center) and ambient variables (temperature, salinity, and nutrient data). Coloured dots in the biplot represent the projection of the different stations. Axis 1 has high negative loadings for PP, Chl *a*, NH_4^+ and $NO_3^- + NO_2^-$, and high positive loadings for temperature and N_2 fixation rates, with values of –0.812, –0.768, –0.936, –0.783, 0.942 and 0.506, respectively (see table S5). Axis 2 has high positive loadings of 0.584, 0.943 and 0.602 for climatological P*, salinity and N_2 fixation rates, respectively. PCA analysis was run in XLSTAT 2017 (Addinsoft, Paris, France, 2017).