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Nitrogen fixation by *Trichodesmium* and small diazotrophs in the subtropical northeast Atlantic

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ABSTRACT: We measured gross and net nitrogen fixation in fractionated samples (organisms >10 µm and <10 µm), and the density of *Trichodesmium*, during a cruise along the northeast Atlantic boundary current system and during 2 mesoscale experiments in the upwelling systems of Cape Silleiro (northwest Iberia) and Cape Ghir (northwest Africa). The density of *Trichodesmium* (<0.5 trichomes l^{-1}) and its associated rates of nitrogen fixation (<0.1 µmol N m^{-2} d^{-1}) were low. Trichomes appeared to accumulate at frontal sites — such as upwelling filaments and the Azores Front. Gross and net rates of nitrogen fixation were always <0.4 nmol N $l^{-1}d^{-1}$ except off the northwest African coast where a gross nitrogen fixation peak of 0.98 nmol N l^{-1} d^{-1} was measured. The <10 µm fraction contributed more to both gross and net nitrogen fixation than did the >10 µm fraction in most of the areas studied. The <10 µm fraction was responsible for 70 to 92% of the total nitrogen fixation in cold nutrient-rich areas. The contribution of small diazotrophs to nitrogen fixation in the upwelling sites suggests that the distribution and activity of these organisms are more widespread than previously thought.

KEY WORDS: Nitrogen fixation · *Trichodesmium* · Upwelling · Subtropical northeast Atlantic · Canary Current Large Marine Ecosystem

INTRODUCTION

Nitrogen is the main nutrient that limits primary production in the open ocean oligotrophic environment where the quasi-permanent stratification of the upper water column prevents mixing with the denser and nutrient-rich deep waters (Falkowski 1997). Dinitrogen (N_2) fixation in the ocean is predominantly attributed to the photoautotrophic *Cyanobacteria*. This process enriches the food web with combined nitrogen through leakage of ammonium and amino acids which serve as a source of nitrogen for autotrophic non-diazotrophic phytoplankton (Mahaffey et al. 2005). Indeed, N_2 fixation is thought to fuel 50% of the primary production in these 'oceanic deserts' (Capone et al. 2005). Nitrogen is removed from the oxygen minimum zones (OMZ) and from sediments through denitrification. Based on present knowledge, the rates of denitrification exceed those of N_2 fixation (Codispoti 2007), suggesting imbalances in the cycling of nitrogen in the ocean. However, some models suggest that areas of N_2 fixation and denitrification are coupled and could result in a homeostatic nitrogen cycle (Deutsch et al. 2007).

Among marine diazotrophic microorganisms, *Trichodesmium* has been considered as the principal N_2 fixer in the ocean. However, recent discoveries recognized unicellular diazotrophic *Cyanobacteria* as

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important contributors to the oceanic nitrogen budget (Zehr et al. 2001). Although the presence of non-cyanobacterial *nifH* genes has been reported in the oligotrophic oceans (Falcón et al. 2004, Langlois et al. 2005, 2008), their contribution to overall N_2 fixation requires further study.

While *Trichodesmium* seems to be restricted to tropical oligotrophic areas (Capone et al. 1997), the unicellular *Cyanobacteria* of Groups A (UCYN-A), B and C may be more widely distributed. In the Atlantic Ocean, Langlois et al. (2008) found unicellular diazotrophs from the equator to ~35°N. Many of the Group A and Group B sequences were highly similar to those found previously in the Pacific Ocean, suggesting that these unicellular diazotrophs have a cosmopolitan distribution (Langlois et al. 2005). Unicellular oceanic diazotrophs have been documented in the North Pacific (Zehr et al. 2001), South Pacific (Moisander et al. 2010), the Mediterranean Sea (Le Moal & Biegala 2009) and the Arabian Sea (Mazard et al. 2004).

Organisms of Group A (UCYN-A) are the most abundant and widely distributed unicellular diazotrophs in the open ocean (Langlois et al. 2005, Church et al. 2008, Langlois et al. 2008, Moisander et al. 2010). Although present mainly in tropical latitudes, UCYN-A occur in waters with a wide range of temperatures (Moisander et al. 2010); they are found in surface waters but also occur at greater depths where temperature and light intesity are lower and the concentration of inorganic nitrogen is higher (Montoya et al. 2004, Moisander et al. 2010). Organisms of Groups B and C seem to occupy more narrow ranges of temperature (Langlois et al. 2005, 2008).

An increasing number of studies have reported on the distribution and abundance of diazotrophs, but only few have also measured N_2 fixation.

Diazotrophy in the ocean is thought to be limited by iron and/or phosphorus (Mills et al. 2004, Moore et al. 2009). The northeast Atlantic is expected to have high rates of N_2 fixation because of the recurrent input of iron and phosphorus through aeolian transport from the nearby Sahara desert (Prospero 1981). However, N_2 fixation in this area could also be restricted as a result of the cold, nutrient-rich upwelling waters from the Iberian–Canary Current which extend hundreds of kilometres offshore through upwelling filaments (Álvarez-Salgado et al. 2007). Both the availability of combined nitrogen and the low temperatures could prevent N_2 fixation, limiting its relevance in upwelling regions—as happens in the cold, high-latitude seas (Gruber & Sarmiento 1997). The majority of the N_2 fixation studies conducted in the northeast Atlantic used a geochemical approach (e.g. Mahaffey et al. 2003, Álvarez & Álvarez-Salgado 2007, Bourbonnais et al. 2009). The role of diazotrophy in nitrogen cycling in the northeast Atlantic Ocean remains unclear and more direct measurements of N_2 fixation are needed.

We measured N_2 fixation by size-fractionated plankton from the northeast Atlantic — in samples from oligotrophic open ocean waters and from 2 active coastal upwelling regions of the Canary Current Large Marine Ecosystem (Arístegui et al. 2009): Cape Silleiro (northwest Iberia) and Cape Ghir (northwest Africa). The aims of this study were (1) to provide a first estimate of the diazotrophic activity in 2 active upwelling areas of the northeast Atlantic, and (2) to compare the relative contribution of *Trichodesmium* with that of smaller diazotrophs. We combined the 2 most commonly used methods to measure (1) gross N_2 fixation, using the acetylene reduction assay (ARA) (Stal 1988), and (2) net N_2 fixation, using the $^{15}N_2$ tracer technique as employed by Montoya et al. (1996) (Gallon et al. 2002, Mulholland et al. 2004). The difference between the rates obtained by these 2 approaches is thought to be a proxy for the release of dissolved organic nitrogen (DON) (Gallon et al. 2002, Mulholland et al. 2004). DON fuels an important amount of autotrophic production (Berman & Bronk 2003, Bronk et al. 2007), and some diazotrophs may release up to 50% of their recently fixed N_2 as DON (Mulholland & Bernhardt 2005); therefore, the potential influence of DON in supporting primary production and global nitrogen cycling should not be overlooked (Bronk et al. 1994, Mulholland 2007).

MATERIALS AND METHODS

Sampling and hydrographic measurements

The sampling was carried out within the framework of the project 'Shelf–Ocean Exchanges in the Canaries– Iberian Large Marine Ecosystem' (CAIBEX) on board the R/V 'Sarmiento de Gamboa' during the summer of 2009. The cruise was divided into 3 legs: 2 meso-scale experiments off Cape Silleiro, northwest Iberia (6 to 24 July), and off Cape Ghir, northwest Africa (16 August to 5 September), and a large-scale open-ocean grid (CAIBOX, 25 July to 14 August) connecting the 2 Cape regions (Fig. 1). Cape Silleiro and Cape Ghir are sites where upwelling filaments typically develop (Arístegui et al. 2009). However, the filaments of Cape Silleiro did not develop at the time of the cruise, so the sampling

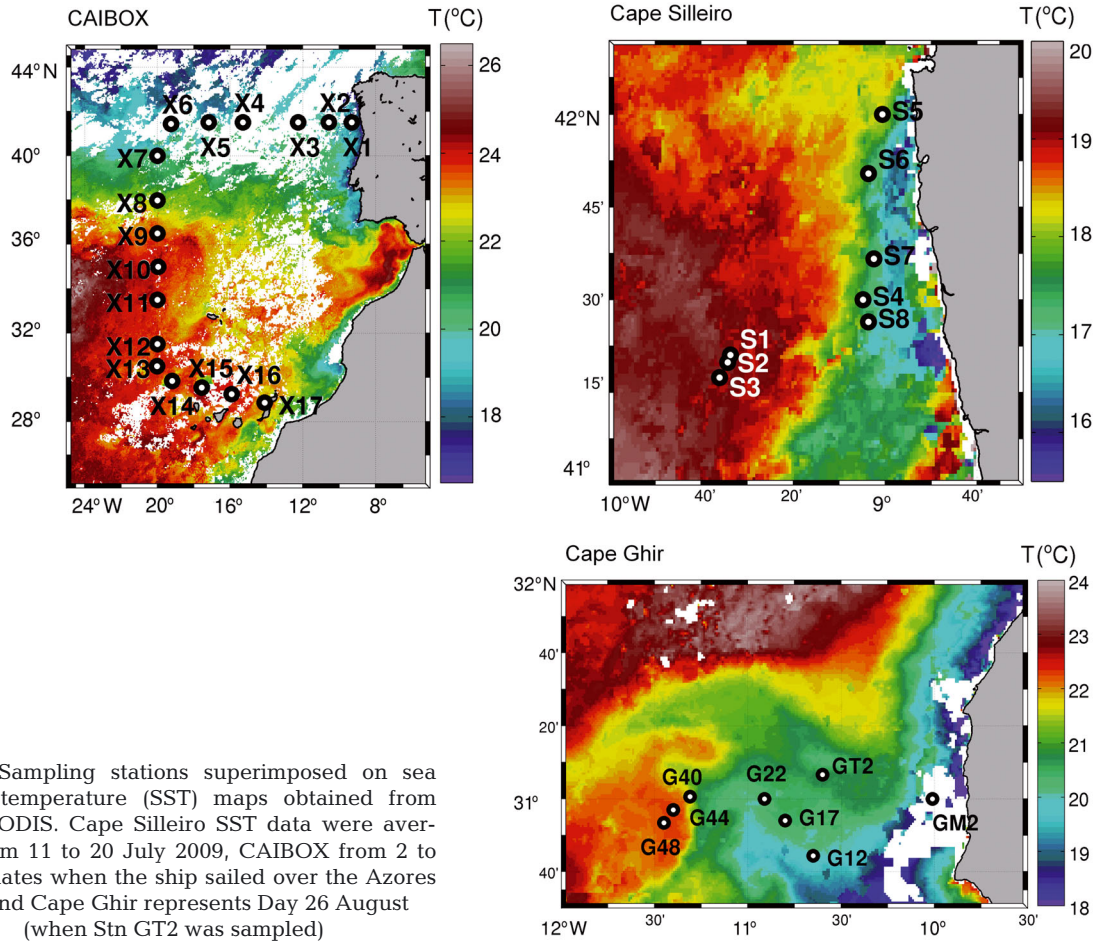

Fig. 1. Sampling stations superimposed on sea surface temperature (SST) maps obtained from Aqua-MODIS. Cape Silleiro SST data were averaged from 11 to 20 July 2009, CAIBOX from 2 to 4 July (dates when the ship sailed over the Azores Front), and Cape Ghir represents Day 26 August (when Stn GT2 was sampled)

stations corresponded only to sites of coastal upwelling (Stns S05 to S08) and to the open ocean (Stns S01 to S03). A strong upwelling filament developed during the Cape Ghir cruise, allowing tracking and sampling of the structure during 6 consecutive days. Samples were collected along the path of the filament at Stns G12, G17, G22, and G40, G44, G48, as well as at 2 stations outside the filament (Stns GM2 and GT2). Finally, 17 additional stations were sampled along the box-grid of the CAIBOX cruise, connecting the northwest Iberia upwelling with the Canary Islands waters through a meridional section at 20°W (Fig.1).

More detailed descriptions of the hydrographic features found during these cruises may be found elsewhere (Carracedo et al. 2011, in press, C. Troupin et al. unpubl. data). Temperature, salinity, fluorescence and photosynthetically active radiance (PAR) data were recorded by a CTD SeaBird 911 plus, a Sea-Tech fluorometer and a Li-Cor PAR sensor, all mounted on a General Oceanics 24 bottle rosette sampler.

Plankton net casts and counts of *Trichodesmium*

At each station, a plankton net of 50 µm mesh size was deployed 2 or 3 times from the deep chlorophyll maximum (DCM) and hauled vertically to the surface at a mean speed of 20 m min^{-1}. Typically, 20 to 60 m^3 were filtered by the net, depending on the depth of the DCM and the number of tows. The sample was concentrated to 240 ml, of which 60 ml were fixed with 10% formaldehyde to a final concentration of 4%. The formaldehyde was buffered with phosphate-buffered saline (PBS, Sigma Aldrich) and adjusted to pH 8.5. The samples were stored in the dark at ambient temperature until used for counting *Trichodesmium* in the laboratory with an inverted microscope. The number of free trichomes ranged from 1 to 1200 per sample. The colonial tufts or puffs were present in low numbers (typically 1 to 4 per sample).

The other 180 ml of the plankton net sample were used for measuring N_2 fixation in the >50 µm fraction (see next section).

Measurements of N_2 fixation

Water from the near-surface (5 m) was collected at each station between 08:00 h and 12:00 h (UTC). N_2 fixation was measured by the ARA technique (Stal 1988) and the $^{15}N_2$ stable isotope technique (Montoya et al. 1996). The incubations for both techniques were done in on-deck incubators cooled with surface seawater. Neutral density screens (Lee Filters) were used to reproduce the incident PAR light measured at 5 m depth. ARA incubations were 3 to 4 h, and $^{15}N_2$ incubations were for 24 h.

For the ARA, triplicate samples of bulk seawater, each of 2 l, were filtered through 25 mm Whatman GF/F filters to obtain total N_2 fixation rates. The <10 µm fraction was obtained by pre-filtering whole seawater through 47 mm white polycarbonate filters of pore size 10 µm (GE-Osmonics Poretics) and subsequently filtering onto GF/F filters. The activity of the >10 µm fraction was computed by subtracting the rates of the <10 µm fraction from the total N_2 fixation rates. All GF/F filters were placed in 10 ml crimp-top vials (Varian/Chrompack), humidified with 0.5 ml of filtered (GF/F) seawater, and sealed with a rubber stopper and an aluminium cap using a seal crimper. After sealing, 2 ml of acetylene were injected into each of the samples using gas-tight syringes (Hamilton). Acetylene was generated from calcium carbide (CaC_2, Sigma Aldrich) by adding Milli-Q water in a reaction flask (Stal 1988). The gas was recovered in tedlar gas bags (volume 1 l) with polypropylene valves (SKC). The ethylene contamination of the generated acetylene was <12 ppm.

Blank GF/F filters were incubated with the same volume of filtered seawater and acetylene. A set of 3 blanks was made once every 5 stations. As we corrected ARA rates with their respective blanks every 5 stations, we obtained specific detection limits for each set of stations. The detection limit of the ARA technique ranged between 0.0185 and 0.1268 nmol ethylene, defined as 3 times the standard deviation of the difference between the ethylene produced after the incubation period and the ethylene content of the blanks.

We divided the 180 ml of sample left over from the plankton net casts (see above) into 3 parts, each filtered through a GF/F filter and processed for the ARA as described above. It was assumed that this activity corresponds to the N_2 fixation attributed to *Trichodesmium* or to endosymbiont *Cyanobacteria*, such as *Richelia intracellularis*, which is the typical endosymbiont of the diatoms *Rhizosolenia* spp. or *Hemiaulus* spp., which range in size from 15 to 100 µm and from 15 to 35 µm, respectively.

After incubation, 10 ml of headspace were sampled and transferred to pre-evacuated Hungate tubes, which were finally sealed with thermofusible glue and stored in the dark at ambient temperature until analysis. In previous tests, the retention of gas in Hungate tubes was found to be greatly enhanced after sealing with thermofusible glue, so this approach was used in the present study. Acetylene and ethylene were measured using a gas chromatograph (Agilent Technologies, model HP 5890) equipped with a flame ionization detector (FID), fitted with a Varian (Middelburg) wide-bore column (ref. CP7584) packed with CP-PoraPLOT U (27.5 m length, 0.53 mm inner diameter, 0.70 mm outer diameter, 20 µm film thickness). The column flow rate was 4 ml min^{-1} at a pressure of 5 psi. Helium was used as a carrier gas at a flow rate of 30 ml min^{-1}. Hydrogen and airflow rates were set at 30 ml min^{-1} and 365 ml min^{-1}, respectively. Helium and hydrogen were obtained from Carburos Metálicos (Air Products Group). Oven, injection and detector temperatures were set at 52, 120 and 170°C, respectively. Acetylene reduction rates were calculated using acetylene as an internal standard (Stal 1988) and converted to N_2 fixation rates using a factor of 4:1. Daily rates were computed from hourly rates multiplied by the number of light hours in each specific date and geographic position.

For the $^{15}N_2$ technique, unfiltered surface and <10 µm seawater (pre-filtered with polycarbonate filters as detailed above) was transferred to transparent polycarbonate bottles (volume 1.24 l, Nalgene). The bottles were completely filled using silicone tubing to prevent the introduction of air bubbles. They were then sealed with septum screw-caps before trace additions of $^{15}N_2$ (2 ml 99 atom %; Tracer Tec) were injected through the septum using a gas-tight syringe. Enrichments varied from 9.8 to 11.2%. The pressure across the septum was equilibrated by allowing the excess water to escape through a syringe tip piercing the septum. Finally, the bottles were placed in the on-deck incubator for 24 h. After the incubation, samples were filtered through precombusted GF/F filters, wrapped in precombusted aluminium foil, and stored at –20ºC until analysis. The particulate organic nitrogen (PON) content and isotopic ratio of samples was measured with a Thermo Flash EA 1112 elemental analyser interfaced by a Conflo III with a Thermo Delta V Advantage IRMS. N_2 fixation estimated by $^{15}N_2$ was calculated according to Montoya et al. (1996). Considering a minimum acceptable change of $\delta^{15}N$ between the initial and the final PON sample of 4‰, the incubation

time and the detection limit of the elemental analyser used (0.75 µg N), we can set our detection limit for the $^{15}N_2$ technique at 0.001 nmol N l^{-1} d^{-1} (see Montoya et al. 1996).

The potential release of DON was taken as the difference between gross (ARA) and net ($^{15}N_2$) N_2 fixation (Gallon et al. 2002, Mulholland et al. 2004). N_2 fixation rates could be underestimated by injecting $^{15}N_2$ bubbles (Montoya et al. 1996) instead of adding $^{15}N_2$ dissolved in seawater or culture medium (Mohr et al. 2010). Hence, using the latter method might decrease the difference with the results obtained by the ARA, but whether or not this was indeed the case has hitherto not been investigated systematically. The difference between ARA and $^{15}N_2$-estimated rates is a good proxy for the release of DON, but the actual release may be smaller than reported here.

Nutrient sampling and analysis

Samples for analysis of nitrate and nitrite (NO_3^- + NO_2^-) and phosphate (HPO_4^{2-}) were collected at the same stations where N_2 fixation had been measured. In the Cape Silleiro and CAIBOX cruise legs, samples drawn into 50 ml polyethylene containers were immediately analysed on board using a Prescop Alpkem autoanalyser with detection limits of 0.1 µM for NO_3^- and 0.02 µM for NO_2^- and HPO_4^{2-}. In the Cape Ghir leg, samples were recovered in 15 ml polyethylene tubes and stored frozen at –20°C. These samples were analysed in the land-based laboratory using an AA3 Bran+Luebbe autoanalyser with detection limits of 0.01, 0.003 and 0.024 µM for NO_3^-, NO_2^- and HPO_4^{2-}, respectively. Recommendations for automated seawater nutrient analysis by Grasshoff et al. (1983) were followed.

RESULTS

Distribution and abundance of *Trichodesmium*

The density of *Trichodesmium* was low across the whole area of study; the organisms were present mainly as single trichomes. Only a few colonies were found, either as puffs or tufts (Fig. 2). Trichomes typically contained an average of 100 cells. The number of trichomes observed during the CAIBOX cruise increased from the Galician coast to the south, ranging from 0 to a maximum of 0.43 trichomes l^{-1} at Stn X15. A peak of 0.25 trichomes l^{-1} was found at Stn X8, coinciding with the Azores Front (AF), which was situated at ~37° 30 N as concluded from our CTD profiles (data not shown) and from sea-surface temperature (SST) Aqua-MODIS imagery (Fig.1). A very low number of only 0.034 trichomes of *Trichodesmium* l^{-1} was found at the last station of the CAIBOX transect (Stn X17). *Trichodesmium* densities off Cape Silleiro were low (average of 0.031 trichomes l^{-1}) and did not appear to be related to the distribution of upwelled or open-ocean waters. The abundance of single trichomes was also low off Cape Ghir, where only a maximum of 0.38 trichomes l^{-1} was found at Stn GT2; this station was situated at the edge of the upwelling filament.

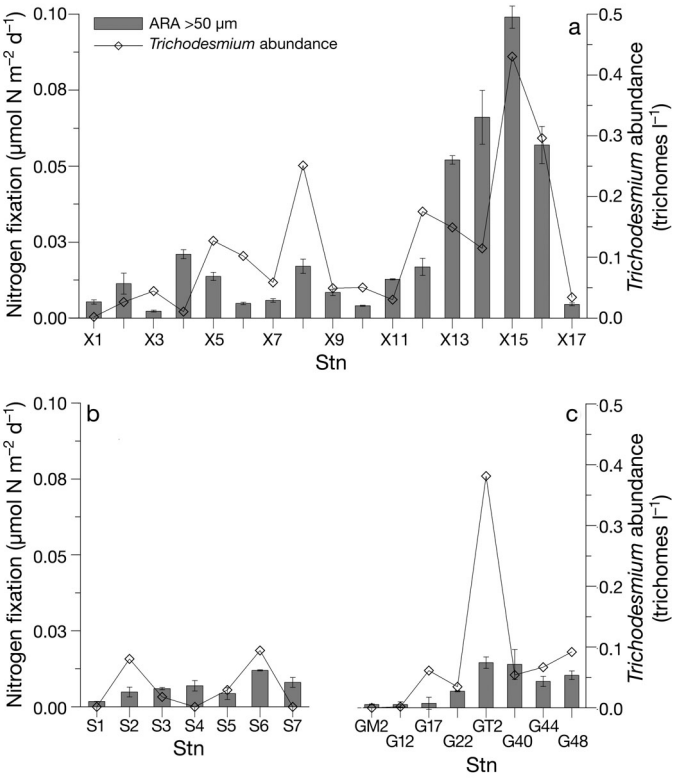

Fig. 2. Density of *Trichodesmium* (trichomes l^{-1}) and gross N_2 fixation rates of the >50 µm fraction (µmol m^{-2} d^{-1}) during (a) CAIBOX, (b) Cape Silleiro and (c) Cape Ghir cruise legs

N_2 fixation and potential DON release

Gross rates of N_2 fixation measured from net samples (>50 µm) are presented as areal rates (i.e. µmol N m^{-2} d^{-1}, Fig. 2) because the net tows sampled the whole water column above the DCM. The rates of acetylene reduction associated with the plankton net samples were low across the studied area. The rates of acetylene reduction increased towards the south-

ern part of the CAIBOX (south of the AF), coinciding with higher temperatures and lower concentrations of inorganic nutrients (Table 1), with maximum rates ranging from 0.05 to 0.1 µmol N m^{-2} d^{-1} between Stns X13 and X16. The nitrogenase activity of the >50 µm fraction decreased abruptly to 0.01 µmol N m^{-2} d^{-1} at Stn X17, coinciding with a decrease in the density of *Trichodesmium*. Because no *Cyanobacteria*–diatom symbioses were found during the inspection of the samples recovered from the plankton net, N_2 fixation in the >50 µm fraction was attributed to *Trichodesmium,* although the presence of other diazotrophs that might have been trapped due to clogging of the plankton net cannot be excluded (Proctor 1997). Also, *Trichodesmium* densities correlated well with N_2 fixation in the >50 µm fraction during the CAIBOX and Cape Ghir cruise legs (r^2 = 0.75 and 0.84, n = 17 and 8, respectively, both $p < 0.01$), but the correlation was not significant in Cape Silleiro (r^2 = 0.58, p = 0.17, n = 7). The lack of a significant correlation could be due to the small range of variability in both parameters.

The AF divided the CAIBOX area into 2 parts characterized by 0.07 ± 0.02 nmol N l^{-1} d^{-1} and 0.11 ± 0.05 nmol N l^{-1} d^{-1} average rates of gross N_2 fixation (Stns X1 to X8 and X9 to X17, respectively). The differences between the rates of N_2 fixation in both areas were statistically significant (*t*-test, p = 0.04, n = 8). These rates were also significantly related to the SST (r^2 = 0.56, $p < 0.01$; Fig. 3a). In this transect, the contribution of the >10 and <10 µm fractions to gross N_2 fixation was similar (40 to 60%), although the small fraction was responsible for ~60 to 95% of total net N_2 fixation (Table 2). Following the slight decrease in SST, N_2 fixation associated with the >10 and <10 µm fractions decreased abruptly in the last 2 stations near the coasts of the Canary Islands (Stns X16 and X17).

Gross N_2 fixation by the >10 µm fraction in the CAIBOX (open ocean) was similar to that off Cape Silleiro and Cape Ghir, while the activity of the <10 µm fraction was higher in the Cape regions than in the oceanic area sampled. Net N_2 fixation by all fractions was higher off Cape Silleiro and Cape Ghir than in the open ocean (Fig. 3b,c). A peak of gross N_2 fixation of 0.98 nmol N l^{-1} d^{-1} was observed for the <10 µm fraction at Stn G22 in Cape Ghir (not graphically represented as it exceeds the other rates by more than 2-fold; Fig. 3c). Most of the N_2 fixation was attributed to the <10 µm fraction. This fraction contributed similarly to gross and net N_2 fixation in both upwelling regions (~70 and ~90% at Cape Silleiro and Cape Ghir, respectively; Table 2). Overall, the contribution of the >10 µm fraction ranged from 0 to ~60% (Fig. 4a), whereas it ranged between ~50 and 100% for the <10 µm fraction (Fig. 4b).

Differences between gross and net N_2 fixation resulted in deviations from the theoretical C_2H_4:N_2 ratio of 4:1. The highest average ratios occurred in the southern part of the CAIBOX grid, for both the >10 and <10 µm fractions (Fig. 4). We estimate that 41.13 and 76.43% of the total gross N_2 fixed in the northern and southern part of the CAIBOX transect (north and south of the AF, respectively) was potentially released as DON. On average, almost all of the N_2 fixed by the >10 µm fraction north of the AF was potentially lost as DON (99.65%, Table 2), whereas the potential loss was only 28.53% south of the AF. The potential loss by the <10 µm fraction was higher south of the AF (Table 2). In Cape Silleiro and Cape Ghir the total potential loss of recently fixed N_2 as DON ranged between ~44 and 54%. The contribution of the <10 µm fraction was the greatest in both cases (64.07 and 89.73% in Cape Silleiro and Cape Ghir, respectively; Table 2). While the >10 µm and <10 µm fractions contributed similarly to total net N_2 fixation in Cape Silleiro and Cape Ghir, the average C_2H_4:N_2 ratios were similar for the <10 µm fraction (Fig. 4b), and the average C_2H_4:N_2 ratio for the >10 µm fraction off Cape Silleiro was higher (8.43) than off Cape Ghir (6.38) (Fig. 4a).

Table 1. Average and standard deviation values of sea surface temperature (SST; ºC), nitrate plus nitrite (NO_3^- + NO_2^-; µM) and phosphate (HPO_4^{2-}; µM). The CAIBOX cruise leg is divided into 2 parts (north and south) by the Azores Front (AF)

	SST (ºC)	NO_3^- + NO_2^-	HPO_4^{2-}
CAIBOX north of AF (Stns X1 to X8)	19.48 ± 0.61	0.16 ± 0.20	0.03 ± 0.02
CAIBOX south of AF (Stns X9 to X17)	23.45 ± 0.74	0.09 ± 0.05	0.03 ± 0.01
Cape Silleiro	16.63 ± 1.61	0.67 ± 0.70	0.05 ± 0.06
Cape Ghir	19.66 ± 1.28	2.22 ± 3.41	0.68 ± 0.31

DISCUSSION

N_2 fixation in the ocean has long been assumed to be restricted to the (sub)tropical, oligotrophic waters where the water temperature is well above 20°C (Stal 2009). Although the N_2 fixation measured during this study was lower than predicted by geochemical methods and by modelling (summarised in Mahaffey et

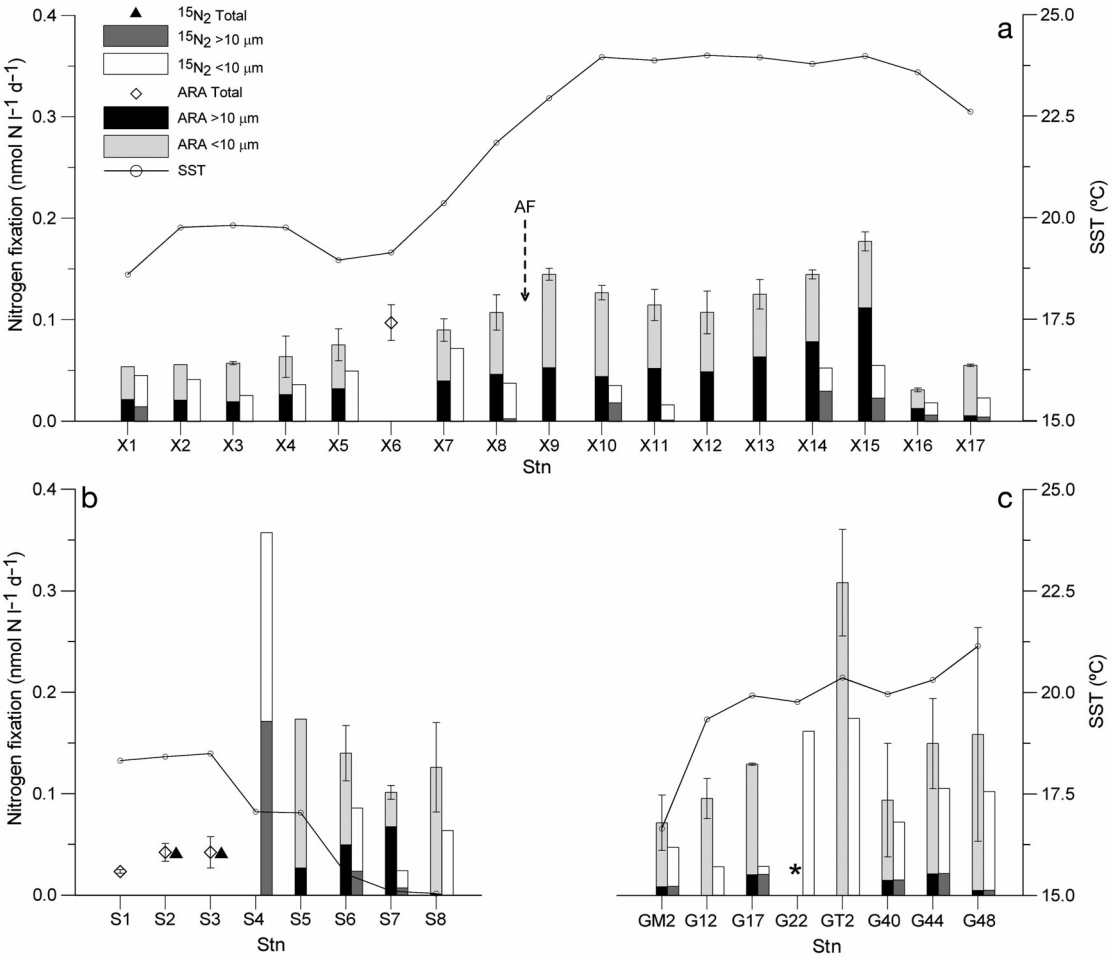

Fig. 3. Fractionated gross N_2 fixation (left bars) and net N_2 fixation (right bars) rates in (a) CAIBOX, (b) Cape Silleiro and (c) Cape Ghir cruise legs. The line represents sea surface temperature (SST; °C). ARA = acetylene reduction assay. Where fractionated rates are not available, only total rates are provided. Standard deviation bars are presented where available. The asterisk (*) in (c) represents a peak of 0.98 nmol N l^{-1} d^{-1}; it is not represented graphically because it exceeds the rest of the rates by more than 2-fold. The location of the Azores Front (AF) is shown by an arrow in (a)

al. 2005), it occurred throughout the studied area, including the upwelling sites.

The density of *Trichodesmium* was low (<0.5 trichomes l^{-1}) and peaked only at frontal sites (Figs. 1 & 2). Taking into account the buoyancy of *Trichodesmium* (Villareal & Carpenter 2003), it is plausible that trichomes accumulated in these frontal areas, where water density may change abruptly in small spatial scales due to differences in temperature, salinity and concentrations of organic matter. These *Trichodesmium* densities are similar to those obtained by other authors for the same latitudinal range as our CAIBOX transect (Fernández et al. 2010, González-Taboada et al. 2010). Discrepancies between N_2 fixation rates by organisms in the >50 µm range and *Trichodesmium* densities at some stations (Fig. 2) could have been caused by the presence of cyanobionts in copepods (Proctor 1997), the variable physiological status of *Trichodesmium* cells (LaRoche & Breitbarth 2005), or even the presence of aggregates of unicellular diazotrophs in matrices of organic compounds potentially trapped in filters or nets with larger mesh sizes (N. S. R. Agawin et al. unpubl. data).

The measurements indicate that the contribution of the >50 µm fraction to total gross N_2 fixation was always <1% (Table 2). We calculated an average cell-specific N_2 fixation rate of 0.06 ± 0.1 pmol N cell^{-1} d^{-1}, which is much lower than the ~0.22 pmol N cell^{-1} d^{-1} reported by Mulholland et al. (2006). The low densities of *Trichodesmium* and the low diazotrophic activity that we found suggest that the populations we encountered had drifted from elsewhere rather than grown actively *in situ* (LaRoche & Breitbarth 2005).

Table 2. Summary of average and standard deviation of gross and net N_2 fixation, percentage of total N_2 fixation lost as dissolved organic nitrogen (DON), and percentage contribution to total gross and total net N_2 fixation by each fraction (>10 µm and <10 µm). Average rates or contributions by the >50 µm fractions have been included only where applicable (as no net N_2 fixation by the >50 µm fraction was assayed)

	Average gross N_2 fixation (nmol N l^{-1} d^{-1})			Average net N_2 fixation (nmol N l^{-1} d^{-1})			Fixed N_2 lost as DON (% of total N_2 fixation)			Contribution to total gross N_2 fixation (%)			Contribution to total net N_2 fixation (%)	
	>10 µm	<10 µm	>50 µm (× 10^{-4})	Total	>10 µm	<10 µm	Total	>10 µm	<10 µm	>10 µm	<10 µm	>50 µm	>10 µm	<10 µm
CAIBOX north of AF (Stns X1 to X8)	0.03 ± 0.01	0.04 ± 0.01	1.47 ± 1.00	0.07 ± 0.02	0.002 ± 0.001	0.04 ± 0.02	41.13	99.65	24.2	40.25	59.75	0.22	5.67	94.33
CAIBOX south of AF (Stns X9 to X17)	0.05 ± 0.03	0.06 ± 0.02	3.87 ± 3.29	0.11 ± 0.05	0.01 ± 0.01	0.02 ± 0.01	76.43	28.53	58.12	42.28	57.72	0.44	35.72	64.28
Cape Silleiro	0.04 ± 0.03	0.10 ± 0.05	3.11 ± 8.93	0.09 ± 0.06	0.05 ± 0.08	0.08 ± 0.07	44.08	42.74	64.07	29.65	70.35	0.45	26.34	73.66
Cape Ghir	0.01 ± 0.01	0.24 ± 0.31	4.03 ± 8.23	0.25 ± 0.3	0.03 ± 0.08	0.08 ± 0.06	54.06	14.85	89.73	7.62	92.38	0.17	12.77	87.23

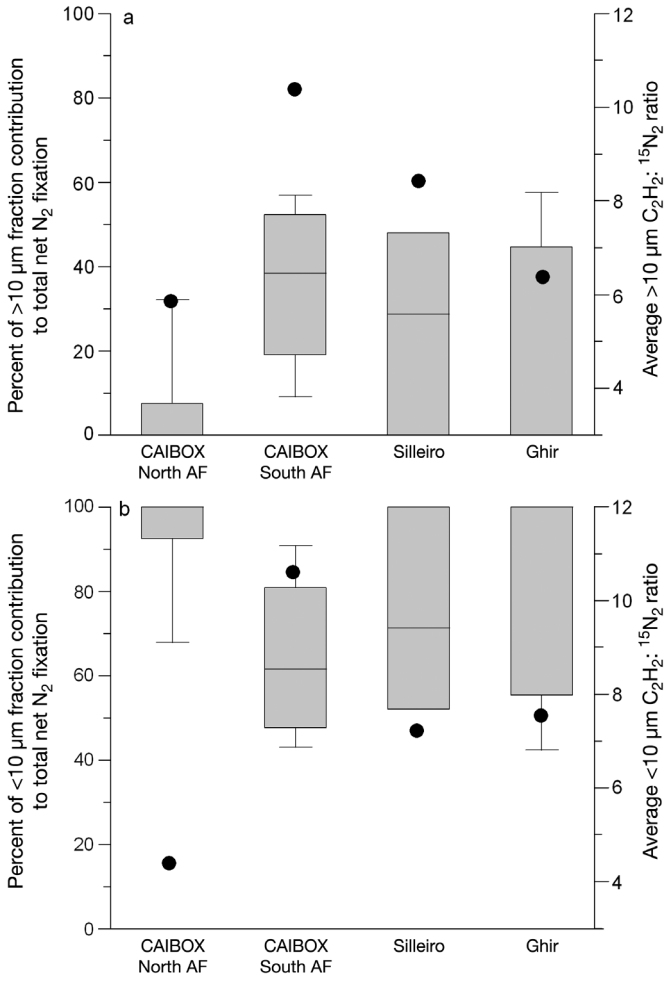

Fig. 4. Box plot summary of the (a) >10 µm fraction and (b) <10 µm fraction contribution to total net N_2 fixation rates (%). Associated measured C_2H_4:$^{15}N_2$ ratios are presented as black circles. Error bars represent the range of values recorded. The upper and lower limits of the boxes represent the upper and lower quartiles, respectively. Bands crossing the boxes represent the median

Although the contribution of unicellular diazotrophs to the oceanic nitrogen budget is not precisely known, their N_2 fixation rates may locally exceed those of *Trichodesmium* (Montoya et al. 2004, Bonnet et al. 2009). N_2 fixation by organisms of the smaller size fraction (<10 µm), potentially containing unicellular *Cyanobacteria* and heterotrophic diazotrophs, predominated over that attributed to the >10 µm fraction at all stations, confirming results obtained by other authors (Voss et al. 2004, Montoya et al. 2007) for lower latitudes of the eastern Atlantic Ocean.

In the north Atlantic Ocean, measurements of N_2 fixation have been conducted mainly in the western basin (e.g. Capone et al. 2005), thereby biasing

extrapolations to the whole of the north Atlantic. The few studies measuring N_2 fixation and abundance of diazotrophs across the whole tropical Atlantic have revealed uneven geographic distributions, with higher numbers of *Trichodesmium* in the western North Atlantic while N_2 fixation associated with unicellular diazotrophs increased towards the east (Voss et al. 2004, Montoya et al. 2007, Goebel et al. 2010, Turk et al. 2011). Indeed, the densities of *Trichodesmium* reported in the literature for the northeast Atlantic typically range from 0 to 100 trichomes l^{-1} (Tyrrell et al. 2003, Moore et al. 2009, Fernández et al. 2010, González-Taboada et al. 2010; present study), while densities in the northwest Atlantic easily reached 100 to 1000 trichomes l^{-1} (Capone et al. 1997, Mahaffey et al. 2003).

Few studies have addressed N_2 fixation in upwelling areas. Staal et al. (2007) measured N_2 fixation from 14°N to 13°S along the west African coast (1 to 20°W). They did not find N_2 fixation in the areas affected by the coastal upwelling (roughly 20 to 15°N). These authors found average N_2 fixation rates ranging from 2.2 to 3.7 µmol N m^{-2} d^{-1}, which is in the same range as reported here. Integrating our volumetric ARA values to the depth of the mixed layer (MLD), we calculate an average total ARA-derived N_2 fixation rate of 2.14 ± 0.99 and 6.08 ± 7.73 µmol m^{-2} d^{-1} in the upwelling areas of Cape Silleiro and Cape Ghir, respectively.

Staal et al. (2003) argued that a high temperature is needed to prevent oxygen deactivation of nitrogenase. This would be even more crucial for unicellular *Cyanobacteria* because of their higher surface-to-volume ratio. In contrast to this paradigm, Raimbault & Garcia (2008) found N_2 fixation rates as high as 3.6 nmol N l^{-1} d^{-1} off the Chilean upwelling system, where the water temperature was ~15°C. These authors did not detect the presence of *Trichodesmium* and therefore they attributed the measured N_2 fixation rates to nanoplanktonic and picoplanktonic diazotrophs. In our study, N_2 fixation in the small size fraction (<10 µm) was always <0.4 nmol N l^{-1} d^{-1} except for a peak of 0.98 nmol N l^{-1} d^{-1} at 1 station off Cape Ghir (Fig. 3c). The contribution of the small size fraction was generally higher in these colder and nutrient-richer waters of the northwestern Iberian and northwestern African upwelling systems than it was in the open-ocean (CAIBOX). Molecular biological studies performed during the CAIBEX cruises have confirmed that 66% of the <3 µm clones sequenced belonged to the UCYN-A group (N. S. R. Agawin et al. unpubl. results). Moreover, we cannot exclude the possibility that heterotrophic diazotrophs were present in the <10 µm fraction, although their importance for oceanic N_2 fixation is debated (Riemann et al. 2010).

The contribution of >10 µm and <10 µm diazotrophs to gross and net N_2 fixation appeared to be highly variable across the studied areas (Table 2). The percentage contribution of each fraction to total release of DON did not correlate to either water temperature or concentrations of inorganic nutrients. DON release by diazotrophs may be influenced by physical and hydrographic factors (temperature, nutrients, light, turbulence), and biological factors such as the actual physiological state of the cells, interactions with the surrounding planktonic community (virus, bacteria, grazers), or varying direct uptake of DON by other phytoplankton.

Studies performed to date indicate that the DON released by *Trichodesmium* may vary greatly among environments (see summary tables in Mulholland et al. 2006). Also, *Trichodesmium* might release DON when experiencing abrupt changes in temperature and/or light (Mulholland 2007), which might have been the case in our study area, where hydrographic features such as upwelling filaments and fronts were found. Bronk (1999) found a higher release of DON in cultures of non-diazotrophic unicellular *Cyanobacteria* in nitrogen-replete conditions, in contrast to the release of dissolved organic carbon (DOC) which usually increases in nutrient-depleted situations. However, studies on the dynamics of DON release by unicellular diazotrophic *Cyanobacteria* are lacking in the literature (Mulholland 2007), and the mechanism of release of DON may be different in diazotrophic and non-diazotrophic *Cyanobacteria*.

The difference between N_2 fixation derived from ARA and from $^{15}N_2$ assimilation has been interpreted as the release of DON (Gallon et al. 2002, Mulholland et al. 2004, 2006). Notwithstanding this, new insight into N_2 fixation techniques has demonstrated that the use of $^{15}N_2$ bubbles potentially underestimates N_2 fixation rates (especially in short incubations) due to slow dissolution of the $^{15}N_2$ into the sample water (Mohr et al. 2010). The use of $^{15}N_2$-saturated water, instead of bubbles, might decrease the difference between the ARA and $^{15}N_2$ approaches to some extent, but it will probably not invalidate the subtraction of ARA and $^{15}N_2$ fixation as a proxy for DON release (see 'Materials and Methods'). Nevertheless, this aspect deserves attention in future studies.

We have demonstrated that organisms in the <10 µm fraction are more important than *Trichodesmium* for the fixation of N_2 in the subtropical

northeast Atlantic Ocean. Gross rates of N_2 fixation found in the upwelling zones were similar to those in the open ocean. This finding increases the latitudinal and habitat range of diazotrophic organisms, even when the rates were invariably low (<0.4 nmol N l^{-1} d^{-1}).

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