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que el Consejo de Doctores del Departamento en sesión extraordinaria tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "*Magnitude and controls of*  $N_2$  *fixation in the subtropical Northeast Atlantic*" presentada por el/la doctorando/a D/Dª Mar Benavides Gorostegui y dirigida por el Dr. Javier Arístegui Ruiz y la Dra. Nona Sheila Agawin Romualdo.

Y para que así conste, y a efectos de lo previsto en el Artº 73.2 del Reglamento de Estudios de Doctorado de esta Universidad, firmo la presente en Las Palmas de Gran Canaria, a 3 de Diciembre de 2012.



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#### Magnitude and controls of $N_{\rm 2}$ fixation in the

#### subtropical Northeast Atlantic

Magnitud y controles de la fijación de nitrógeno en el Nordeste Atlántico subtropical

Tesis doctoral presentada por D<sup>a</sup> Mar Benavides Gorostegui para optar al grado de Doctor por la Universidad de Las Palmas de Gran Canaria.

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A mi abuelo Víctor

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#### Abstract

Biological N<sub>2</sub> fixation contributes significantly to new production in the oligotrophic subtropical gyres, which cover  $\sim 50\%$  of the world's oceans. It was generally assumed that diazotrophy was restricted to the warm  $(>20^{\circ}C)$  and stratified surface waters of the tropical and subtropical oceans (<200 m). Nevertheless, recent evidence indicates that diazotrophic organisms are more diverse than it was previously thought, and able to fix  $N_2$  at higher latitudes and depths where the temperatures are colder. In the last decade intense research has been devoted to the study of N<sub>2</sub> fixation rates and the diversity of diazotrophs in the different oceanic basins of the world. However, most of the studies have been concentrated in the western Atlantic Ocean, whereas its eastern half has been seldom studied. In this thesis N2 fixation and dissolved nitrogen release rates, as well as the diversity of diazotrophic organisms over different areas of the subtropical Northeast Atlantic, were studied considering the effect of various environmental factors (e.g. temperature, aerosols, nutrients). We found that N2 fixation rates in the eastern subtropical Atlantic are generally low compared to the western basin, although the eastern subtropical region provides >70% of the N<sub>2</sub> fixed in the whole North Atlantic subtropical latitudinal band. Size-fractionated experiments showed that unicellular diazotrophs (<10 µm fraction) predominate in these waters, as they typically contributed  $\sim$ 20-90% to total N<sub>2</sub> fixation. This was further confirmed by whole-cell hybridization analyses, which indicated that the predominant diazotrophic forms usually ranged from  $<1 \mu m$  to  $3 \mu m$  in size. Moreover, analyses of the *nifH* gene revealed that UCYN-A diazotrophs are especially abundant in these waters. The deposition of atmospheric dust over the Canary Islands waters had a differential effect on N<sub>2</sub> fixation rates by different groups: the diazotrophic activity of unicellular diazotrophs was enhanced after a dust deposition peak, while other diazotrophs such as Trichodesmium seemed to be unaffected. Also, proxies of atmospheric dust presence were significantly correlated with N<sub>2</sub> fixation rates in a long transect over the subtropical North Atlantic, confirming the enhancing effect of atmospheric dust on diazotrophic activity. The release of recently fixed N<sub>2</sub> as DON was measured using stable isotope techniques. We found that cultured unicellular diazotrophs do not release DON significantly (only  $\sim 1\%$  of the total N<sub>2</sub> fixed), while natural populations release  $\sim$ 23%. Finally, the comparison of N<sub>2</sub> fixation rates with new production rates indicated that the relative contribution of diazotrophy to biological production in our study area is low (<1%), at least in surface waters (<100 m). This result can change however if N<sub>2</sub> fixation measurements are extended to higher depths, where recently discovered heterotrophic diazotrophs thrive.

#### Resumen

La fijación de N<sub>2</sub> biológica contribuye significativamente a la producción nueva en los giros subtropicales oligotróficos, que cubren ~50% del océano global. Hasta hace poco se asumía que la actividad diazotrófica estaba restringida a las aguas superficiales de los océanos tropicales y subtropicales (<200 m), pero sin embargo, estudios recientes demuestran que los organismos diazótrofos son más diversos de lo que se creía, y pueden fijar N<sub>2</sub> a latitudes y profundidades donde las temperaturas son más frías. En la última década se ha invertido mucho esfuerzo científico en medir tasas de fijación de N<sub>2</sub> y estudiar la diversidad de diazótrofos por todos los océanos. A pesar de ello, la mayoría de estos estudios se han centrado en el Atlántico Noroeste, quedando la mitad Nordeste mucho menos estudiada. En esta tesis nos hemos centrado en el Atlántico Nordeste subtropical para aportar nuevas medidas de tasas de fijación de N2, exudación de nitrógeno disuelto y diversidad de diazótrofos en esta zona, teniendo en cuenta el efecto de varios factores ambientales (e.g. temperatura, aerosoles, nutrientes). Hemos observado que las tasas de fijación de N2 en el Atlántico Nordeste subtropical son generalmente bajas, en comparación con la cuenca Oeste. A pesar de ello, la zona del Atlántico Este subtropical aporta >70% del N<sub>2</sub> fijado en todo el rango latitudinal del Atlántico Norte subtropical. Mediante experimentos en los que las muestras se fraccionaban según tamaño, vimos que los diazótrofos unicelulares (<10 μm) predominan en estas aguas, ya que aportan un  $\sim$ 20-90% a la fijación de N<sub>2</sub> total. Mediante análisis de hibridación observamos que la mayoría de las células tenían un tamaño entre <1 µm y 3 µm. También, mediante análisis del gen *nifH* vimos que las cianobacterias unicelulares del grupo A (UCYN-A) son la forma predominante en esta aguas. Además, estudiamos la deposición de polvo atmosférico sobre las islas Canarias, y comprobamos que afecta de forma distinta a distintos grupos de diazótrofos. Tras un evento de deposición de polvo atmosférico, la fijación de N<sub>2</sub> en diazótrofos unicelulares aumentó ~90%, mientras que Trichodesmium pareció no verse afectado. En un transecto cruzando el Atlántico Norte sobre el paralelo 24.5°N, también vimos que el AOD 550 nm (un indicador de presencia de polvo en la atmósfera) se correlaciona significativamente con las tasas de fijación de N<sub>2</sub>, de manera que podemos confirmar que los aerosoles tienen un efecto sobre la actividad diazotrófica. También hemos medido la exudación de DON mediante técnicas de isótopos estables. En diazótrofos unicelulares cultivados bajo condiciones óptimas de luz y temperatura, la exudación de DON apenas representa  $\sim 1\%$  del N<sub>2</sub> fijado total. Sin embargo, poblaciones naturales de diazótrofos unicelulares exudan ~23%. En último lugar, comparamos las tasas de fijación de N<sub>2</sub> con tasas de producción nueva en nuestra zona de estudio, y comprobamos que las primeras aportan <1% a las segundas, al menos en aguas superficiales (<100 m). Este resultado podría verse modificado si las estimas de fijación de  $N_2$  se extendieran a mayores profundidades, donde habitan los recientemente descubiertos fijadores de N2 heterotróficos.

PART I: Introduction

#### **General introduction**

#### Oceanic N<sub>2</sub> fixation and its protagonists

The ocean is the largest ecosystem on Earth. It covers 71% of the Earth's surface and it provides roughly half of its primary production (Barange et al., 2010). Despite the high productivity of the oceans in a global basis, there are wide regions where biological productivity is low, the so-called 'ocean deserts' (Fig. 1). In the open ocean subtropical gyres, the water column remains stratified due to intense solar heating throughout the year. Stratification hinders the upwelling of nutrient-rich deep waters to the surface and hence restricts primary production in the upper water column, where enough light is available for photosynthesis.



Fig. 1. Global distribution of Chlorophyll *a* (Chl a). Dark blue areas are the least biologically productive of the ocean. Extracted from the National oceanic and atmospheric administration (NOAA) website (http://www.noaanews.noaa.gov/stories2008/20080305\_oceandesert.html).

Open ocean biological production is largely limited by the availability of nitrogen (Falkowski, 1997). There, the main sources of nitrogen are the upwelling and diffusion of deep waters enriched in nitrate ( $NO_3^{-}$ ), the deposition of atmospheric nitrogenous compounds and the biological fixation of atmospheric N<sub>2</sub> (Gruber, 2008). Although dinitrogen ( $N_2$ ) is the main component of our atmosphere (78%), it is mainly unavailable to primary producers. This is caused by the high amount of energy required to break the triple bond linking both nitrogen atoms of the N<sub>2</sub> molecule. Only a restricted group of organisms are able to carry out biological N<sub>2</sub> fixation: the N<sub>2</sub> fixers or *diazotrophs* (from the greek *dis* -bis-, *azōos* -inert-, and *trophikos* -nutrition-). These organisms contain a nitrogenase enzyme complex, composed of two proteins: dinitrogenase (an iron-molybdenum protein encoded by the *nifDK* gene), and dinitrogenase-reductase (an iron protein encoded by the *nifH* gene) (Postgate, 1982). Molybdenum is sometimes replaced by vanadium or iron in some alternative nitrogenases. The nitrogenase enzyme complex allows diazotrophs to reduce N<sub>2</sub> to ammonia (NH<sub>3</sub>) under the following stoichiometry:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$
 (1)

As previously mentioned, the biological fixation of  $N_2$  requires a high amount of energy (16 ATP). Where it occurs, this process enriches the food web through the leakage of ammonium ( $NH_4^+$ ) and amino acids that serve as a source of nitrogen for autotrophic non-diazotrophic phytoplankton (Karl et al., 2002).

Studying the nitrogen cycle is of major interest. Microbes control the oceanic nitrogen cycle through a set of gain and loss processes, and sustains the marine trophic web (Fig. 2). Inorganic nitrogen is fixed (or 'gained') through N<sub>2</sub> fixation and the assimilation of  $NO_{3^-}$ , nitrite ( $NO_{2^-}$ ) and  $NH_{4^+}$ , and remineralized (or 'lost') through ammonification, denitrification and anaerobic ammonium oxidation (anammox).

The study of  $N_2$  fixation has become of great interest not only because it fuels primary production in large oceanic areas (Capone et al., 2005), but also because of its critical role in balancing the oceanic nitrogen cycle. Indeed,  $N_2$ fixation versus denitrification and anammox are the processes thought to maintain the fixed nitrogen reservoir balance in the ocean (Codispoti, 2007). Present estimates indicate that nitrogen loss via denitrification and anammox in suboxic and anoxic zones far exceed rates of  $N_2$  fixation in surface waters (Table 1). However, current improvements in the methodology used to estimate  $N_2$  fixation (Mohr et al., 2010) points toward a smaller difference between fixed nitrogen gains and losses, which could eventually balance the oceanic fixed nitrogen budget (Großkopf et al., 2012).



Fig. 2. The marine nitrogen cycle. Modified from http://bit.ly/wC3xZo

Recent studies have shown that planktonic diazotrophs are more diverse (Fig. 3) and more widely distributed than previously thought (Zehr et al., 2001, 2008; Moisander et al., 2010; Riemann et al., 2010; Farnelid et al., 2011). **Filamentous heterocystous cyanobacteria**, like *Anabaena*, *Aphanizomenon* and *Nodularia*, are frequently present in estuaries as well as in semi-enclosed seas, like the Baltic Sea, but are rare in open ocean waters (Zehr, 2011).

**Non-heterocystous filamentous cyanobacteria** such as *Katagnymene* and *Trichodesmium* have been observed, however, in open ocean regions (e.g. Langlois et al., 2005), although the species of *Trichodesmium* are undoubtedly the most abundant forms. This cyanobacterium is ubiquitous in the tropical and subtropical oceans, where it often forms massive blooms. The high abundances found in these regions lead to the thought that it was the principal N<sub>2</sub> fixer in the oceans. Its diazotrophic activity was discovered in the early 60s (Dugdale et al., 1961) and, ever since, *Trichodesmium* has been the focus of intensive research. As a result, we now have fairly good knowledge of its nutritional (e.g. Berman-Frank et al., 2001a; Dyhrman et al., 2002), temperature (e.g. Breitbarth et al., 2007) and physical (Davis and McGillicuddy, 2006) controls, global distribution (Luo et al., 2012), and potential responses to increased atmospheric CO<sub>2</sub> levels (e.g. Hutchins et al., 2007). A recent review by Bergman et al. (2012) covers all of these aspects.

Another group frequently targeted in  $N_2$  fixation studies are **symbiotic diazotrophs**, such as the cyanobacteria *Richelia* and *Calothrix* which are symbionts of the diatoms *Rhizosolenia*, *Hemiaulus* or *Chaetoceros* (e.g. Foster et al., 2011).

Process	Codispoti et al. (2001)	Galloway et al. (2004)	Gruber (2004)
Sources			
Pelagic Na fixation	117	106	120
Benthic $N_2$ fixation	15	15	15
River input	76	48	80
Atmospheric deposition	86	33	50
Total	294	202	265
	·		
Sinks			
Organic nitrogen export	1		1
Benthic denitrification	300	206	180
Pelagic denitrification	150	116	65
Sediment burial	25	16	25
N <sub>2</sub> O loss to atmosphere	6	4	4
Total	482	342	275
Sources - sinks	-188	-140	-10

Table 1. Global estimates of nitrogen sinks and sources. Modified from Gruber (2008). All rates are in Tg N y $^{-1}$ .

These symbiotic relationships may contribute significantly to the oceanic carbon 'biological pump'. Indeed, diatom-diazotroph associations have been recently identified as the predominant cause for particulate matter export peaks during the summer in the North Pacific Ocean (Karl et al., 2012). Other less studied symbiosis of the pelagic habitat are those of anaerobic diazotrophic bacteria and copepods (Proctor, 1997).

Recently, the application of molecular biology techniques has allowed the identification of a new wide set of diazotrophs through the detection of the nifH gene. Zehr et al. (1998; 2001) were the first to report the presence of unicellular diazotrophic cyanobacteria (UCYN) in the North Pacific Ocean. In the following years, UCYN of groups A, B and C (UCYN-A, UCYN-B and UCYN-C) were detected throughout the Atlantic (Langlois et al., 2005, 2008) and the Pacific Oceans (Needoba et al., 2007), where they occasionally exceeded Trichodesmium N<sub>2</sub> fixation rates (Falcón et al., 2004; Montoya et al., 2004; Benavides et al., in press). The discovery of UCYN-A was especially remarkable. This yet-uncultivated small organism (<1  $\mu$ m) is now recognized to be the most abundant marine diazotrophic cyanobacterium (Luo et al., 2012). In comparison to Trichodesmium and other diazotrophs which need warm waters to grow (generally  $>20^{\circ}$ C), UCYN-A have been detected at higher latitudes and depths (Moisander et al., 2010), and are even present in upwelling systems where the temperatures are often below 17°C and fixed nitrogen concentrations are high (Sohm et al., 2011b; N.S. Agawin, unpublished results).

A remarkable peculiarity of UCYN-A is their photoheterotrophic metabolism. These organisms lack genes for the oxygen-evolving photosystem II and carbon fixation (Zehr et al., 2008), hence they are believed to rely on compounds produced by other organisms (Tripp et al., 2010). Indeed, Thompson et al. (2012) in a very recent study found UCYN-A in symbiosis with a prymnesiophyte, confirming earlier suspicions of their mode of life. These authors have proposed the name *Candidatus atelocyanobacterium* thalassa for the UCYN-A.



Fig. 3. Phylogenetic diversity of the *nifH* gene from marine planktonic diazotrophs. From Riemann et al. (2010).

Besides UCYN, the global diazotrophic domain is further extended to **non-cyanobacterial diazotrophs**, which are found in all four clusters of the *nifH* gene (Chien and Zinder, 1996). They belong mainly to  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -proteobacteria, and less frequently to Archaea and anaerobic bacteria (Riemann et al., 2010). Recent evidence indicates that non-cyanobacterial *nifH* is more abundant and diverse than UCYN in global ocean surface waters

(Farnelid et al., 2011), while heterotrophic N<sub>2</sub> fixation dominates in the South Pacific Ocean Gyre (Halm et al., 2011). Non-cyanobacterial *nifH* is also the most abundant below 200 m depth in the Sargasso Sea (Hewson et al., 2007), and N<sub>2</sub> fixation rates up to 0.3 nmol L<sup>-1</sup> d<sup>-1</sup> associated with heterotrophs have been measured in hypoxic basins in the Southern California Bight (Hamersley et al., 2011). All this suggests that typical *nifH* measurements in the upper water column underestimate the real importance of non-cyanobacterial diazotrophy (Riemann et al., 2010). Nevertheless, although measurements of non-cyanobacterial *nifH* abundance and distribution are increasing, the contribution of these organisms to global N<sub>2</sub> fixation remains unknown.

#### Controls and limitations

There are several environmental factors that control oceanic N<sub>2</sub> fixation, but the extent to which each one of these limits or enhances N<sub>2</sub> fixation varies among diazotrophic species. Overall, the most obvious limiting factor is oxygen (which is a byproduct of photosynthesis). Oxygen deactivates the nitrogenase enzyme complex, inhibiting diazotrophic activity (Postgate, 1982). Photosynthetic diazotrophic organisms have developed different strategies to overcome this issue. Some filamentous cyanobacteria bear heterocysts that create a microanaerobic environment and thus are well adapted to N<sub>2</sub> fixation in oxygenated waters. However, most oceanic photosynthetic diazotrophic cyanobacteria are non-heterocystous (Zehr, 2011), and must find other solutions to fix N<sub>2</sub> in the presence of oxygen. Single-celled diazotrophs such as Crocosphaera or Cyanothece confine  $N_2$  fixation to the night, when photosynthesis and subsequent oxygen production are absent. Trichodesmium lacks heterocysts but surprisingly fixes CO<sub>2</sub> and N<sub>2</sub> simultaneously during the day (Capone et al., 1997; Berman-Frank et al., 2001b). This cyanobacterium is able to fix N<sub>2</sub> in the presence of oxygen by a combined spatial and temporal segregation strategy. Temporal segregation strategies such as transient modifications of the nitrogenase enzyme complex or its rapid turnover have been discussed (Capone et al., 1997). Another possible strategy is the reduction of cellular oxygen by 'overfixation' of carbon, which likely explains the high carbon-to-nitrogen (C:N) ratios found in these diazotrophs (Mulholland, 2007). Moreover, Berman-Frank et al. (2001b) found that N<sub>2</sub> fixation takes place in a different time frame than photosynthesis during the day. Spatial segregation is provided by structural differences along the trichomes: the filaments have zones where cells are light and less granulated. These cells termed diazocytes are the place where the nitrogenase is found (Bergman et al., 2012). Once N<sub>2</sub> is fixed, it is rapidly distributed along the whole trichome (Finzi-Hart et al., 2009). There is a wide body of literature addressing N<sub>2</sub> fixation limitation by **temperature**. Because high water temperatures prevent oxygen dissolution and enhance cell respiration, temperature is thought to constrain the global distribution of diazotrophic cyanobacteria (Staal et al., 2003; Stal, 2009). For example, the optimum temperature for the growth of *Trichodesmium* has been estimated at  $27^{\circ}C$  (Breitbarth et al., 2007), which confines its growth to the tropical and subtropical oceans. Although UCYN are also most abundant in the tropical and subtropical oceans (Luo et al., 2012), the presence of UCYN-A and heterotrophic diazotrophs at higher latitudes and depths (Moisander et al., 2010), in cold-water upwelling systems (Sohm et al., 2011b), and even in the Arctic (Blais et al., 2012) indicates that the diazotrophic latitudinal range is wider.

 $N_2$  fixation may also be limited by the in situ availability of **fixed nitrogen** (e.g. Krishnamurthy et al., 2007). Additions of  $NO_3$ - suppress the expression of *nif* genes, and the production of diazocytes in *Trichodesmium* (Mulholland et al., 2001; Holl and Montoya, 2005). In the case of UCYN, Dekaezemacker and Bonnet (2011) studied the effect of  $NO_3$ - and  $NH_4$ + additions on the diazotrophic activity of two cultured strains of *Crocosphaera*. They found that  $N_2$  fixation rates decrease with increasing concentrations of  $NH_4$ +, while the rates were not affected by any additions of  $NO_3$ -. This may explain why UCYN are usually found at greater depths in the water column than *Trichodesmium*, and even in coastal upwelling systems (Sohm et al., 2011b).

UCYN-A may rely on dissolved organic matter (DOM) as a nutrient source (Tripp et al., 2010), and therefore their nitrogenase activity is likely unaffected by high inorganic nitrogen in situ concentrations. Indeed, UCYN-A are dominant in the northwest African upwelling system off Cape Ghir (~30- $31^{\circ}$ N) (N.S.R. Agawin, unpublished results), where NO<sub>3</sub>- concentrations are usually >2  $\mu$ M (Benavides et al., 2011).

On the other hand, the availability of other nutrients such as **iron and phosphorus** influences  $N_2$  fixation. Diazotrophs have very high iron cellular quotas (e.g. Berman-Frank et al., 2001a). More importantly, iron is the major cofactor of the nitrogenase reductase, which makes  $N_2$  fixation directly dependent on iron availability. Iron reaches the open ocean mainly through the deposition of atmospheric dust, although this flux can be overpassed by vertical diffusive mixing (Rijkenberg et al., 2012).

Due to the proximity of the Sahara desert, the eastern Atlantic receives the greatest inputs of desert dust in the world's ocean (Prospero, 1981; Fig. 4). The distribution of Saharan dust deposition over the North Atlantic (Fig. 4) closely matches the distribution of diazotrophic activity (Fig. 5), suggesting a connection between iron availability and  $N_2$  fixation.



Fig. 4. Global distribution of average dust deposition (g m<sup>-2</sup> y<sup>-1</sup>). From Jickells et al. (2005).

This has been further confirmed experimentally by adding dust to seawater samples and by correlations between  $N_2$  fixation rates and in situ dissolved iron concentrations (e.g. Mills et al., 2004; Moore et al., 2009; Fernández et al., 2010).



Phosphorus is needed for energy-carrying compounds such as ATP or NADPH. Phosphorus is available at low concentrations in the open ocean and frequently limits  $N_2$  fixation in *Trichodesmium* (e.g. Hynes et al., 2009) and UCYN such as *Crocosphaera* (Dyhrman and Haley, 2006). To overcome this limitation, *Trichodesmium* bears enzymatic systems capable of uptaking organic phosphorus (phosphonates; Dyhrman et al., 2006) or inorganic phosphate (PO<sub>4</sub><sup>3-</sup>) (Dyhrman et al., 2002). *Crocosphaera* cannot uptake organic phosphorus forms but is capable of inducing a high affinity PO<sub>4</sub><sup>3-</sup> binding system when phosphorus supply is low (Dyhrman and Haley, 2006).

**Physical forcing** controls the availability of inorganic nutrients in the upper ocean through mixing, upwelling and vertical diffusion. In contrast to autotrophic phytoplankton species, whose growth depends on  $NO_3$ -transported from deep waters or  $NH_4^+$  regenerated in situ, diazotrophic organisms are capable of growing on  $N_2$  as the only source of nitrogen. Theoretically, this sets out a different (with less competition) scenario for these organisms, which would grow better in the core of warm and nutrient-poor stratified waters, than in cold nutrient-rich waters.

Although the role of physical forcing on N<sub>2</sub> fixation has been seldom studied, previous research indicates that mesoscale features and associated density fronts influence N<sub>2</sub> fixation rates and diazotrophic organism diversity to some extent (Sohm et al., 2011c). Holl et al. (2007) measured greater N<sub>2</sub> fixation rates in the core of warm-core eddies than in the core of cold-core eddies off the west coast of Australia. In an anticyclonic eddy in the North Pacific Ocean close to station ALOHA (22°45' N, 158°W), Fong et al. (2008) found relatively high concentrations of filamentous cyanobacteria and UCYN across the eddy, and enhanced N<sub>2</sub> fixation rates towards its edge. Church et al. (2009) gathered monthly measurements at station ALOHA from 2004 to 2007 and detected an increase in  $N_2$  fixation rates coinciding with periods of positive sea surface height anomaly (SSHA). Davis and McGillicuddy (2006) found high abundances of Trichodesmium associated with anticyclonic eddies in a cross-Atlantic cruise. Similarly, Benavides et al. (2011) found N<sub>2</sub> fixation peaks associated with density fronts and high concentrations of the filamentous diazotroph Trichodesmium at the edge of an upwelling filament in the Northeast Atlantic. Also, because *Trichodesmium* is a buoyant organism (Villareal and Carpenter, 2003) adapted to stratified warm waters, high turbulence is known to prevent its proliferation (Carpenter and Price, 1976). All these studies suggest that physical forcing exerts a clear influence on N<sub>2</sub> fixation activity and diazotrophic organisms' distribution.

#### Measuring N<sub>2</sub> fixation

There are two main approaches to estimate  $N_2$  fixation: the 'geochemical' methods, and the 'biological' (or 'direct') methods.

**Geochemical methods** rely on the study of the horizontal and vertical distribution of particulate and dissolved nitrogen pools that have a chemical signature indicative of N<sub>2</sub> fixation. There are two principal geochemical methods based on the estimation of the N\* and  $\delta^{15}N$  parameters.

The N\* parameter is based on the relative concentrations of NO<sub>3</sub>- and PO<sub>4</sub><sup>3-</sup> in seawater. Redfield et al. (1963) established that carbon, nitrogen and phosphorus are taken up and remineralized at a constant ratio of 106:16:1 (C:N:P). N<sub>2</sub> fixation and denitrification are not paralleled by equivalent inputs or losses of phosphorus. Therefore, N<sub>2</sub> fixation raises N:P ratios, while denitrification lowers them, i.e.: nitrogen production in excess of the Redfield stoichiometry (N:P>16) is indicative of N<sub>2</sub> fixation, and nitrogen removal in deficit of the Redfield stoichiometry (N:P<16) is indicative of N\* parameter, which measures the concentration of NO<sub>3</sub>- in excess (or deficit) of that expected from the remineralization of PO<sub>4</sub><sup>3-</sup> at Redfield stoichiometries, following the general formulation below:

$$N^* = [NO_3^{-}] - 16[PO_4^{3-}]$$
 (2)

where  $[NO_{3}]$  and  $[PO_{4}]$  are the concentrations of  $NO_{3}$  and  $PO_{4}$ , respectively. This parameter was firstly introduced in the 90s by Michaels et al. (1996). The equation was amended by adding constants that bring the global N:P ratio to 16 and the intercept to zero (Michaels et al., 1996; Gruber and Sarmiento, 1997). Extensive nutrient databases from projects such as WOCE or GEOSECS have been used to estimate the distribution and magnitude of N\* in the oceans.

The second geochemical method most commonly used is based on the  $\delta^{15}N$  parameter. This parameter measures the relative abundance of the nitrogen isotopes  $^{14}N$  and  $^{15}N$  in a sample with respect to standard atmospheric N<sub>2</sub> (Equation 3):

$$\delta^{15}N = \left[ (^{15}N/^{14}N)_{\text{sample}} / (^{15}N/^{14}N)_{\text{standard}} - 1 \right] \times 1000$$
(3)

Atmospheric N<sub>2</sub> has a  $\delta^{15}N \sim 0.6\%_0$ , while NO<sub>3</sub><sup>-</sup> has a  $\delta^{15}N \sim 5\%_0$  (Karl et al., 2002). The  $\delta^{15}N$  of a given dissolved or particulate nitrogen pool is given by the isotopic composition of its source and the isotopic fractionation experienced along its assimilation. Therefore, organic nitrogen produced by N<sub>2</sub> fixation is depleted in  $\delta^{15}N$ , while higher  $\delta^{15}N$  values are expected in organic nitrogen produced by the assimilation of NO<sub>3</sub><sup>-</sup>.

In general, geochemical methods have the disadvantage that N<sub>2</sub> fixation signatures (N\*>2.5 µmol kg<sup>-1</sup> or low  $\delta^{15}$ N values) can be caused by processes other than N<sub>2</sub> fixation. For example, high N:P fixed nitrogen reaches the ocean via atmospheric deposition of anthropogenic materials derived from fossil fuel combustion and agriculture fertilizers (Zamora et al., 2010). Moreover, this anthropogenic atmospheric nitrogen has low  $\delta^{15}$ N values too (Hastings et al., 2003). Other misinterpretations stem from isotopic fractionation during nitrogen cycling (Altabet, 1988). Despite these drawbacks, the geochemical methods have the advantage that they can be used in large oceanic regions at higher spatial resolutions than it is possible using biological methods.

The **biological methods** measure the actual amount of N<sub>2</sub> fixed by in situ diazotrophic organisms during a given period of incubation. In oceanographic cruises, incubations are generally performed on deck on light-adapted incubators cooled with surface seawater, or alternatively using in situ free-floating arrays. There are two principal biological methods: the acetylene reduction assay (ARA) and the assimilation of the stable isotope <sup>15</sup>N<sub>2</sub>.

The ARA measures N<sub>2</sub> fixation indirectly relying on the fact that the nitrogenase enzyme is capable of reducing acetylene, a triple-bonded molecule structurally comparable to N<sub>2</sub>. The reduction of acetylene to ethylene can be directly compared with the reduction of N<sub>2</sub> to NH<sub>4</sub><sup>+</sup>. To convert ethylene produced to N<sub>2</sub> fixation fixed, a conversion ratio must be applied. The common theoretical ratios used are 3:1 or 4:1 (C<sub>2</sub>H<sub>4</sub>:N<sub>2</sub>), but empirically obtained ratios are usually higher (Mulholland et al., 2004, 2006; Benavides et al., 2011). The use of the ratio 3:1 or 4:1 depends on whether the recycling of hydrogen is considered or not. Hydrogen is an obligate byproduct of N<sub>2</sub> fixation (see Equation 1). Two electrons are used when reducing acetylene to ethylene, while eight electrons are used to reduce N<sub>2</sub> to 2NH<sub>3</sub>, yielding a conversion factor of 4:1 (Equation 5). The by-produced hydrogen can be recycled through an uptake hydrogenase enzyme, using two electrons less and yielding a 3:1 ratio (Equation 6).

$$C_2H_2 + 2e^- + 2H^+ \rightarrow C_2H_4 \tag{4}$$

$$N_2 + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2$$
 (5)

$$N_2 + 6e^- + 6H^+ \rightarrow 2NH_3$$
 (6)

Hydrogen is only recycled under a limited availability of reducing equivalents. The lack of reducing equivalents implies that oxygen cannot be efficiently scavenged from the cell through respiration. This means that the suboxic conditions needed for  $N_2$  fixation would not be met. Thus, this is not usually the case in cyanobacteria and a ratio of 4:1 is more likely (Jensen and Cox, 1983; Stal, 1988).

The ARA has been successfully used in a variety of marine environments and cultures of diazotrophic organisms. However, the method has a number of methodological issues that should be considered:

The use of  $N_2$  by the nitrogenase enzyme is inhibited in the presence of saturating acetylene. Therefore, it is plausible that the use of acetylene to assay the nitrogenase enzyme activity may alter the cell's nitrogen metabolism itself (Capone, 1993). This problem can be overcome using short-term incubations (Stal, 1988). Another usual problem is the low dissolution of acetylene and ethylene into water samples. This is usually solved using a high headspace-to-liquid volume ratio. An additional issue is gas diffusion limitation in cell suspension samples (cultures or natural planktonic samples) caused by  $O_2$  and  $CO_2$  gradients. This is why sample filtration onto slightly humidified filters is usually preferred for planktonic samples, which allows a direct contact of the organisms with the gas phase (Staal et al., 2001).

The other principal biological method is the stable isotope tracer  ${}^{15}N_2$ . This method was first introduced by Burris and Miller (1941), but it was not until years later that isotope ratio mass spectrometers (IRMS) were sensitive enough to measure open ocean low N<sub>2</sub> fixation rates accurately, and high purity  ${}^{15}N_2$  was commercially available. The protocol established by Montoya et al. (1996) has been widely used in the last decade, yielding a large amount of N<sub>2</sub> fixation data, which are particularly abundant in the North Atlantic and North Pacific Oceans (Luo et al., 2012). Briefly, the method consists on injecting a volume of  ${}^{15}N_2$  to a seawater sample, which is incubated for a given period (on deck or in situ), and finally filtered through glass fiber filters. The filters are later analyzed by IRMS and the amount of  ${}^{15}N_2$  transferred from the aqueous phase to the particulate cell material can be determined.

Recently, the method has been proven to underestimate  $N_2$  fixation rates

to a variable extent. One of the major assumptions of the  ${}^{15}N_2$  methods is that the atom %  ${}^{15}N$  enrichment remains constant throughout the incubation period (Fry, 2006). However, this condition is not met when  ${}^{15}N_2$  is injected as a gas bubble. Mohr et al. (2010) found that the  ${}^{15}N$  atom % enrichment increases with time as the  ${}^{15}N_2$  bubble dissolves into the water sample (Fig. 6). When the 'bubble method' is applied, the theoretical complete dissolution of the volume of  ${}^{15}N_2$  injected to the sample is calculated using the gas dissolution equations provided by Weiss (1970). However, if the  ${}^{15}N_2$  bubble is not fully dissolved at the start of the incubation, the real  ${}^{15}N$  enrichment of the source N<sub>2</sub> pool is lower than theoretically expected, which in turn miscalculates N<sub>2</sub> fixation rates according to the following equation:

 $N_2$  fixation = [(at%XS PON)/ (at%XS source x incubation time)] x [PON] (7)

where at%XS is the atom % enrichment in excess of natural  $^{15}N$  abundance (0.36%) of the particulate organic pool (PON) or the N<sub>2</sub> source, and [PON] is the concentration of particulate organic nitrogen.



Fig. 6. Measured dissolved  ${}^{15}N_2$  concentrations as percentage of calculated concentration assuming complete dissolution when  ${}^{15}N_2$  is added as bubble (white circles) or dissolved in seawater (grey circles) over a 24 h incubation period. The grey circles show that the  ${}^{15}N_2$  enrichment is constant when  ${}^{15}N_2$  is added dissolved in seawater instead of as a bubble. Adapted from Mohr et al. (2010).

Mohr et al. (2010) used cultures of the unicellular diazotrophic cyanobacterium *Crocosphaera* to compare N<sub>2</sub> fixation rates obtained by both methods. The resulting N<sub>2</sub> fixation rates using the bubble method were underestimated by 60% compared to the dissolved method. This significant finding emphasized the urgent need to apply the new method on field samplings and to recalculate previously published N<sub>2</sub> fixation rates measured using the bubble method. With this purpose, a workshop was held at the GEOMAR - Helmholtz Centre for Ocean Research in Kiel (Germany) in February 2012. Experts on the field presented recent results of experiments where both methods were compared and discussed on the best approach to prepare dissolved <sup>15</sup>N<sub>2</sub> to be used in field trips.

To date, only a few comparisons of the two methods have been published. Wilson et al. (2012) made comparisons with natural waters at station ALOHA. These authors measured a 2 - 6 fold increase of N<sub>2</sub> fixation rates when <sup>15</sup>N<sub>2</sub> was added dissolved in seawater compared to when it was added as a bubble. In the Atlantic Ocean, Großkopf et al. (2012) measured large underestimations too. There are several reasons why the bubble method may underestimate true  $N_2$  fixation rates: (1) the water temperature -high temperatures preclude the dissolution of gasses-, (2) the agitation of the incubation bottles, (3) the volume of the incubation bottle, (4) the volume of  $^{15}N_2$  injected, (5) the duration of the incubation, (6) the time the incubation starts relative to the onset of nitrogenase activity -which differs between diazotroph species-, and (7) the DOM coating of the <sup>15</sup>N<sub>2</sub> bubble (Mohr et al., 2010). Adding to these factors, Großkopf et al. (2012) found that underestimations are lower when the community is dominated by Trichodesmium (62%), and higher when diazotrophs other than Trichodesmium (symbionts, unicellular cvanobacteria, heterotrophic diazotrophs) predominate (up to 570%). All these factors vary widely among the previously published works, making the recalculation of N<sub>2</sub> fixation rather impossible (Großkopf et al., 2012).

Less attention has been paid to another important factor that underestimates N<sub>2</sub> fixation rates: the release of recently fixed N<sub>2</sub>. Glibert and Bronk (1994) measured dissolved organic nitrogen (DON) release in natural populations of *Trichodesmium*. These authors found that on average 50% of the N<sub>2</sub> *Trichodesmium* fixed was released as DON. This 'loss' of fixed N<sub>2</sub> is remarkable and remains unaccounted for because, commonly, only particulate materials are recovered in field and culture N<sub>2</sub> fixation experiments, leading to underestimations of the true N<sub>2</sub> fixation rate (Bronk et al., 1994; Glibert and Bronk, 1994). The unpopularity in measuring DON release probably stands on its difficulty. A simple procedure was proposed by Gallon et al. (2002) and Mulholland et al. (2004). These authors used the difference between ARAderived and  ${}^{15}N_2$  bubble-derived N<sub>2</sub> fixation rates as a proxy for recently fixed nitrogen release. Alternatively, DON release can be estimated by measuring the  ${}^{15}N$  atom % enrichment of the DON pool. Slawyk and Raimbault (1995) proposed a method consisting of extracting the  ${}^{15}N$  of dissolved inorganic and organic nitrogen pools in several steps under high pH and high temperature conditions. These harsh conditions may break DON into smaller molecules and underestimate true DON release rates (McCarthy and Bronk, 2008). Bronk and Glibert (1991) obtained good results using ion retardation columns, which unfortunately are no longer commercially available in its previous quality and therefore the method is no longer applicable. Trials with columns manufactured in the laboratory have not been successful either (D. Bronk, personal communication). Thus, a reliable method to estimate DON release rates from recently fixed N<sub>2</sub> remains unavailable, but the magnitude of this process seems to be significant and should not be overlooked.

As we have seen above, the geochemical and biological methods are strikingly different. Not surprisingly, the N<sub>2</sub> fixation rates derived from each of these groups of methods are strikingly different too. In the North Atlantic Ocean N<sub>2</sub> fixation rates derived from geochemical methods range from 0.84 to ~90 Tg N y<sup>-1</sup> (see Table 1 in Mahaffey et al., 2005), while the average rate derived from biological methods has been recently estimated at 12.7 Tg N y<sup>-1</sup> (see Table S3 in the supplementary information of Großkopf et al., 2012). In the near future, rates estimated by both groups of methods may become closer as the dissolved <sup>15</sup>N<sub>2</sub> method is applied in different oceanic regions and higher N<sub>2</sub> fixation rates are obtained (Mohr et al., 2010; Großkopf et al., 2012).

#### The relationship with climate

Current CO<sub>2</sub> partial pressure (pCO<sub>2</sub>) levels are expected to double by the end of this century (IPCC, 2007). The main consequences of this increase which will affect planktonic organisms are (1) seawater acidification, (2) increase of seawater temperature, (3) water column stratification, (4) expansion of oxygen minimum zones (OMZ), and (4) desertification and associated increase of desert dust inputs to the ocean (Boyd and Doney, 2002; Jickells et al., 2005; Stramma et al., 2008).

While seawater acidification (the increase of  $CO_2$  dissolved in seawater) can either enhance photosynthetic activity in some species, or be harmful for others (as the calcifying coccolithophores; Doney et al., 2009), seawater warming and stratification will likely enhance the proliferation of diazotrophic

cyanobacteria such as *Trichodesmium*. Indeed, culture experiments have revealed that the length of *Trichodesmium* filaments and its N<sub>2</sub> fixation rates increase when exposed to increasing levels of  $CO_2$  (e.g. Levitan et al., 2007). Also, heterotrophic N<sub>2</sub> fixation in hypoxic zones (Hamersley et al., 2011), suggests that the predicted expansion of OMZ (Stramma et al., 2008) will likely enhance global N<sub>2</sub> fixation rates too, either by in situ heterotrophic N<sub>2</sub> fixation, or by the enhancement of diazotrophy in other oceanic areas in order to balance increased denitrification occurring in the OMZ (Deutsch et al., 2007).

Trichodesmium and other diazotrophs would be further beneficiated by an increased availability of iron through desert dust deposition in the oceans. Michaels et al. (2001) proposed a theoretical climate-based  $N_2$  fixation feedback cycle (Fig. 7).



Fig. 7. The climate-based N<sub>2</sub> fixation feedback cycle. From Michaels et al. (2001).

In this conceptual model, desertification causes a global increase of  $N_2$  fixation rates as a result of iron limitation relieve through increased desert dust deposition, which lowers atmospheric  $CO_2$  levels decreasing global temperatures. Global cooling may in turn decrease desert dust inputs diminishing  $N_2$  fixation and raising  $CO_2$  levels, which would again raise global temperatures. Conclusively, diazotrophic responses to atmospheric  $CO_2$  levels are prone to have an important role in carbon and nitrogen and subsequent productivity in the future oceans.

#### Thesis objectives and outline

Although the North Atlantic basin holds the greatest amount of marine  $N_2$  fixation data available worldwide, most of these data correspond to its western basin, while measurements in the eastern basin are scarce.

The aim of this thesis was to investigate extensively  $N_2$  fixation and its controls in the subtropical Northeast Atlantic, in order to expand our knowledge of the magnitude and factors that regulate  $N_2$  fixation and the diversity of diazotrophic organisms in these waters. According to this, the specific objectives of our work were outlined to answer the following questions:

1. What is the magnitude and spatial distribution of  $N_2$  fixation and recently fixed  $N_2$  release in the subtropical Northeast Atlantic? What is the relative importance of the different planktonic size fractions? **Chapter 1.** 

These questions were answered by measuring gross and net N<sub>2</sub> fixation rates in >10  $\mu$ m and <10  $\mu$ m organisms in two coastal upwelling zones and one openocean area over the Canary Current. Sample-fractioning allowed us to compare the relative importance of symbiont and filamentous diazotrophs (>10  $\mu$ m) with that of unicellular diazotrophs (<10  $\mu$ m). Dissolved nitrogen release was assessed as the difference between gross and net N<sub>2</sub> fixation rates.

2. How much dissolved organic nitrogen (DON) is released by healthy unicellular diazotrophs?: a culture-based experimental approach. Chapter 2.

To answer this question, we measured DON release in cultures of the marine diazotrophic unicellular cyanobacterium *Cyanothece* sp. Miami BG 043511 using stable isotopes techniques.

3. Do oceanic unicellular diazotrophs release DON? How does it compare to DON release in other diazotrophs? What controls this activity? Does it have an specific geographical trend? **Chapter 3**.

To answer this question we measured DON release in >10  $\mu m$  and <10  $\mu m$ 

organisms in the subtropical North Atlantic over the 24.5°N parallel. In order to identify which factors were controlling the diazotrophic activity, we compared fractionated N<sub>2</sub> fixation and DON release rates with factors such as temperature, salinity, sea surface height anomalies, nutrient concentrations and atmospheric dust loads.

# 4. How does Saharan dust deposition affect $N_2$ fixation rates and the diversity of diazotrophic organisms in the subtropical Northeast Atlantic? Chapter 4.

To answer this question we measured  $N_2$  fixation rates and the abundance of *Trichodesmium* and unicellular diazotrophs, together with atmospheric dust deposition and its iron content in the Canary Islands.

# 5. What is the molecular diversity of diazotrophs in the subtropical Northeast Atlantic? Chapter 5.

To answer this question we analyzed the diversity of the *nifH* gene through nested-PCR techniques, and the abundance of unicellular diazotrophs through Fluorescent In Situ Hybridization techniques in two active upwelling zones and an open-ocean area over the Canary Current.

# 6. What is the contribution of $N_2$ fixation to new production and the excess nitrogen signal in the subtropical Northeast Atlantic? Chapter 6.

To answer this question we measured the uptake of nitrate and ammonium over the Canary Current and compared it to  $N_2$  fixation rates measured in the same region. We also compared the observed  $N_2$  fixation activity with the excess nitrogen signal previously observed in this area.

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"[...] But, he thought, I keep them with precision. Only I have no luck anymore. But who knows? Maybe today. Everyday is a new day. It is better to be lucky. But I would rather be exact. Then when luck comes you are ready."

The old man and the sea Ernest Hemingway

### Chapter 1

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

Benavides M, Agawin NSR, Arístegui J, Ferriol P, Stal LJ (2011). Aquatic Microbial Ecology 65:43-53

#### Abstract

We measured gross and net nitrogen fixation in fractionated samples (organisms >10  $\mu$ m and <10  $\mu$ m), 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<sup>-1</sup>) and its associated rates of nitrogen fixation (<0.1  $\mu$ mol N m<sup>-2</sup> d<sup>-1</sup>) 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<sup>-1</sup>d<sup>-1</sup> except off the northwest African coast where a gross nitrogen fixation peak of 0.98 nmol N  $L^{-1}$  d<sup>-1</sup> was measured. The <10  $\mu$ m fraction contributed more to both gross and net nitrogen fixation than did the >10  $\mu$ m fraction in most of the areas studied. The  $<10 \ \mu m$  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.

#### 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 important contributors to the
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oceanic nitrogen budget (Zehr et al., 2001). Although the presence of noncyanobacterial *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 and 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 intensity 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<sub>2</sub> fixation because of the recurrent input of iron and phosphorus through aeolian transport from the nearby Sahara desert (Prospero, 1981). However, N<sub>2</sub> fixation in this area could also be restricted as a result of the cold, nutrient-rich upwelling waters from the Iberian-Canary Current system, which extend hundreds of kilometers offshore through upwelling filaments (Álvarez-Salgado et al., 2007). Both the availability of combined nitrogen and the low temperatures could prevent N<sub>2</sub> fixation, limiting its relevance in upwelling regions—as happens in the cold, high-latitude seas (Gruber and 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 and 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<sub>2</sub>

fixation are needed.

We measured N<sub>2</sub> fixation by size-fractionated plankton from the Northeast Atlantic in samples from oligotrophic open ocean waters and from two 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 two active upwelling areas of the Northeast Atlantic, and (2) to compare the relative contribution of *Trichodesmium* with that of smaller diazotrophs. We combined the two 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 two 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 and Bronk, 2003; Bronk et al., 2007), and some diazotrophs may release up to 50% of their recently fixed  $N_2$  as DON (Mulholland and 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 three legs: 2 mesoscale 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 two 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 stations corresponded only to sites of coastal upwelling (stations S05 to S08) and to the open ocean (stations S01 to S03).

A strong upwelling filament developed during the Cape Ghir cruise, allowing tracking and sampling of the structure during six consecutive days. Samples were collected along the path of the filament at stations G12, G17, G22, and G40, G44, G48, as well as at two stations outside the filament (stations GM2 and GT2). Finally,

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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°N (Fig.1).



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 station GT2 was sampled).

More detailed descriptions of the hydrographic features found during these cruises may be found elsewhere (Carracedo et al., 2012; Troupin et al., 2012). 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  $\mu$ m mesh size was deployed two or three times from the deep chlorophyll maximum (DCM) and hauled vertically to the surface at a mean speed of 20 m min<sup>-1</sup>. Typically, 20 to 60 m<sup>3</sup> 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 phosphatebuffered 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  $\mu m$  fraction (see next section).

## Measurements of N<sub>2</sub> fixation

Water from the near-surface (5 m) was collected at each station between 08:00 h and 12:00 h (UTC). N<sub>2</sub> fixation was measured by the ARA technique (Stal, 1988) and the <sup>15</sup>N<sub>2</sub> 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 <sup>15</sup>N<sub>2</sub> 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<sub>2</sub> fixation rates. The <10  $\mu$ m fraction was obtained by pre-filtering whole seawater through 47 mm white polycarbonate filters of pore size 10  $\mu$ m (GE-Osmonics Poretics) and subsequently filtering onto GF/F filters. The activity of the >10  $\mu$ m fraction was computed by subtracting the rates of the <10  $\mu$ m fraction from the total N<sub>2</sub> 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<sub>2</sub>, 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 three 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 three 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<sub>2</sub> 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  $\mu$ m and from 15 to 35  $\mu$ m, respectively.

After incubation, 10 mL of headspace were sampled and transferred to preevacuated 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<sup>-1</sup> at a pressure of 5 psi. Helium was used as a carrier gas at a flow rate of 30 mL min<sup>-1</sup>. Hydrogen and airflow rates were set at 30 mL min<sup>-1</sup> and 365 ml min<sup>-1</sup>, 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  $\mu m$  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  $-20^{\circ}$ C until analysis. The particulate organic nitrogen (PON) content and isotopic ratio of samples was measured with a Thermo Flash EA 1112 elemental analyzer interfaced by a Conflo III with a Thermo Delta V Advantage IRMS. N<sub>2</sub> 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<sup>-1</sup> d<sup>-1</sup> (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 analyzed on board using a Prescop Alpkem autoanalyzer with detection limits of 0.1 µmol L<sup>-1</sup> for  $NO_3^-$ , and 0.02 µmol L<sup>-1</sup> for  $NO_2^-$  and  $HPO_4^{2-}$ . In the Cape Ghir leg, samples were recovered in 15 mL polyethylene tubes and stored frozen at  $-20^{\circ}$ C. These samples were analyzed in the land-based laboratory using an AA3 Bran+Luebbe autoanalyser with detection limits of 0.01, 0.003 and 0.024 µmol L<sup>-1</sup> 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<sup>-1</sup> at station X15. A peak of 0.25 trichomes L<sup>-1</sup> was found at station X8, coinciding with the Azores Front (AF), which was situated at  $\sim 37^{\circ}$  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<sup>-1</sup> was found at the last station of the CAIBOX transect (station X17).

*Trichodesmium* densities off Cape Silleiro were low (average of 0.031 trichomes L<sup>-1</sup>) 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<sup>-1</sup> was found at station GT2; this station was situated at the edge of the upwelling filament.

## N<sub>2</sub> fixation and potential DON release

Gross rates of N<sub>2</sub> fixation measured from net samples (>50 µm) are presented as areal rates (i.e.  $\mu$ mol N m<sup>-2</sup> d<sup>-1</sup>, 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 southern 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  $\mu$ mol N m<sup>-2</sup> d<sup>-1</sup> between stations X13 and X16. The nitrogenase activity of the >50  $\mu$ m fraction decreased abruptly to 0.01  $\mu$ mol N m<sup>-2</sup> d<sup>-1</sup> at station X17, coinciding with а 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<sub>2</sub> fixation in the  $>50 \mu m$  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  $\mu$ m fraction during the CAIBOX and Cape Ghir cruise legs (r<sup>2</sup> = 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 two parts characterized by  $0.07 \pm 0.02$  nmol N L<sup>-1</sup> d<sup>-1</sup> and  $0.11 \pm 0.05$  nmol N L<sup>-1</sup> d<sup>-1</sup> average rates of gross N<sub>2</sub> fixation (stations X1 to X8 and X9 to X17, respectively). The differences between the rates of N<sub>2</sub> fixation in both areas were statistically significant (*t*-test, p = 0.04, n = 8). These rates were also significantly related to the SST (r<sup>2</sup> = 0.56, p < 0.01; Fig. 3a). In this transect, the contribution of the >10 and <10 µm fractions to gross N<sub>2</sub> fixation was similar (40 to 60%), although the small fraction was responsible for ~60 to 95% of total net N<sub>2</sub> fixation (Table 2). Following the slight decrease in SST, N<sub>2</sub> fixation associated with the >10 and <10 µm fractions decreased abruptly in the last 2 stations near the coasts of the Canary Islands (stations X16 and X17).



Fig. 2. Density of *Trichodesmium* (trichomes L<sup>-1</sup>) and gross N<sub>2</sub> fixation rates of the > 50  $\mu$ m fraction ( $\mu$ mol m<sup>-2</sup> d<sup>-1</sup>) during (a) CAIBOX, (b) Cape Silleiro and (c) Cape Ghir cruise legs.

Gross N<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> fixation of 0.98 nmol N L<sup>-1</sup> d<sup>-1</sup> was observed for the <10 µm fraction at station G22 in Cape Ghir (not graphically represented as it exceeds the other rates by more than two-fold; Fig. 3c). Most of the N<sub>2</sub> fixation was attributed to the <10 µm fraction. This fraction contributed similarly to gross and net N<sub>2</sub> 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).

Table 1: Average and standard deviation values of sea surface temperature (SST;  ${}^{\circ}C$ ), Nitrate plus nitrite (NO<sub>3</sub>·+ NO<sub>2</sub>·; µmol L·1) and phosphate (HPO<sub>4</sub><sup>2</sup>·; µmol L·1). CAIBOX cruise leg is divided in two parts (north and south) by the Azores Front (AF).

	SST (ºC)	NO <sub>3</sub> <sup>-</sup> + NO <sub>2</sub> <sup>-</sup> (μmol L <sup>-1</sup> )	HPO4 <sup>2-</sup> (µmol L <sup>-1</sup> )
CAIBOX North of AF (stations X1 to X8)	$19.48\pm0.61$	$0.16\pm0.20$	$0.03\pm0.02$
CAIBOX South of AF (stations X9 to X17)	$23.45 \pm 0.74$	$0.09\pm0.05$	$0.03\pm0.01$
Cape Silleiro	$16.63 \pm 1.61$	$0.67\pm0.70$	$0.05\pm0.06$
Cape Ghir	$19.66\pm1.28$	$2.22\pm3.41$	$0.68\pm0.31$

We estimate that 41.13 and 76.43% of the total gross N<sub>2</sub> 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<sub>2</sub> fixed by the >10  $\mu$ m 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  $\mu$ m fraction was higher south of the AF (Table 2). In Cape Silleiro and Cape Ghir the total potential loss of recently fixed N<sub>2</sub> as DON ranged between ~44 and 54%.

The contribution of the <10  $\mu$ m fraction was the greatest in both cases (64.07 and 89.73% in Cape Silleiro and Cape Ghir, respectively; Table 2). While the >10  $\mu$ m and <10  $\mu$ m fractions contributed similarly to total net N<sub>2</sub> fixation in Cape Silleiro and Cape Ghir, the average C<sub>2</sub>H<sub>4</sub>:N<sub>2</sub> ratios were similar for the <10  $\mu$ m fraction (Fig. 4b), and the average C<sub>2</sub>H<sub>4</sub>:N<sub>2</sub> ratio for the >10  $\mu$ m fraction off Cape Silleiro was higher (8.43) than off Cape Ghir (6.38) (Fig. 4a).





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<sup>-1</sup> d<sup>-1</sup>; it is not represented graphically because it exceeds the rest of the rates by more than two-fold. The location of the Azores Front (AF) is shown by an arrow in (a).

## 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 modeling (summarized in Mahaffey et

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

The density of *Trichodesmium* was low (<0.5 trichomes L<sup>-1</sup>) and peaked only at frontal sites (Figs. 1, 2). Taking into account the buoyancy of *Trichodesmium* (Villareal and 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<sub>2</sub> 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 and 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. unpublished results).

The measurements indicate that the contribution of the >50  $\mu$ m fraction to total gross N<sub>2</sub> fixation was always < 1 % (Table 2). We calculated an average cell-specific N<sub>2</sub> fixation rate of 0.06 ± 0.1 pmol N cell<sup>-1</sup> d<sup>-1</sup>, which is much lower than the ~0.22 pmol N cell<sup>-1</sup> d<sup>-1</sup> 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 and Breitbarth, 2005).

Although the contribution of unicellular diazotrophs to the oceanic nitrogen budget is not precisely known, their N<sub>2</sub> fixation rates may locally exceed those of *Trichodesmium* (Montoya et al., 2004; Bonnet et al., 2009). N<sub>2</sub> fixation by organisms of the smaller size fraction (<10  $\mu$ m), potentially containing unicellular cyanobacteria and heterotrophic diazotrophs, predominated over that attributed to the >10  $\mu$ m 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).



Fig. 4. Box plot summary of the (a) >10  $\mu$ m fraction and (b) <10  $\mu$ m fraction contribution to total net N<sub>2</sub> fixation rates (%). Associated measured C<sub>2</sub>H<sub>4</sub>:<sup>15</sup>N<sub>2</sub> 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.

Indeed, the densities of *Trichodesmium* reported in the literature for the Northeast Atlantic typically range from 0 to 100 trichomes L<sup>-1</sup> (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<sup>-1</sup> (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°N). 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<sub>2</sub> fixation rates ranging from 2.2 to 3.7  $\mu$ mol N m<sup>-2</sup> d<sup>-1</sup>, 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<sub>2</sub> fixation rate of 2.14 ± 0.99 and 6.08 ± 7.73  $\mu$ mol m<sup>-2</sup> d<sup>-1</sup> 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 and Garcia (2008) found N<sub>2</sub> fixation rates as high as 3.6 nmol N L<sup>-1</sup> d<sup>-1</sup> 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<sub>2</sub> fixation rates to nanoplanktonic and picoplanktonic diazotrophs. In our study, N<sub>2</sub> fixation in the small size fraction (< 10 µm) was always < 0.4 nmol N L<sup>-1</sup> d<sup>-1</sup> except for a peak of 0.98 nmol N L<sup>-1</sup> d<sup>-1</sup> at one 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  $\mu$ m clones sequenced belonged to the UCYN-A group (N. S. R. Agawin et al. unpublished results). Moreover, we cannot exclude the possibility that heterotrophic diazotrophs were present in the <10  $\mu$ m fraction, although their importance for oceanic N<sub>2</sub> fixation is debated (Riemann et al., 2010).

The contribution of >10  $\mu$ m and <10  $\mu$ m diazotrophs to gross and net N<sub>2</sub> 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  $\mu$ m fraction are more important than *Trichodesmium* for the fixation of N<sub>2</sub> in the subtropical Northeast Atlantic Ocean. Gross rates of N<sub>2</sub> 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<sup>-1</sup> d<sup>-1</sup>).

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	Average gross N2 fixation (nmol N L <sup>-1</sup> d <sup>-1</sup> )		Average net N2 fixation (nmol N L <sup>-1</sup> d <sup>-1</sup> )		Fixed $N_2$ lost as DON (% of total $N_2$ fixation)		Contribution to total gross N2 fixation (%)			Contribution to total net N2 fixation (%)				
	>10	<10	>50 (x10 <sup>-4</sup> )	Total	>10	<10	Total	>10	<10	>10	<10	>50	>10	<10
CAIBOX North of AF	0.03	0.04	1.47	0.07	0.002	0.04	41.1	99.7	24.2	40.25	59.8	0.2	5.7	94.3
CAIBOX South of AF	0.05	0.06	3.87	0.11	0.01	0.02	76.4	28.5	58.12	42.28	57.8	0.4	35.7	64.3
Cape Silleiro	0.04	0.10	3.11	0.09	0.05	0.08	44.1	42.7	64.07	29.65	70.4	0.5	26.3	73.7
Cape Ghir	0.01	0.24	4.03	0.25	0.03	0.08	54.1	14.9	89.73	7.62	92.4	0.2	12.8	87.2

"Contra el optimismo no hay vacunas"

Mario Benedetti

# Chapter 2

Dissolved organic nitrogen and carbon release by a marine unicellular diazotrophic cyanobacterium

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## Abstract

Dinitrogen (N<sub>2</sub>) fixation rates may be underestimated when recently fixed N<sub>2</sub> is released as dissolved organic nitrogen (DON). DON release (DONr) is substantial in the filamentous cyanobacterium *Trichodesmium* but has never been reported in unicellular diazotrophic cyanobacteria. In this study we used axenic cultures of the marine unicellular diazotroph *Cyanothece* sp. Miami BG 043511 to measure dissolved organic matter release under N<sub>2</sub>-fixing conditions. DONr was measured as the transfer of <sup>15</sup>N<sub>2</sub> from the culture medium to the extracellular DON pool. On average, the DON released represented ~1% of the total N<sub>2</sub> fixed. The average release of dissolved organic carbon (DOC) as determined by <sup>14</sup>C, represented ~2% of the total carbon fixed. These results suggest that cultured populations of unicellular diazotrophs do not release much dissolved organic matter, but it cannot be excluded that DONr is important in the field when grazers and bacteria are present, or when the organism is exposed to environmental stresses such as turbulence, excess light, temperature changes or nutrient limitation.

## Introduction

The release of fixed dissolved organic carbon (DOC) by healthy marine plankton has been studied since the 1960s (e.g. Fogg, 1962). Release of organic matter in an oligotrophic environment might seem disadvantageous for phytoplankton as it represents a loss of energy and promotes growth of heterotrophic bacteria that compete for nutrients. Hence, these organisms may be outcompeted by those that do not release DOC. The organic carbon may serve as a substrate for autotrophic and heterotrophic plankton (Wood and Van Valen, 1990). Therefore, the release of organic compounds by healthy cells has often been regarded as a paradox in biological oceanography, although it may also have advantages. For example, organic compounds help chelating elements with low availability and solubility such as trace metals (Barbeau, 2006). The release of dissolved organic compounds also helps to protect the photosynthetic apparatus in high-light regimes by dissipating excess energy, and shortens the phytoplankton's lag-phase after nutrient-impoverished periods (Wood and Van Valen, 1990; Wannicke et al., 2009).

DOC release (DOCr) by phytoplankton has been widely studied during the past decades (Fogg, 1962; Sharp, 1977; Wood and Van Valen, 1990). Diazotrophic plankton such as blooming filamentous cyanobacteria are also known to release high amounts of DOC when the bloom decays, feeding grazers

PART II: Results

and stimulating bacterial production (Sellner, 1997). DOCr has also been observed in unicellular cyanobacteria (Bertilsson et al., 2005). However, little is known about the magnitude and ecological significance of the release of dissolved organic nitrogen (DONr) by oceanic diazotrophic cyanobacteria. Most marine dinitrogen (N<sub>2</sub>) fixation field-studies have been conducted by measuring the <sup>15</sup>N enrichment of particulate nitrogen (PN) after a certain time of incubation. The filtrates are usually discarded and therefore the N<sub>2</sub> fixed and subsequently released as dissolved organic or inorganic nitrogen (DON or DIN) is not taken into account. Thus, the amount of N<sub>2</sub> that has been fixed is potentially underestimated (Bronk et al., 1994; Gallon et al., 2002; Mulholland, 2007).

Global oceanic denitrification exceeds N<sub>2</sub> fixation by ~200 Tg N y<sup>-1</sup> (Mahaffey et al., 2005; Codispoti, 2007). Recent research indicates that this disequilibrium could be less severe when N<sub>2</sub> fixation measurements are expanded to higher latitudes and greater depths (e.g. Moisander et al., 2010), and when these measurements are indeed accurate, which has been questioned recently (Mohr et al., 2010). However, less attention has been paid to the potential underestimation of global oceanic N<sub>2</sub> fixation rates caused by not taking into account DONr.

Estimating DONr is also important because it is a source of new nitrogen for autotrophic and heterotrophic plankton (Berman and Bronk, 2003; Bronk et al., 2007). For instance, transfer of fixed  $N_2$  from large diazotrophic cyanobacteria to picoplankton has been described for the Baltic Sea (Ohlendieck et al., 2000) and for the Southwest Pacific (Garcia et al., 2007). N<sub>2</sub> fixed by the filamentous cyanobacterium Trichodesmium can also sustain the growth of larger autotrophic plankton, such as diatoms (Lee Chen et al., 2010), or even promote harmful algae blooms, as for example those of Karenia brevis off the Florida shelf (Bronk et al., 2004). Agawin et al. (2007) studied interactions between N<sub>2</sub>-fixing and non-N<sub>2</sub>-fixing cyanobacteria in a coupled modeling-chemostat culture experiment and found that the DON released by the N<sub>2</sub>-fixing unicellular cyanobacterium *Cyanothece* induced a four-fold increase of the abundance of the non-diazotrophic Synechococcus than it would have achieved in monoculture. This suggests that organic compounds exuded by diazotrophs can fuel primary production and may exert considerable control on the composition of the plankton community in the oligotrophic ocean.

Among marine diazotrophs, *Trichodesmium* was long thought to be the principal  $N_2$  fixer in the oceans (e.g. Capone et al., 1997). However, in the last decade molecular techniques revealed that unicellular diazotrophic

cyanobacteria are more abundant and more widely distributed than *Trichodesmium* (Luo et al., 2012), and their N<sub>2</sub> fixation rates are often higher (Falcón et al., 2004; Garcia et al., 2007; Moisander et al., 2010). Field populations of *Trichodesmium* are known to release up to ~50% of the recently fixed N<sub>2</sub> as DON (Glibert and Bronk, 1994), but in cultures the release is much less (Mulholland et al., 2004). Due to the wider geographical distribution of unicellular diazotrophs, it is necessary to study their DONr activity in order to estimate the potential underestimation of N<sub>2</sub> fixation rates. However, direct estimates of DON release by unicellular diazotrophs have not been reported (Mulholland, 2007).

DONr rates can be estimated as the difference between gross and net  $N_2$  fixation rates, measured by the acetylene reduction assay (ARA) and  ${}^{15}N_2$  assimilation into biomass, respectively (Gallon et al., 2002; Mulholland et al., 2004). Alternatively, one can measure the  ${}^{15}N$  atom % enrichment of the DON pool and use common tracer equations to estimate DON release rates (e.g. Glibert and Bronk, 1994; Mulholland et al., 2004). Both approaches have been used to indirectly estimate DONr by unicellular diazotrophs. Benavides et al. (2011) performed size-fractionated paired ARA and  ${}^{15}N_2$  assimilation measurements in the eastern North Atlantic and estimated that the <10 µm diazotrophs potentially released up to 67% of their recently fixed N<sub>2</sub>. These facts suggest that unicellular diazotrophs in the field release DON similarly as *Trichodesmium*. In this paper we used cultures of *Cyanothece* sp. Miami BG 043511 (hereafter *Cyanothece*) as a model organism to test whether the release of dissolved organic matter in unicellular diazotrophic cyanobacteria may be an important process.

# Materials and methods

#### Culture conditions and experimental setup

 $N_2$  fixation and DONr (experiment #1, 10 days), carbon fixation and DOCr (experiment #2, 8 days) were examined in this study. Cultures of *Cyanothece* sp. Miami BG 043511 (formerly classified as *Synechococcus*) were obtained from the Culture Collection Yerseke (strain CCY 0408). Cultures were grown in 250 mL transparent polypropylene tissue flasks with filter screw caps (VWR) in an illuminated incubator (Snijders ECD01E) at 27°C under a 12-12 h light-dark cycle and a light irradiance of ~50 µmol photons m<sup>-2</sup> s<sup>-2</sup>. Standard YBCII medium devoid of a source of combined nitrogen (Chen et al., 1996) was used in experiments #1 and #2. In both experiments two replicate cultures were inoculated with 1% of an exponentially growing stock culture. Experiment #1 lasted 10 days and experiment #2 lasted 8 days. Therefore, 20 and 16 culture replicates were prepared for experiment #1 and #2, respectively. Every experiment day, two replicates were sacrificed for the assays and analyses described below.

#### Chlorophyll, biomass, cell abundance and cell size

During both experiments, chlorophyll *a* (Chl *a*) concentrations, cell abundance and cell size were monitored daily. For Chl *a* analysis, culture aliquots were filtered onto 25 mm GF/F filters. Chl *a* was extracted with 90% cold acetone for 24 h and subsequently analyzed by means of a Cary Eclipse fluorescence spectrophotometer, previously calibrated with pure Chl *a* (Sigma), and concentrations calculated using the equations in Ritchie (2006). Cell counts and cell size measurements were performed daily in triplicate vortexed fresh samples using a Multisizer 3 Coulter Counter (Becton Dickinson). Bacterial contaminant numbers were checked daily by phase contrast microscopy and did not exceed 3% of the cell number of the cyanobacteria throughout the experiments.

#### N<sub>2</sub> fixation

Net N<sub>2</sub> fixation was assayed once a day in the middle of the dark period using the stable isotope tracer method by Montoya et al. (1996). For this technique, 125 mL glass vials were filled with culture to overflow, closed with rubber stoppers (allowing the excess culture to escape by a sterile syringe tip piercing the septum) and crimp-sealed with aluminum caps. Trace additions of <sup>15</sup>N<sub>2</sub> (500  $\mu$ L; 98.3 at.% <sup>15</sup>N, Euriso-top, Saint-Aubin, France) were made using a gas-tight syringe (Hamilton) and the samples were incubated for 3 h. The <sup>15</sup>N<sub>2</sub> incubated samples were filtered onto pre-combusted GF/F filters (6 h, 450°C) to obtain the <sup>15</sup>N enrichment and PN concentration.

## DONr and intracellular DON production (inDONp)

In experiment #1, DONr was estimated as the transfer of  $^{15}$ N from the culture medium to the extracellular DON pool, using the ammonium diffusion technique by Slawyk and Raimbault (1995) to isolate the labeled DON, and equations in Slawyk et al. (1998). In this experiment, we also investigated the inDONp (i.e. N<sub>2</sub> fixed as DON but not released during the incubation period),

computed as inDONp = [( $R_{inDONf} / R_{N2} x t$ )] x inDONf, where  $R_{inDONf}$  represents the excess <sup>15</sup>N enrichment of the intracellular DON pool at the end of the incubation period,  $R_{N2}$  represents the initial <sup>15</sup>N<sub>2</sub> enrichment of the culture medium, t is the incubation time and inDONf is the final intracellular DON concentration (i.e. after the 3 h incubation period).

After incubation and filtration, the filtrate was recovered and stored frozen for extracellular DON analysis. The intracellular DON content of cells was obtained by gently filtering (vacuum pressure <100 mm Hg) culture aliquots through 3  $\mu$ m pore size, 25 mm polycarbonate filters, adding 15 mL of boiling Milli-Q water and recovering the filtrate (combined thermal and osmotic shock; Thoresen et al., 1982). For the analysis of <sup>15</sup>N-labelled DON aliquots of the extracellular and intracellular filtrates were poisoned with 1%  $HgCl_2$  after 3 h of incubation with  ${\rm ^{15}N_2}.$  These filtrates were then stored in sterile 50 mL polyethylene tubes at room temperature and in the dark until analysis. To separate DIN (i.e.: nitrate + nitrite + ammonium) from DON and analyze the <sup>15</sup>N content of the latter, we followed the 3-step ammonium diffusion methodology proposed by Slawyk and Raimbault (1995). In step (1), filtrate subsamples were covered with a screw cap provided with a needle tip and half a GF/F filter humidified with 0.25 N H<sub>2</sub>SO<sub>4</sub>. MgO and DeVarda alloy (DA) were added to the samples and were subsequently incubated at  $50^{\circ}$ C for one week in order to strip off the DIN as  $(NH_4)_2$ SO<sub>4</sub>. In step (2), the remaining nitrogen in the subsamples was only DON, which was oxidized to DIN by adding a solution composed of K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>, NaOH and H<sub>3</sub>BO<sub>3</sub>. Finally, in step (3) the remaining nitrogen was removed by repeating step (1).

PN, particulate carbon (PC) and <sup>15</sup>N enrichments were analyzed by means of a Thermo Flash EA 1112 elemental analyzer connected to a Thermo Delta Advantage IRMS. DON concentrations were computed by subtracting DIN from total dissolved nitrogen (TDN). The concentrations of TDN were determined by autoclave destruction and the persulfate oxidation method (Valderrama, 1981). DIN was analyzed with a SEAL Technicon Autoanalyzer II, following recommendations by Kirkwood (1995). Detection limits were 0.1, 0.03 and 0.16 µmol L<sup>-1</sup> for nitrate, nitrite and ammonium, respectively.

#### Potential underestimation of N<sub>2</sub> fixation rates

Due to the slow dissolution of gaseous  ${}^{15}N_2$  in water,  $N_2$  fixation rates are underestimated to a variable extent when  ${}^{15}N_2$  is added to the sample as a bubble (Mohr et al., 2010), indicating that previously published  $N_2$  fixation rates are likely underestimated. This important observation was published

PART II: Results

while the experiments reported in this paper were in progress. For consistency, we decided not to apply the new method provided by Mohr et al. (2010), which consists of adding  ${}^{15}N_2$  dissolved in the culture medium or seawater sample. Alternatively, we provide a second set of N<sub>2</sub> fixation, DONr and inDONp rates estimated using the % dissolution of a  ${}^{15}N_2$  bubble after our period of incubation. After 3 h, the % dissolution of a  ${}^{15}N_2$  bubble into YBCII medium at 27°C is 28.8% (W. Mohr, personal communication).

### *Carbon fixation, DOCr and intracellular DOC production (inDOCp)*

In experiment #2 we tested carbon fixation, DOCr and intracellular DOC production (inDOCp) by *Cyanothece*. 50 mL of culture were incubated for 3 h in the middle of the light period with 100  $\mu$ L of 1057 kBq mL<sup>-1</sup> NaH<sup>14</sup>CO<sub>3</sub> (American Radiolabeled Chemicals, Inc). The initial CO<sub>2</sub> concentration was measured by automated titration with a Metrohm Dosimat 765 and a Metrohm 780 pH Meter. GF/F and 3  $\mu$ m polycarbonate filters were used as described above for experiment #1.

After incubation and filtration, filters were transferred to polyethylene scintillation vials (Perkin Elmer Pico Prias) and exposed to HCl fumes in a desiccator in order to remove the non-incorporated <sup>14</sup>C. Ultima Gold MV (Perkin Elmer) counting cocktail was added. Non-incorporated <sup>14</sup>C in the filtrate was removed by adding 100  $\mu$ L concentrated HCl to 50 mL samples. The next day, samples were counted using Insta-Gel (Perkin Elmer) counting cocktail. The scintillation counter used was a Packard TRICARB 2300. To avoid radioactive contamination of analysis equipment, parallel culture replicates not incubated with <sup>14</sup>C were used to measure final PC concentrations. However, the replicate culture flask used for <sup>14</sup>C measurements was split into two parts and analyzed separately in order to estimate analytical variability.

Samples for the analysis of the intracellular DOC and extracellular DOC concentrations were taken at the end of incubations and analyzed with a Skalar Formacs TOC continuous flow analyzer, previously calibrated with potassium hydrogen phthalate.

# Results

#### Growth and biomass

Cell number and the biomass indices Chl *a*, PN and PC were used to estimate cell division rate and growth rate of the *Cyanothece* cultures. These

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variables showed similar patterns in all experiments (Figs. 1a-b). Based on the increase of cell number the average division rate was 0.64 d<sup>-1</sup> and the average growth rates were, 0.27, 0.22 and 0.23 d<sup>-1</sup>, in experiment #1 as based on Chl *a*, PN and PC, respectively. In experiment #2 these numbers were 0.41, 0.35, 0.24 and 0.21 d<sup>-1</sup>, respectively.

#### Particulate and dissolved nitrogen pools

The initial and final (before and after the 3 h incubation period with  $^{15}N_2$ ) concentrations of PN, extracellular and intracellular DON and DIN are shown in Table 1 (experiment #1). The initial concentration of the PN pool ranged from 129 to 1095 µmol N L<sup>-1</sup>. Except on days 1 and 2, the final amount of PN was always lower than initial level. However, differences between initial and final PN concentrations were statistically significant (t-test, p = 0.028). The initial concentration of extracellular DON and DIN was always smaller than the final concentration, except for extracellular DON on days 4, 8 and 10. Initial and final extracellular DIN concentrations were statistically different (p = 0.017). while differences between extracellular DON concentrations were not (p = 0.585). Extracellular DON ranged from 10 to 97 µmol N L<sup>-1</sup>, while DIN values were one or two orders of magnitude lower (<1 to 4.5 µmol N L<sup>-1</sup>). In contrast, the initial concentrations of intracellular DON and DIN were always higher than the final concentrations, except intracellular DON on day 5, and intracellular DIN on days 2, 3 and 10. Initial and final intracellular DON concentrations were significantly different (p = 0.027), while intracellular DIN concentrations were not (p = 0.859).

While PN and extracellular DON and DIN concentrations increased as the culture grew (along the 10 days experiment), the intracellular DON and DIN pools showed a different pattern. The intracellular DON pools measured up to ~5 - 14 µmol N L<sup>-1</sup> during the first two and last two days of the experiment, while between days 3 and 8 the concentrations were generally twice as high. Intracellular DIN remained more or less constant with concentrations between <1 and ~2 µmol N L<sup>-1</sup>, only a peak of 3.14 µmol N L<sup>-1</sup> was observed on day 3. The concentration of  $^{15}N_2$  added to the samples (0.16 µmol N L<sup>-1</sup>) was taken into account when comparing the initial and final (pre and post incubation with  $^{15}N_2$ ) total nitrogen concentrations in all pools. The final total nitrogen concentration (sum of all pools) was lower than the initial one except on days 1 and 2. Initial total nitrogen concentrations were on average ~15% higher than final values. This is probably a result of adding the injected  $^{15}N_2$  in the mass balance. Some of this  $^{15}N_2$  is fixed and thus transferred to the particulate and dissolved nitrogen pools, but some is probably unused or not dissolved into the culture medium during the incubation period (Mohr et al., 2010), and thus not accounted for in the final sum.



Fig. 1: Cell number, particulate carbon (PC), Chlorophyll *a* (Chl *a*), and particulate nitrogen (PN) in (a) experiment #1 and (b) experiment #2.

Table 1: Average concentrations of nitrogen in the particulate nitrogen pool (PN) and extracellular and intracellular dissolved organic and inorganic nitrogen pools (exDON, exDIN, inDON and inDIN), at the start and at the end of the incubations performed with additions of  $^{15}N_2$  to *Cyanothece* cultures (experiment #1). The sum of all pools at the start and at the end of the incubations is given. All values are given in µmol N L<sup>-1</sup>. For initial values, the concentration of  $^{15}N_2$  added to the samples is included in the sum as well (0.16 µmol N L<sup>-1</sup>). Standard deviation values are given in brackets.

	Initial						Final					
Day	PN	exDON	exDIN	inDON	inDIN	Total (+ <sup>15</sup> N <sub>2</sub> )	PN	exDON	exDIN	inDON	inDIN	Total
1	129.3 (21.88)	10.23 (0.14)	0.56 (0.13)	9.89 (0.73)	1.35 (0.49)	151.46	137.47 (16.21)	11.46 (0.72)	0.86 (0.47)	5.50 (2.59)	1.22 (0.06)	156.51
2	214.16 (27.83)	10.66 (0.68)	0.78 (0.57)	11.78 (12.65)	1.39 (0.23)	238.93	245.27 (45.36)	44.91 (46.8)	1.39 (0.23)	10.45 (4.24)	1.46 (0.18)	303.47
3	302.25 (50.49)	15.81 (1.27)	0.54 (0.05)	37.21 (12.26)	1.59 (0.69)	360.56	274.72 (29.65)	26.16 (5.96)	1.12 (0.05)	26.19 (5.24)	3.14 (0.66)	331.33
4	436.81 (92.53)	49.61 (2.47)	1.36 (0.03)	34.74 (4.07)	1.86 (0.99)	524.54	388.59 (20.88)	36.37 (2.82)	1.91 (0.61)	32.24 (3.36)	1.37 (0.40)	460.49
5	522.07 (10.3)	33.97 (9.42)	1.53 (1.09)	25.40 (23.29)	1.8 (0.62)	584.93	388.55 (38.89)	60.84 (38.55)	1.72 (0.54)	30.35 (1.24)	1.79 (0.53)	483.26
6	736.19 (101.56)	60.79 (8.52)	0.97 (0.28)	38.88 (11.16)	1.42 (0.03)	838.41	720.52 (17.20)	71.4 (25.56)	1.69 (0.41)	31.26 (11.91)	1.14 (0.39)	826.01
7	908.25 (58.59)	58.54 (19.28)	1.79 (0.13)	45.58 (5.19)	1.37 (0.03)	1018.69	817.93 (106.90)	67.23 (5.78)	1.52 (0.67)	38.08 (1.98)	1.34 (0.25)	926.10
8	1095.11 (57.91)	73.49 (14.7)	1.43 (0.25)	42.95 (8.09)	2.54 (1.9)	1215.68	874.93 (96.95)	61.05 (2.78)	2.64 (0.11)	23.37 (0.96)	1.82 (0.93)	963.81
9	1046.15 (41.66)	56.16 (12.79)	1.24 (0.01)	14.31 (1.89)	2.09 (0.9)	1120.11	1028.53 (55.99)	61.17 (5.85)	2.47 (0.95)	10.99 (3.26)	1.37 (0.17)	1104.53
10	1065.71 (214.14)	96.86 (18.79)	1.55 (0.42)	9.20	0.98	1174.46	883.81 (3.91)	61.62 (5.74)	4.49 (0.04)	6.74 (0.45)	1.36 (0.34)	958.03

The initial and final concentrations of <sup>15</sup>N in the particulate and dissolved pools of *Cyanothece* cultures during experiment #1 are considered separately in Table 2. Unfortunately, the <sup>15</sup>N enrichment of the DIN pools was not measured and therefore these data are lacking from Table 2. Final concentrations of <sup>15</sup>N in the PN pool were larger than the initial ones, except on days 5, 8 and 10. Differences between initial and final <sup>15</sup>N concentrations of the PN pool were not statistically significant (*t*-test, p = 0.117). However, Montoya et al. (1996) considered a minimum acceptable change of  $\delta^{15}$ N between the initial and the final PN (of a sample incubated with <sup>15</sup>N<sub>2</sub>) of 4‰. The difference between the final and initial  $\delta^{15}$ N values of PN in our experiments ranged between 66 and ~1400 (data not shown), hence we are convinced that the N<sub>2</sub> fixation rates presented here are accurate.

The final concentration of <sup>15</sup>N (sum of all pools) was higher than the initial sum in days 1 - 3 indicating an excess recovery of <sup>15</sup>N. This imbalance is probably due to the low intracellular and extracellular DON concentrations measured on these days. However, the average percentage recovery for the rest of the experiment (days 4 – 10) is 99.08% ( $\pm$  10.41, standard deviation).

#### N<sub>2</sub> fixation, DONr and inDONp

When considering the complete dissolution of the  ${}^{15}N_2$  bubble (tracer method by Montoya et al., 1996), net N<sub>2</sub> fixation rates (assimilation of  ${}^{15}N_2$  into biomass) started at 3.66 µmol N L<sup>-1</sup> h<sup>-1</sup> on day 1, peaked at 7.36 µmol N L<sup>-1</sup> h<sup>-1</sup> on day 2, and then decreased to ~2 µmol N L<sup>-1</sup> h<sup>-1</sup> at the end of experiment #1 (Table 3). When only 28.8% dissolution of the  ${}^{15}N_2$  bubble was considered, all rates increased ~2.5-fold and followed the same temporal pattern.

DONr peaked at day 2 with rates of 0.07 and 0.19  $\mu$ mol N L<sup>-1</sup> h<sup>-1</sup>, and at day 8 with rates of 0.06 and 0.14  $\mu$ mol N L<sup>-1</sup> h<sup>-1</sup>, when considering 100% dissolution and 28.8% dissolution of the <sup>15</sup>N<sub>2</sub> bubble, respectively. In the middle of the experiment, DONr rates remained close to 0.03 and 0.08  $\mu$ mol N L<sup>-1</sup> h<sup>-1</sup> (100% and 28.8% <sup>15</sup>N<sub>2</sub> dissolution, respectively), and were lower on days 1 and 10. On average, DONr represented ~1% of the total nitrogen fixed (net N<sub>2</sub> fixed + DON released + intracellular DON produced). It should be noted that the 'total N<sub>2</sub> fixed' includes recently fixed N<sub>2</sub> released as DIN. Unfortunately, the <sup>15</sup>N enrichment in DIN was not measured.

Table 2: Average concentrations of <sup>15</sup>N in the particulate nitrogen (PN) and extracellular and intracellular dissolved organic nitrogen (exDON and inDON) pools, at the start and at the end of the incubations performed with additions of <sup>15</sup>N<sub>2</sub> to *Cyanothece* cultures (experiment #1). The sum of <sup>15</sup>N in all pools at the start and at the end of the incubations is given. All values are given in µmol N L<sup>-1</sup>. For initial values, the concentration of <sup>15</sup>N<sub>2</sub> added to the samples is included in the sum as well (0.16 µmol N L<sup>-1</sup>). A percentage of <sup>15</sup>N recovery is given based on the amount of <sup>15</sup>N in the samples at the start of the incubations with the amount of <sup>15</sup>N recovered in at their end. Standard deviation values are given in brackets.

		Ini	tial						
Day	PN	exDON	inDON	Total (+ <sup>15</sup> N <sub>2</sub> )	PN	exDON	inDON	Total	% recovery
1	0.53 (0.09)	0.04 (0.00)	0.02	0.75	1.06 (0.02)	0.04 (0.0)	0.05 (0.01)	1.15	153.33
2	0.82 (0.1)	0.04	0.02 (2.59)	1.04	2.02 (0.63)	0.17 (0.19)	0.06 (0.07)	2.25	216.35
3	1.15 (0.19)	0.05 (0.01)	0.03 (0.01)	1.39	1.73 (0.03)	0.08 (0.02)	0.12 (0.04)	1.93	138.85
4	1.65 (0.34)	0.15 (0.06)	0.08 (0.02)	2.04	2.06 (0.06)	0.16 (0.04)	0.13 (0.02)	2.35	115.19
5	1.95 (0.04)	0.12 (0.03)	0.1 (0.01)	2.33	1.95 (0.32)	0.14 (0.02)	0.1 (0.09)	2.19	93.99
6	2.74 (0.38)	0.29 (0.06)	0.09 (0.00)	3.28	3.17 (0.01)	0.2 (0.01)	0.12 (0.04)	3.49	106.40
7	3.38 (0.21)	0.25 (0.12)	0.09 (0.04)	3.88	3.42 (0.55)	0.25 (0.02)	0.15 (0.04)	3.82	98.45
8	4.08 (0.22)	0.29 (0.03)	0.12	4.65	3.68 (0.38)	0.24 (0.0)	0.15	4.07	87.53
9	3.87 (0.15)	0.2 (0.03)	0.08	4.31	4.22 (0.19)	0.24 (0.0)	0.06 (0.01)	4.52	104.87
10	3.96 (0.79)	0.26 (0.07)	0.04	4.42	3.51 (0.05)	0.31 (0.14)	0.03 (0.01)	3.85	87.10

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The inDONp rates (transfer of  ${}^{15}N_2$  to the intracellular DON pool) were generally one order of magnitude higher than DONr, with values ranging from 0.003 to 0.2 µmol N L<sup>-1</sup> h<sup>-1</sup> when 100% dissolution of the  ${}^{15}N_2$  bubble was considered, and 0.008 to ~0.5 µmol N L<sup>-1</sup> h<sup>-1</sup> when only 28.8% dissolution is considered. The overall tendency of inDONp rates was to decrease along the experiment, and the lowest rates were observed during the last three days.

## Carbon pools, carbon fixation, DOCr and inDOCp

Rates of net carbon fixation, DOCr and inDOCp, and the concentration of carbon in the particulate and dissolved pools of *Cyanothece* cultures during experiment #2 are given in Table 4. Carbon fixation rates are given either as net carbon fixed (carbon fixed into biomass), or as 'total carbon fixed' (net carbon fixed + DOC released + intracellular DOC produced). Total carbon fixation ranged from 54 to 151 µmol C L<sup>-1</sup> h<sup>-1</sup>, close to net carbon fixation, which ranged from 37 to 101 µmol C L<sup>-1</sup> h<sup>-1</sup>. The rate of DOCr rates ranged from <1 to 3 µmol C L<sup>-1</sup> h<sup>-1</sup>, while the rate of inDOCp was one to two orders of magnitude higher, ranging from 15 to 55 µmol C L<sup>-1</sup> h<sup>-1</sup>. DOCr rates represented only 1 – 4% of the total carbon fixed (average ~2%). Particulate carbon (PC) ranged from 950 to 5600 µmol C L<sup>-1</sup>. Extracellular DOC concentrations ranged from ~70 to 600 µmol C L<sup>-1</sup>. Intracellular DOC concentrations were generally higher than extracellular DOC concentrations, except on the last three days of experiment #2 (days 6 – 8).

## C:N fixation ratios

The ratios provided here come from two different experiments (#1 and #2, see Materials and methods). C:N fixation ratios would be more accurate if both carbon and  $N_2$  fixation rates had been measured simultaneously in the same culture. However, experiment #2 was done independently because samples treated with radioactivity (<sup>14</sup>C) need to be manipulated in a separate laboratory and cannot be further processed by an elemental analyzer or IRMS.

Carbon was fixed at higher rates than nitrogen, as reflected by the high molar ratios of net carbon fixation: net N<sub>2</sub> fixation (mol C: mol N) (Table 5). C:N ratios increased along the experiment and exceeded Redfield in almost all experiment days. When net N<sub>2</sub> fixation rates were calculated considering 100% dissolution of the <sup>15</sup>N<sub>2</sub> bubble, C:N ranged from 8 to ~40.

Table 3: Average rates of net  $N_2$  fixation (A), dissolved organic nitrogen (DON) released (B), intracellular DON produced (C), and total nitrogen fixed (A+B+C) measured considering 100% dissolution of the  ${}^{15}N_2$  bubble, and, alternatively, considering 28.8% dissolution of the  ${}^{15}N_2$  bubble after a 3 h incubation period. All rates are given in µmol N L<sup>-1</sup> h<sup>-1</sup>. The last column includes the percentage of DON released compared to the total N<sub>2</sub> fixed (A+B+C), which is equal for both  ${}^{15}N_2$  bubble  ${}^{6}$  dissolutions. Note that the total N<sub>2</sub> fixed column does not include any N<sub>2</sub> fixed and released as dissolved inorganic nitrogen (DIN). Standard deviation values are given in brackets.

	1	00% dissolution	of <sup>15</sup> N <sub>2</sub> bubble		28.8% dissolution of <sup>15</sup> N <sub>2</sub> bubble				
Day	Net N2 fixation (A)	DONr (B)	inDONp (C)	Total № fixed (A+B+C)	Net N <sub>2</sub> fixation (A)	DONr (B)	inDONp (C)	Total N2 fixed (A+B+C)	% DONr
1	3.66 (0.27)	0.00 (0.00)	0.06 (0.04)	3.72	9.46 (0.68)	0.01 (0.002)	0.16 (0.11)	9.62	0.08
2	7.36 (3.06)	0.07 (0.10)	0.15 (0.10)	7.59	18.9 (7.80)	0.19 (0.2)	0.39 (0.25)	19.25	0.98
3	4.85 (0.52)	0.04 (0.02)	0.10 (0.02)	4.99	12.3 (1.36)	0.09 (0.04)	0.26 (0.04)	12.51	0.72
4	4.28 (0.11)	0.03 (0.03)	0.20 (0.03)	4.51	10.8 (0.31)	0.08 (0.07)	0.49 (0.07)	10.95	0.69
5	3.44 (1.18)	0.03 (0.01)	0.18 (0.01)	3.65	8.62 (2.99)	0.07 (0.02)	0.45 (0.02)	8.75	0.76
6	3.47 (0.38)	0.03 (0.01)	0.06 (0.01)	3.56	8.60 (0.97)	0.08 (0.03)	0.15 (0.03)	8.77	0.95
7	2.76 (1.01)	0.04 (0.01)	0.13 (0.01)	2.93	6.78 (2.47)	0.09 (0.01)	0.33 (0.01)	6.97	1.35
8	3.08 (0.19)	0.06 (0.02)	0.01 (0.02)	3.15	7.51 (0.48)	0.14 (0.05)	0.02 (0.05)	7.79	1.78
9	2.90 (0.08)	0.03 (0.01)	0.00 (0.01)	2.94	7.02 (0.16)	0.08 (0.03)	0.01 (0.03)	7.18	1.09
10	1.83 (0.21)	0.02 (0.01)	0.00 (0.01)	1.85	4.4 (0.52)	0.05 (0.02)	0.01 (0.01)	4.49	1.01

Table 4: Average rates of net carbon fixation (A), rate of release of dissolved organic carbon (DOCr) (B), and rate of production of intracellular DOC (inDOCp) (C), as well as the rate of the total carbon fixed (sum of A, B and C) measured using <sup>14</sup>C. All rates are given in µmol C L<sup>-1</sup> h<sup>-1</sup>. The percentage of carbon fixed which is released as DOC is given. The three final columns represent the concentration of carbon in the particulate carbon (PC), extracellular and intracellular DOC pools (exDOC and inDOC) at the end of the incubation period. Concentrations are expressed in µmol C L<sup>-1</sup>. Standard deviation values are given in brackets.

Day	Net carbon fixed (A)	DOCr (B)	inDOCp (C)	Total carbon fixed (A+B+C)	% DOCr	РС	exDOC	inDOC
1	37.47 (4.1)	2.41 (0.12)	14.55 (3.6)	54.43 (5.8)	4.43	948.92 (0.4)	71.39 (17.7)	299.97 (62.9)
2	61.38 (7.7)	0.70 (0.4)	37.44 (7.6)	99.52 (12.9)	0.70	1119.28 (0.03)	75.42 (1.7)	108.84 (39.3)
3	89.31 (7)	0.96 (0.3)	54.80 (6.32)	145.07 (11.8)	0.66	1651.62 (0.17)	111.60 (6.7)	172.30 (1.9)
4	82.62 (10.3)	1.88 (0.2)	52.50 (1.2)	137 (16.9)	1.37	2117.82 (0.13)	160 (2)	306.50 (8.7)
5	97.6 (4.5)	3.11 (0.8)	46.10 (2)	146.81 (7.8)	2.11	2735.87 (1.41)	274.77 (11.3)	559.24 (203.7)
6	84.7 (1.9)	2.54 (0.2)	46.04 (2.8)	133.28 (3.4)	1.91	4373.13 (0.24)	324 (28.2)	282.50 (172.6)
7	101 (2.7)	2.70 (0.1)	47.52 (12.2)	151.22 (4.1)	1.79	5615.35 (0.1)	459.10 (45)	360.90 (40.4)
8	98.59 (9.01)	2.43 (0.2)	49.34 (14.5)	150.36 (13.8)	1.62	5543 (0.9)	599.24 (36.9)	297.75 (17.1)

When 28.8% dissolution of the  ${}^{15}N_2$  bubble was assumed, net N<sub>2</sub> fixation rates were ~2.5 fold higher. Consequently, when 28.8% dissolution of the  ${}^{15}N_2$  bubble was applied C:N ratios were lower and did not exceed Redfield, except on days 1 and 2. From day 3 on, the C:N ratios ranged from 3 to ~15.

DOCr : DONr ratios give an estimation of the C:N ratio of the dissolved organic matter released. These ratios were also high, ranging from ~10 to 116.3 when 100% dissolution of the  $^{15}\mathrm{N}_2$  bubble was considered for the calculation of N<sub>2</sub> fixation rates, and from ~4 to 46.5 when 28.8% dissolution was assumed. On day 1, the DONr rates were close to zero (Table 3), inflating DOCr : DONr ratios (Table 5).

## Discussion

## Measuring N<sub>2</sub> fixation and nitrogen release: methodological issues

There are a number of methodological issues which affect the estimation of N<sub>2</sub> fixation and DONr rates: (1) potential underestimation of N<sub>2</sub> fixation rates when the <sup>15</sup>N<sub>2</sub> bubble method is used, (2) unsuccessful recovery of all <sup>15</sup>N added at the end of an incubation period, (3) methodological problems related to DON isolation for IRMS analysis, (4) artifacts associated to sample manipulation, and (5) intracellular isotopic dilution. Each will be discussed in detail below.

Table 5: Net carbon fixation : net  $N_2$  fixation, and dissolved organic carbon release (DOCr) : dissolved organic nitrogen release (DONr) ratios (mol C:mol N).  $N_2$  fixation rates were calculated assuming 100% dissolution of the  $^{15}N_2$  bubble or, alternatively, assuming 28.8% dissolution of the  $^{15}N_2$  bubble after a 3 h incubation period.

	100% dissolution of	f <sup>15</sup> N <sub>2</sub> bubble	28.8% dissolution of <sup>15</sup> N <sub>2</sub> bubble		
Day	Net carbon fixation : net N <sub>2</sub> fixation	DOCr : DONr	Net carbon fixation : net N <sub>2</sub> fixation	DOCr : DONr	
1	10.24	3.89	830.1	321.3	
2	8.33	3.19	9.5	3.7	
3	18.42	7.11	27.0	10.6	
4	19.29	7.50	62.7	24.8	
5	28.34	11.11	116.3	46.5	
6	24.44	9.66	75.6	30.5	
7	36.62	14.61	70.6	28.7	
8	32.02	12.87	42.6	17.5	

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(1) Recently, Mohr et al. (2010) demonstrated that the routinely used  $^{15}N_2$  bubble tracer method underestimates N<sub>2</sub> fixation rates due to the slow dissolution of the gas into the medium or the sample seawater. These authors recommended adding <sup>15</sup>N<sub>2</sub> already dissolved in the water instead of as a bubble, as previously done by Glibert and Bronk (1994), in order to provide a constant <sup>15</sup>N enrichment of the source N<sub>2</sub> pool. Mohr et al.'s (2010) study was published while the experiments reported in this paper were being carried out. As the experiments had already started, we chose not to change the methodology to maintain consistency. In order to give an approximation of what the rates would have been like using more realistic dissolution of <sup>15</sup>N<sub>2</sub> after 3 h incubation, we recalculated net N<sub>2</sub> fixation, DONr and inDONp rates assuming 28.8% dissolution of the  ${}^{15}N_2$  (Table 3), which is the dissolution of  ${}^{15}N_2$ observed after 3 h of incubation in YBCII medium at 27°C (W. Mohr, personal communication). When 28.8% dissolution is applied, net  $N_2$  fixation rates explain better the daily PN increase observed (Tables 1 and 3), corroborating that the <sup>15</sup>N<sub>2</sub> bubble method underestimates N<sub>2</sub> fixation rates.

(2) Another problem often encountered during <sup>15</sup>N experiments is that the label is not fully recovered. Bronk and Glibert (1994) demonstrated that the fate of this 'missing' <sup>15</sup>N differs between marine systems. In particular, <sup>15</sup>N transfer from the source pool to the extracellular DON pool seems to be more important in oligotrophic systems, which supports the need to include DONr measurements in routine nitrogen uptake experiments in order to avoid underestimations (Bronk et al., 1994).

(3) Measuring the <sup>15</sup>N enrichment of the DON pool is prone with difficulties. Extracting DON for isotope analysis is difficult and has prompted intensive discussion in the past (Slawyk et al., 1998; Bronk and Ward, 2000; Slawyk et al., 2000). The protocol for extracting DON used here (Slawyk and Raimbault, 1995) (see Materials and methods) could underestimate DONr because the alkaline hot experimental conditions (60°C) of the extraction process may hydrolyze some fraction of the DON as ammonium (McCarthy and Bronk, 2008). The alternative DON isolation method is the ion retardation column (Bronk and Glibert, 1991), which is no longer commercially available in its previous quality and now retains variable amounts of DON, and therefore is not reliable (McCarthy and Bronk, 2008). Hence, consistent DON extraction protocols await development.

(4) Other methodological problems may affect the measurement of DONr, such as the breakage of cells during vacuum filtration and exposure to light and temperature changes during sample manipulation (Feuillade et al., 1990; Wannicke et al., 2009). Vacuum pressure was kept low throughout our

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experiments (<100 mm Hg) and care was taken to avoid temperature and light changes. Light was kept constant in our experiments ( $\sim$ 50 µmol photons m<sup>-2</sup> s<sup>-2</sup>), and was well below natural intensities in tropical and subtropical waters where *Cyanothece* occurs (Langlois et al., 2008). We therefore think that culture settings or sample manipulation did not affect DONr analysis.

(5) The intracellular isotopic dilution in cells with high intracellular DON pools might have affected the measurements of the DONr rates. When the intracellular DON pool and its turnover time are high, isotopic equilibrium between the intracellular pool and the extracellular medium is slow. This causes artificially low DON release rates, as the DON recovered in the extracellular pool is <sup>14</sup>N-rich material, which was inside the cell previously to <sup>15</sup>N<sub>2</sub> addition. (Mulholland et al., 2004).

## Nitrogen versus carbon metabolism in Cyanothece

Diazotrophs are known to fix carbon at rates exceeding the C:N Redfield ratio, (Mulholland et al., 2006; Mulholland, 2007). Reasons why this phenomenon occurs in natural populations include (1) substantial nitrogen and/or carbon release due to methodological problems associated to cell manipulation, (2) excess carbon fixation (this ballast may cause sinking of the cells), and (3) excess carbon fixation to decrease cellular oxygen concentrations which may inhibit the nitrogenase (Mehler reactions), or for ATP production (Mulholland, 2007). Errors in global estimates of  $N_2$  fixation would be lessened if accurate C:N fixation ratios of marine diazotrophs were used instead of theoretical elemental stoichiometries (Mulholland, 2007).

The DON released during our experiments was similar to the DOC released when expressed as a percentage of the total nitrogen or carbon fixed. On average, *Cyanothece* released ~1% of the recently fixed gross N<sub>2</sub> as DON, while ~2% of the gross carbon fixed was released as DOC. In a series of culture experiments, Wannicke et al. (2009) found that cultured *Trichodesmium* released 71% of the fixed N<sub>2</sub> as DON and ammonium and 50% of the carbon fixed as DOC. In our study DOCr/DONr C:N ratios were high and always exceeding Redfield stoichiometry, with the only exception of day 2 when N<sub>2</sub> fixation rates were calculated using 28.8% dissolution of the <sup>15</sup>N<sub>2</sub> bubble (Table 5). These high ratios suggest that the dissolved organic matter released by *Cyanothece* is enriched in carbon.

It should be noted that DOC and DON were treated as independent pools, but the exudate material of natural populations of diazotrophic organisms is normally composed of both DOC and DON. For example, this is the case for amino acids (Gallon et al., 2002). Whereas eukaryotic phytoplankton organisms are known to exude dissolved organic compounds lacking nitrogen such as carbohydrates (Newell et al., 1972), diazotrophic cyanobacterial exudates are commonly rich in nitrogen (Capone et al., 1994; Mulholland, 2007).

## DONr by unicellular diazotrophic cyanobacteria

*Trichodesmium* is thought to release fixed N<sub>2</sub> to provide other trichomes in the colony with nitrogen (Mulholland and Capone, 2000), because not every cell in a trichome and not all trichomes express nitrogenase (Bergman and Carpenter, 1991), or are in the same phase of the cell cycle (Wannicke et al., 2009). Some unicellular cyanobacteria release organic compounds to feed neighboring cells. For example, *Gloeothece* cells live embedded in a mucilaginous sheath that acts as an 'extracellular vacuole' (Flynn and Gallon, 1990). The advantage of releasing DON by free-living unicellular diazotrophs is not clear, if there is any. Possibly free-living unicellular diazotrophs release DON as a response to abiotic factors such as light stress (Wood and Van Valen, 1990). In our study, the DON released represented on average ~1% of the total N<sub>2</sub> fixed by *Cyanothece* during experiment #1 (Table 3). Given that optimum culture conditions were used, the growth of the cells was balanced and DONr was negligible.

Cultured nitrogen-starved eukaryotic phytoplankton have been reported to release dissolved organic compounds when reaching stationary phase (Newell et al., 1972). Contrarily, in our study similar levels of DONr and DOCr were observed throughout the experiment (Tables 3, 4). A decrease in DONr rates was observed during the last three days of experiment #1, when the cells were reaching the stationary phase (Fig. 1). We hypothesize that this decrease of DONr rates might have been caused by the increasing availability of DIN and DON in the medium during the last days of the experiment #1, and/or the simultaneous uptake of DON by *Cyanothece* during the experiment (Bronk and Glibert, 1993). Similarly, Bronk (1999) observed a decrease in DONr rates in *Synechococcus* cultures when the cells became nutrient limited. *Cyanothece* cells were incubated under a 12-12h light-dark regime and DONr rates were measured during the dark phase. However, we were unable to quantify how much of the DON released during the dark phase was taken up during the next light period.

The percentages of fixed  $N_2$  released as DON obtained in this study are low (~1% of total  $N_2$  fixation), even lower than those obtained with cultures of

*Trichodesmium* IMS101 (~8%; Mulholland et al., 2004), and much lower than in field studies of *Trichodesmium* (~50%; Glibert and Bronk, 1994). To our knowledge, there are no reports on DONr by unicellular diazotrophs (Mulholland, 2007). Recently, we measured DONr by the <10  $\mu$ m planktonic fraction in surface waters of the North Atlantic Ocean and estimated that on average ~20% of the recently fixed N<sub>2</sub> was released extracellularly as DON (M. Benavides, submitted). The <10  $\mu$ m fraction in natural waters may contain a variety of different diazotrophs, probably mostly UCYN-A and *Crocosphaera* which are dominant in this area of the Atlantic (Langlois et al., 2008). Unfortunately, we cannot discern which diazotrophs release DON and which do not from a <10  $\mu$ m planktonic sample. However, this difference in DONr dynamics between natural and cultured of unicellular diazotrophs is comparable to that observed by Glibert and Bronk (1994) and Mulholland et al. (2004) in natural and cultured populations of *Trichodesmium*, respectively.

The constantly growing evidence of the diversity and wide distribution of oceanic unicellular diazotrophs emphasize the need for accurate  $N_2$  fixation rates and to provide better estimates of DONr by these organisms. Among the oceanic unicellular diazotrophs, UCYN-A are the most abundant (Luo et al., 2012). These cyanobacteria lack the genetic material to fix carbon, which makes them depend on compounds produced by other organisms (Tripp et al., 2010). Therefore, it is unlikely that they release any of their recently fixed  $N_2$ unless being under some sort of environmental or predatory stress. However, recent research on extracellular polysaccharides (EPS) production by *Crocosphaera* (Sohm et al., 2011a) suggests that the DONr dynamics in oceanic UCYN-B diazotrophs could be important.

We chose *Cyanothece* as a model organism because it is easier to grow in cultures, facilitating experiments. This diazotroph is similar to *Crocosphaera* in size ( $\sim$ 2-6 µm) and in their nitrogenase activity patterns. Thus, their organic matter excretion metabolism could be indeed comparable. However, UCYN-A and *Crocosphaera* are much more abundant in the open ocean than *Cyanothece* (Luo et al., 2012). Therefore, DONr should be measured in these other species before we draw any conclusion of the importance of DONr in underestimating global N<sub>2</sub> fixation rates.

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"Que otros se enorgullezcan por lo que han escrito, yo me enorgullezco por lo que he leído"

José Luis Borges

# Chapter 3

Longitudinal variability of size-fractionated  $N_2$  fixation and DON release along 24.5  $^{\rm o}N$  in the subtropical North Atlantic

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# Abstract

Dinitrogen (N<sub>2</sub>) fixation and associated dissolved organic nitrogen (DON) release rates were measured on fractionated samples (>10 µm and <10  $\mu$ m) in the North Atlantic. Net N<sub>2</sub> fixation rates (N<sub>2</sub> assimilation into biomass) ranged from 0.01 to 0.4 nmol N L<sup>-1</sup> h<sup>-1</sup>, and DON release rates ranged from 0.001 to 0.09 nmol N L<sup>-1</sup> h<sup>-1</sup>. On average, DON release represented  $\sim$ 14% and  $\sim$ 23% of >10 µm and <10 µm gross N<sub>2</sub> fixation (assimilation into biomass plus DON release), respectively. This implies that by obviating DON release, true N<sub>2</sub> fixation rates are underestimated. Net N<sub>2</sub> fixation rates by both size fractions were higher in the east and decreased significantly towards the west ( $r_s$ =-0.487, p = 0.002, and  $r_s = -0.496$ , p = 0.001, for the >10 µm and <10 µm fractions, respectively). The sum of both fractions correlated with aerosol optical depth at 550 nm (AOD 550 nm) ( $r_s = 0.382$ , p = 0.017), suggesting an enhancement of diazotrophy as a response to aerosol inputs. In contrast, DON release rates were constant among size fractions and did not correlate with longitude or AOD 550 nm. We also compared  $N_2$  fixation rates obtained using the  ${}^{15}N_2$ dissolved and bubble methods. The first gave rates 50% higher than the latter, which supports the finding that previously published  $N_2$  fixation rates are likely underestimated. We suggest that by combining N<sub>2</sub> fixation and DON release measurements using dissolved <sup>15</sup>N<sub>2</sub> global N<sub>2</sub> fixation rates could increase enough to balance oceanic fixed nitrogen budget disequilibria.

# Introduction

Most of the nitrogen (N) needed for primary production reaches the euphotic zone through upwelling and diffusion of cold nutrient-rich waters from the deep sea. However, in the subtropical gyres of the oceans, the entrance of nutrients from the deep to the upper layer is hindered by the strong water column stratification maintained almost continuously throughout the year by solar heating of the ocean surface and the circulation patterns derived from trade winds (Falkowski, 1997). In these oligotrophic systems, atmospheric nitrogen (N<sub>2</sub>) fixation, which consists of the reduction of N<sub>2</sub> to ammonium (NH<sub>4</sub><sup>+</sup>) by diazotrophic organisms, is important because it provides a new source of N to these stratified N-limited systems (Capone et al., 2005).

Besides its importance in fueling primary production, there is a high interest in obtaining an accurate estimate of the global  $N_2$  fixation rate that can balance fixed N losses, presently estimated at ~200 Tg N y<sup>-1</sup> (Mahaffey et al., 2005; Codispoti, 2007). A recent revision of the  ${}^{15}N_2$  tracer method used to measure  $N_2$  fixation indicates that previously measured rates could be underestimated to a great extent (Mohr et al., 2010). This finding raises the

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question whether a wide application of the revised methods in the global oceans will reconcile gains and losses, balancing the oceanic N budget (Großkpof et al., 2012; Wilson et al., 2012).

Another potentially important source of underestimation in measured  $N_2$  fixation rates is the release of recently fixed  $N_2$ . Glibert and Bronk (1994) reported that the filamentous non-heterocystous diazotrophic cyanobacterium *Trichodesmium* released ~50% of its recently fixed  $N_2$  in the form of dissolved organic nitrogen (DON) and Capone et al. (1994) found evidence that the DON released was largely in the form of amino acids. The DON release activity of widespread unicellular diazotrophic cyanobacteria (UCYN) (Moisander et al., 2010) has not been studied directly (Mulholland, 2007) however. This DON flux is generally unaccounted for in  $N_2$  fixation studies, leading to potential underestimations of total  $N_2$  fixation rates.

Finally, global  $N_2$  fixation rates can be miscalculated when regional estimates are extrapolated to larger oceanic basins or the global ocean. The North Atlantic Ocean comprises the greatest quantity of  $N_2$  fixation and diazotrophic diversity data available worldwide, and therefore  $N_2$  fixation rates obtained here have been frequently used to calculate global oceanic rates. Nevertheless, the heterogeneity of diazotroph abundance, diversity and their associated  $N_2$  fixation rates (Luo et al., 2012) makes global rates computed out of regional measurements subject to overestimations (Hansell et al., 2004). As we move forward in the investigation of oceanic N disequilibrium, more detailed spatial and temporal variability of diazotroph assemblages and their diazotrophic activity are needed.

With the aim of covering the longitudinal variability of  $N_2$  fixation rates in the subtropical North Atlantic and to avoid underestimations, we measured fractionated  $N_2$  fixation and DON release rates at 40 stations over the 24.5°N parallel using the improved  ${}^{15}N_2$  tracer technique (Glibert and Bronk, 1994; Mohr et al., 2010).

#### **Materials and methods**

#### Hydrographic measurements and nutrients

Sampling was performed crossing the North Atlantic Ocean from the northwest (NW) African coast (Cape Jubi) to the Bahamas on-board the R/V *Sarmiento de Gamboa* from 27 January to 15 March 2011. The major part of the cruise was conducted over 24.5°N (World Ocean Circulation Experiment - WOCE- section A05) (Figure 1a). Temperature, salinity and fluorescence data were recorded with a SeaBird 911 plus CTD equipped with redundant

temperature and salinity sensors and a Sea-Tech fluorometer, all mounted on a General Oceanics 24 Niskin bottle rosette sampler. At each station, temperature and salinities were measured from the surface down to 20 m above the seafloor. Water samples were collected at each station in order to calibrate salinity values using a Guildline AUTOSAL model 8400B salinometer with a precision better than 0.002 for single samples.

Samples for nutrient analyses were collected from the rosette Niskin bottles in 15 mL polypropylene tubes and immediately frozen until analysis ashore. The concentrations of nitrate ( $NO_3^-$ ), nitrite ( $NO_2^-$ ),  $NH_4^+$ , phosphate ( $PO_4^{3-}$ ) and silicon (SiO<sub>2</sub>) were determined with a Technicon segmented-flow autoanalyzer. Standard methods were modified to obtain a detection limit of 2 nmol L<sup>-1</sup> (Raimbault et al., 1990; Kérouel and Aminot, 1997).

# Sea surface height anomalies (SSHA) and atmospheric dust

Daily sea level height anomaly (SSHA) data (the difference between the total SSH and the average SSH for this time of year) was downloaded from the archiving, validation, and interpretation of satellite oceanographic remote sensing service (AVISO, http://www.aviso.oceanobs.com/). The daily data were averaged for each three  $N_2$  fixation stations.

Aqua-MODIS aerosol optical depth at 550 nm (AOD 550 nm) can be used as a proxy for dust presence in the atmosphere (Kaufman et al., 2005). In order to assess the effect of atmospheric dust on  $N_2$  fixation rates, we used AOD 550 nm data obtained from the national aeronautics and space administration (NASA) Goddard earth sciences data and information services center Giovanni (NASA GES DISC) online database. The spatial distribution of AOD 550 nm during our cruise is plotted in Figure 1b.

#### Fractionated net N<sub>2</sub> fixation and DON release rates

Rates of N<sub>2</sub> fixation and DON release were measured in the > 10  $\mu$ m and < 10  $\mu$ m size fraction. The size fractionation was done at the start of the incubation. While pre-fractionation may not be the best choice due to potential associated artifacts such as cell disruption and cascade effects (Havens, 2001), it was chosen here to allow us discern between DONr rates by each size fraction. It is now obvious that >10  $\mu$ m diazotrophs such as *Trichodesmium* release DON (Glibert and Bronk, 1994), while if this activity is significant in the widespread unicellular diazotrophs is unknown (Mulholland, 2007).



Fig.1. (a) Map of stations sampled along the transect. CTD stations are represented as crosses. Stations were N<sub>2</sub> fixation experiments were performed during the day and during the night are represented as open and closed circles, respectively. (b) Aerosol optical depth (AOD) at 550 nm averaged from 28 January to 9 March 2011. The image was downloaded from the national aeronautics and space administration (NASA) Goddard earth sciences data and information services center Giovanni (NASA GES DISC) online database.

Therefore, pre-fractionation is necessary to study DONr dynamics by each size fraction separately. We sampled one station per day but alternated between day (0900-1200) and night samplings (1900-2100, local time) (Figure 1a). This approach allowed us to study the diazotrophic activity of organisms that fix N<sub>2</sub> in the light (e.g. *Trichodesmium*, heterotrophic diazotrophs), and organisms that only fix N<sub>2</sub> in the dark to avoid oxygen deactivation of the nitrogenase enzyme system (e.g. *Crocosphaera*).

 $N_2$  fixation and DON release rates were measured using <sup>15</sup>N-labeled  $N_2$  gas during 3 to 4 h incubations, which is the average incubation time frequently used in other DONr studies (e.g. Glibert and Bronk, 1994; Bronk, 1999). In this kind of experiments, incubation times must be long enough to allow sufficient <sup>15</sup>N label to reach the DON pool (according to the detection limit of the isotope ratio mass spectrometer -IRMS- equipment used), and short enough to protect the cells from usual bottle-effects.

There are two general approaches used to add the labeled gas to a sample: the addition of water that was saturated with  ${}^{15}N_2$  (e.g. Glibert and Bronk, 1994; Mohr et al., 2010), and the addition of a bubble (Montoya et al., 1996). In this study we used the addition of saturated water in fractionated seawater samples and the bubble method in whole (unfiltered) seawater samples (see below). With the addition of a bubble, the N<sub>2</sub> fixation rate is potentially underestimated due to slow dissolution of the gas bubble in water (Mohr et al., 2010). This is particularly true in short-term incubations where the gas bubble may not have sufficient time to equilibrate with the sample. If the gas bubble does not equilibrate, the actual atom percent (atom %) enrichment of the available N<sub>2</sub> pool will be lower than calculated resulting in an underestimation of the fixation rate.

To prepare the  ${}^{15}N_2$ -saturated water, surface seawater (~5 m) was recovered from the flow-through system of the ship and filtered through a 47 mm GF/F filter. This was done during the upcast of the CTD to ensure that the  ${}^{15}N_2$ -saturated water was the same as the sample water. This filtered seawater was decanted into 0.5 L transparent polycarbonate bottles (Nalgene) and degassed as outlined in Mohr et al. (2010). Each bottle was filled to overflow, closed with a septum screwcap and 5 mL of  ${}^{15}N_2$  (99 at.%  ${}^{15}N_3$ ; Tracetec) were injected using a Hamilton gas-tight syringe. The bottles were vigorously shaken for 10 - 20 min and then kept in the on-deck incubators until the rosette was back onboard (0.5 - 2.5 h depending on the station depth) to ensure that the  ${}^{15}N$ -enriched seawater had the same temperature as the sample at the time of mixing, in order to protect the organisms from thermal shocks.

To check the real <sup>15</sup>N enrichment of the <sup>15</sup>N<sub>2</sub>-amended seawater added to

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the samples, replicate samples of the  ${\rm ^{15}N_2}\-$ amended seawater prepared onboard were taken in 10 mL crimp vials (Chrompack), filled to overflow, and sealed with teflon-lined stoppers and aluminum caps using a seal crimper. These were stored at room temperature in the dark until being analyzed by membrane inlet mass spectrometry (MIMS) in S. Joye's lab. MIMS analysis showed that the real enrichment of the  ${\rm ^{15}N_2}\-$ amended seawater was  ${\rm \sim 100\%}$  (99 ± 2%) of that expected from complete dissolution of the  ${\rm ^{15}N_2}\-$ bubble.

To collect water used to measure fractionated rates, near surface (~5 m) seawater was collected with a 30 L Niskin bottle at each station. The samples were pre-fractionated by filtering two 2 L replicates through a 10 µm Nitex custommade sieve. The  $<10 \,\mu m$  fraction was recovered in 2.4 L transparent polycarbonate bottles (Nalgene). The >10  $\mu$ m fraction was recovered from the sieve by gently washing and concentrating the biological material using a water sprayer filled with filtered (GF/F) seawater from the same station. Then 200 mL of <sup>15</sup>N<sub>2</sub>-enriched filtered seawater was added to all bottles, they were filled to their neck with filtered seawater from the same station and placed in the on-deck incubators for 3 - 4 hr. The incubators were connected to the ship's flow-through system and covered with neutral density screens (Lee Filters). After the incubation, the samples were filtered through precombusted 25 mm GF/F filters, which were subsequently stored in sterile cryovials (VWR) and frozen. The GF/F filtrates were then filtered through 0.2 µm polycarbonate filters (GE-Osmonics Poretics) using gentle vacuum pressure (<100 mm Hg) to remove bacteria prior to isolating the dissolved N pools. Rigorous care was taken to avoid light stress in the samples. The filtrates stored frozen in triplicate 50 mL polypropylene tubes (VWR) and used to measure the concentrations of  $NO_3^-$ ,  $NO_2^-$ ,  $NH_4^+$  and total dissolved N (TDN) as described below.

#### Whole seawater net N<sub>2</sub> fixation rates

Parallel to seawater collection for fractionation, whole seawater was transferred to 2.4 L transparent polycarbonate bottles. The bottles were completely filled using silicone tubing to prevent the introduction of air bubbles. They were then sealed with septum screw-caps before 2 mL of <sup>15</sup>N-labeled N<sub>2</sub> gas were injected through the septum. The pressure across the septum was equilibrated by allowing the excess water to escape through a sterile syringe tip piercing the septum. The bottles were placed in the on-deck incubator for the same incubation period. After the incubation, samples were also filtered through precombusted GF/F filters, stored in cryovials, and frozen until IRMS analysis ashore. N<sub>2</sub> fixation rates measured with the bubble method were calculated as outlined in Montoya et al. (1996).

# Chemical analyses and rate calculations

Nutrient pools in filtrates were analyzed in D. Bronk's lab after the cruise. Concentrations of NH<sub>4</sub><sup>+</sup> were analyzed in duplicate with the manual phenol hypochlorite method (Hansen and Koroleff, 1983) using a Shimadzu UV-1601 spectrophotometer (detection limit of ~0.03 µmol L<sup>-1</sup>). Concentrations of NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> were determined with a Lachat QuikChem 8500 autoanalyzer (detection limits of 0.1 µmol L<sup>-1</sup> and 0.03 µmol L<sup>-1</sup>, respectively). TDN concentrations were analyzed on a Shimadzu TOC-V equipped with a TNM module. DON concentrations were calculated by subtracting NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> concentrations from TDN concentrations; propagation of error calculations were used to estimate errors associated with DON concentrations (Bronk et al., 2000).

We also isolated the NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup> and TDN pools to determine their <sup>15</sup>N atom % enrichment so that we could quantify rates of DON release. To isolate the NH<sub>4</sub><sup>+</sup> pool, we used solid phase extraction (SPE) columns (C<sub>18</sub>) (Dudek et al., 1986) and then spotted the isolated NH<sub>4</sub><sup>+</sup> on a filter prior to mass spectrometry analysis. The atom % of the particulate organic N (PON) and NH<sub>4</sub><sup>+</sup> pools were analyzed on a Europa GEO 20/20 IRMS with an automated nitrogen and carbon analyzer for solids and liquids (ANCA-SL).

To isolate the TDN pool, 15 mL samples were oxidized to  $NO_3^-$  via persulfate oxidation (Valderrama, 1981). The  $NO_3^-$  was then converted to nitrous oxide ( $N_2O$ ) using denitrifying bacteria lacking  $N_2O$ -reductase activity (Sigman et al., 2001). Isotope ratios of  $N_2O$  were then measured using a ThermoFinnigan GasBench + PreCon trace gas concentration system interfaced to a ThermoScientific Delta V Plus IRMS at the University of California Davis Stable Isotope Facility (Davis, CA). The same approach without the persulfate oxidation was used to isolate the  $NO_3^-$  pool.

The  $^{15}\mathrm{N}$  atom % enrichment of each of these pools was used to measure DON release rates as follows:

atom%TDN x (TDN)=(atom%NO<sub>3</sub><sup>-</sup> x (NO<sub>3</sub><sup>-</sup>))+(atom%NH<sub>4</sub><sup>+</sup> x (NH<sub>4</sub><sup>+</sup>))+(atom%DON x (DON)) (1)

where (TDN), (NO<sub>3</sub><sup>-</sup>),(NH<sub>4</sub><sup>+</sup>) and (DON) are the concentrations of TDN, NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup> and DON, respectively.

Solving Equation (1), the  $^{15}\mathrm{N}$  atom % enrichment of the DON pool was calculated as follows:

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$$atom\%DON = \frac{\left(atom\%TDN \ x \ [TDN]\right) - \left(\left(at\%NO_3^{-} \ x \ [NO_3^{-}]\right) + \left(at\%NH_4^{+} \ x \ [NH_4^{+}]\right)\right)}{[DON]}$$
(2)

 $N_{\rm 2}$  fixation and DON release rates were then calculated using standard tracer equations:

$$rate = \frac{atom\% \text{ excess target pool}}{atom\% \text{ excess source pool x time}} x \text{ target pool concentration}$$
(3)

where atom% excess is the <sup>15</sup>N% enrichment over natural abundance.

 $N_2$  fixation rates obtained from  ${}^{15}N_2$  incorporation into biomass were considered 'net'  $N_2$  fixation rates. The sum of net  $N_2$  fixation rates and DON release rates were considered 'gross'  $N_2$  fixation rates (Mulholland et al., 2004).

#### Results

#### Hydrographic features and nutrient concentrations

Low values of SST and SSS of ~ $19^{\circ}$ C and 36.8 respectively, were observed near the coastal upwelling off NW Africa (Figure 2). From east to west, SST increased up to 24.26°C at 54.03°W, and SSS up to 37.54 at 37.57°W. Moving westwards, SST presented sharp decreases of 1 - 2°C coincident with strong negative SSHA (<-12 cm). In contrast, SSS decreased until 62°W (station 104), where it reached 36.28. From 62°W to the west, SSS increased in two steps coincident with positive or low values of SSHA, and finally reached ~36.74.

The surface concentrations of  $NO_{3^{-}} + NO_{2^{-}}$ ,  $PO_{4^{3^{-}}}$  and  $SiO_{2}$  were generally maximum at the eastern end of the transect (coinciding with the NW African coastal upwelling), and decreased towards the west. The lowest concentrations were observed west of ~45°W, coinciding with the oligotrophic Sargasso Sea.

Along with SST and SSS increments and nutrients high values, the first seven  $N_2$  fixation stations show positive SSHA values, which then decrease considerably until station 71 (44.5°W). From this position to the west, high and predominantly negative SSHA dominate. The overall variability of SSHA along the transect can be observed in Figure 3.

# Dissolved ${}^{15}N_2$ versus ${}^{15}N_2$ bubble net $N_2$ fixation rates

The sum of net N<sub>2</sub> fixation rates in the >10  $\mu$ m and <10  $\mu$ m fractions measured using the dissolved method (Mohr et al., 2010) was compared to net N<sub>2</sub> fixation rates (in whole seawater samples collected at the same stations) using the <sup>15</sup>N<sub>2</sub> bubble classic method (Montoya et al., 1996). The difference between both rates tended to increase towards the east (Figure 4a). Indeed, the percent underestimation of the bubble method was significantly correlated with longitude (r<sub>s</sub> = 0.518, p =0.001). The average percent underestimation of the bubbles could slow its dissolution in seawater (Mohr et al., 2010), we checked for any significant correlations between percentage underestimations and DON concentrations. The concentrations of DON in >10  $\mu$ m and <10  $\mu$ m samples did not correlate significantly with the percentage underestimation of N<sub>2</sub> fixation rates, but followed a similar longitudinal distribution (Figure 4b).



Fig. 2: Values of (a) sea surface height anomalies (SSHA), (b) sea surface temperature (SST) and sea surface salinity (SSS), and (c) surface concentrations of nitrate plus nitrite  $(NO_{3^-} + NO_{2^-})$ , phosphate  $(PO_{4^{3^-}})$  and silicon (SiO<sub>2</sub>) (all in µmol L<sup>-1</sup>) at stations were N<sub>2</sub> fixation experiments were performed.

# $N_2$ fixation and DON release estimated by the dissolved <sup>15</sup> $N_2$ method

Both >10 and <10  $\mu$ m organisms presented a similar range of N<sub>2</sub> fixation rates (~0.01to ~0.44 nmol N L<sup>-1</sup> h<sup>-1</sup>), although >10  $\mu$ m rates were statistically different from <10  $\mu$ m rates (Wilcoxon test, p<0.0001). On average, net N<sub>2</sub> fixation rates of organisms >10  $\mu$ m were slightly higher than those <10  $\mu$ m (0.16 and 0.1 nmol N L<sup>-1</sup> h<sup>-1</sup>, respectively). Both rates showed a tendency to decrease towards the west (Figure 5), showing significant relationships with longitude (Spearman's correlation rank coefficient r<sub>s</sub> = -0.487, p = 0.002, and r<sub>s</sub> = -0.496, p = 0.001, for the >10  $\mu$ m and <10  $\mu$ m fractions, respectively). N<sub>2</sub> fixation rates of organisms >10  $\mu$ m measured during the day were not significantly different from those measured during the night (Wilcoxon test, p = 0.936), while the differences between rates of organisms <10  $\mu$ m measured during the day and the night were almost statistically significant (Wilcoxon test, p = 0.059).

DON release rates in organisms >10  $\mu$ m and <10  $\mu$ m ranged from 0.001 to ~0.09 nmol N L<sup>-1</sup> h<sup>-1</sup> (Figure 6a). Considering gross N<sub>2</sub> fixation as net N<sub>2</sub> fixation plus DON release (see Materials and methods), DON release represented ~14% of >10  $\mu$ m gross N<sub>2</sub> fixation and ~23% of <10  $\mu$ m gross N<sub>2</sub> fixation (Figure 6b). Significant differences in DON release were not found between >10  $\mu$ m and <10  $\mu$ m fractions, neither between day and night (Wilcoxon test, p>0.05). Also, DON release rates did not show a clear trend with longitude (r<sub>s</sub> = 0.203, p = 0.391, and r<sub>s</sub> = -0.046, p = 0.848, for the >10  $\mu$ m and <10  $\mu$ m fractions, respectively).

#### Discussion

#### Dissolved versus bubble methods

Glibert and Bronk (1994) were the first to use dissolved  ${}^{15}N_2$  to measure  $N_2$  fixation in *Trichodesmium* colonies, although they did not perform  ${}^{15}N_2$  bubble versus dissolved  ${}^{15}N_2$  comparisons. Mohr et al. (2010) observed that  $N_2$  fixation rates in cultures of *Crocosphaera watsonii* were 40% higher when the dissolved method was used, compared to the bubble method. Recently, Wilson et al. (2012) compared both methods in natural waters of the North Pacific Ocean (station ALOHA), and obtained rates that were 2 – 3.5 fold higher with the dissolved method than they were with the bubble method. Großkopf et al. (2012) observed that both methods differ by 62% when the diazotrophic community is dominated by *Trichodesmium*, and up to 570% when dominated by diazotrophs other than *Trichodesmium* (symbionts of diatoms, UCYN, heterotrophic diazotrophs).



Fig. 3: West to east maps of sea surface height anomalies (SSHA) averaged every three  $N_{\rm 2}\, fixation$  stations

Our N<sub>2</sub> fixation rates estimated by the dissolved method were ~50% higher than those estimated with the bubble method. This underestimation is at the lower end of the few comparisons available in the literature.

Adding to differences in the diazotrophic community, the factors influencing  $N_2$  fixation differences between the dissolved and bubble methods include (1) temperature -which affects gas dissolution into water-, (2) the agitation of the incubation bottles, (3) the volume of the incubation bottle, (4) the volume of  $^{15}N_2$  injected, (5) the duration of the incubation, (6) the time the incubation starts relative to the onset of nitrogenase activity -which differs between diazotroph species-, and (7) the DOM coating of the  $^{15}N_2$  bubble (Mohr et al., 2010). These factors vary widely among the previously published  $N_2$  fixation data measured using the bubble method, which makes the recalculation of rates difficult if not impossible (Großkopf et al., 2012).

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Fig. 4: (a) N<sub>2</sub> fixation rates obtained as the sum of >10 µm and <10 µm rates using the  ${}^{15}N_2$  dissolved method, and by incubation of whole seawater with a  ${}^{15}N_2$  bubble. (b) DON concentrations (in µmol L<sup>-1</sup>) in the >10 µm and <10 µm fractions. Error bars represent the standard deviation of the mean.

With regards to DOM, we observed that the differences between the dissolved and bubble methods were greater in the eastern part of the transect, coinciding with higher DON concentrations (Figure 4b). Correlations between DON and the difference between the dissolved and bubble methods were not statistically significant (data not shown), but their longitudinal trend was similar. The difference between both methods would have probably correlated better with dissolved organic carbon (DOC), but unfortunately those data are not available.

This study does not include diazotrophic organisms' abundance and distribution, so we cannot analyze how this affected the percentage underestimation of the bubble method with regards to the dissolved method. However, the compilation of diazotroph abundance data shows that *Trichodesmium* clearly dominates in the tropical Northwest Atlantic, while UCYN are somewhat more abundant in the eastern than in the western side of the basin (Luo et al., 2012) and at higher latitudes (Moisander et al., 2010).



Fig. 5: >10  $\mu$ m and <10  $\mu$ m net N<sub>2</sub> fixation rates in stations sampled during (a) the day, and (b) during the night. Error bars represent the standard deviation of the mean.

This distribution of diazotrophs and percentage underestimation by the bubble method versus the dissolved method agrees with Großkopf et al. (2012), although our percentage underestimation values are much lower.

Additionally, we must note that the sum of fractionated N<sub>2</sub> fixation rates (>10  $\mu$ m + <10  $\mu$ m) may be not directly comparable to whole seawater N<sub>2</sub> fixation rates. Sample fractionation is not always successful. For example, if <10  $\mu$ m organisms are retained in the >10  $\mu$ m fraction due to clogging of the mesh used for fractionation. However, due to the oligotrophic character of the area of study, seawater samples flowed easily through the mesh used for fractionation, and thus significant clogging or cell disruption may not be significant.

#### DON release

DON release rates were not significantly different between fractions, nor were they different between day and night, or significantly correlated with

longitude. This lack of spatial and daily variability in both size fractions suggests that the release of a percentage of recently fixed N<sub>2</sub> is inherent to natural assemblages of marine diazotrophs, although we cannot discern if this release was active or passive, via predation, viral infection, or cell death. Axenic cultures of *Cyanothece* sp. Miami BG 043511 grown in optimum conditions released ~1% of their recently fixed N<sub>2</sub> as DON (M. Benavides et al., in revision). This is much lower than the ~23% DON release obtained in this study for the <10  $\mu$ m fraction However, *Cyanothece* or other UCYN belonging to group C (UCYN-C) diazotrophs are less frequent in the North Atlantic Ocean, and therefore it can be expected that the <10  $\mu$ m diazotrophs (Langlois et al., 2008). However, whether or not the DON release activity of any of these diazotrophs is significant is unknown.



Fig. 6: (a) >10  $\mu$ m and <10  $\mu$ m DON release rates. (b) Percentage contribution of DON release to gross N<sub>2</sub> fixation (N<sub>2</sub> fixation + DON release) by the >10  $\mu$ m and <10  $\mu$ m fractions.

Previous studies of diazotrophic DON release have focused in the filamentous cyanobacterium *Trichodesmium* (Glibert and Bronk, 1994). These

authors found that Trichodesmium released 50% of their recently fixed N<sub>2</sub> as DON, implying that true N<sub>2</sub> fixation rates were substantially underestimated if DON release was not taken into account. As previously mentioned, the significance of the DON release activity of UCYN is unknown (Mulholland, 2007), but evidence is accumulating. The difference between gross  $N_2$  fixation rates obtained using the acetylene reduction assay and net N<sub>2</sub> fixation rates obtained using the <sup>15</sup>N<sub>2</sub> method has been used as a proxy to estimate the release of recently fixed N<sub>2</sub> (Gallon et al., 2002; Mulholland et al., 2004). Benavides et al. (2011) measured both rates on fractionated samples (>10 µm and <10  $\mu$ m) over the Canary Current and observed that the <10  $\mu$ m fraction potentially released  $\sim 60\%$  of their recently fixed N<sub>2</sub>. In this study, we measured the  $^{15}$ N enrichment of the DON pool and found that both the >10  $\mu$ m and <10 µm fractions DON release rates represent a significant percentage of gross N<sub>2</sub> fixation. Now that the correction of N<sub>2</sub> fixation rates using the dissolved <sup>15</sup>N<sub>2</sub> method is in the spotlight, we call attention to the inclusion of DON release measurements in routine field samplings. The application of the dissolved method combined with DON release rates could perhaps raise global N<sub>2</sub> fixation rates enough to balance denitrification, balancing the oceanic fixed N budget.

Furthermore, estimating DON release is not only important because its oversight potentially underestimates  $N_2$  fixation rates, but also because DON can fuel autotrophic production. Despite DON has been classically regarded as a refractory pool, recent investigations indicate that some fractions of this pool are labile and have short turnover times. This fact is further confirmed by the membrane transporters capable of uptaking some DON fractions such as amino acids which some phytoplankton species bear (see review by Bronk et al., 2007), or by the transfer of DON from filamentous diazotrophic cyanobacteria to picoplankton (Ohlendieck et al., 2000).

# Longitudinal variability of N<sub>2</sub> fixation

The tropical and subtropical Northeast Atlantic receives the highest inputs of Saharan dust worldwide (Prospero, 1981). N<sub>2</sub> fixation rates are enhanced in the presence of iron-rich desert dust (Mills et al., 2004), and therefore higher rates could be expected in the eastern side of the basin. However, the impact of cold nutrient-rich waters from the coastal upwelling off NW Africa, plus the deposition of atmospheric anthropogenic fixed N can preclude diazotrophy in this area (Benavides et al., 2011). The correlation between diazotrophy and dust inputs is however still argued in the literature. For example, using a biogeochemical model Deutsch et al. (2007) obtained higher N<sub>2</sub> fixation rates in the North Pacific than in the North Atlantic. They argued that, due to the low inputs of atmospheric dust to the North Pacific, resident diazotrophs could have adapted to lower iron requirements, or either subsurface supply was enough to meet the requirements of diazotrophic organisms.

A recent compilation of previously published data shows that total  $N_2$  fixation rates are higher in the western than in the eastern basin, and that the distribution of different groups of diazotrophs also varies longitudinally (Luo et al., 2012). Although studies on *nifH* gene abundance and diversity in the North Atlantic have been numerous in the last decade, there is still a wide gap in the area comprised by 20 - 40°N and 40 - 80°W (see Figure 3 in Luo et al., 2012). This gap impedes a comprehensive assessment of the spatial distribution of different diazotroph species in this basin. However, the figure cited above shows that, in general, small diazotrophs belonging to UCYN groups A, B and C are more abundant in the eastern than in the western basin, and that consequently, their contribution to total N<sub>2</sub> fixation rates increase to the east, where they dominate over larger diazotrophs such as *Trichodesmium* (Voss et al., 2004; Montoya et al., 2007; Benavides et al., 2011). Instead, *Trichodesmium* blooms are recurrent in the western North Atlantic where the highest N<sub>2</sub> fixation rates in this basin are found (Luo et al., 2012).

In contrast, in this study both >10  $\mu$ m and <10  $\mu$ m N<sub>2</sub> fixation rates were highest close to the NW African coast and decreased westwards (Figure 5). This longitudinal variability could also be affected by nutrient availability in the upper ocean controlled by diffusion of nutrient-rich deep waters through the thermocline. In the northern hemisphere, anticyclonic eddies promote positive SSHA, deepening the thermocline and hence enhancing organic matter accumulation and bacterial production within their cores (e.g. Baltar et al., 2010). On the contrary, cyclonic eddies foster negative SSHA, which uplifts the thermocline and promotes the upwelling of deeper nutrient-rich cold waters, enhancing primary production and chlorophyll concentrations (e.g. Arístegui et al., 1997). In contrast to autotrophic phytoplankton species, which depend on  $NO_3^-$  upwelled from the deep sea or  $NH_4^+$  regenerated in situ for their growth, diazotrophic organisms are capable of growing on N<sub>2</sub> as the only source of N. Theoretically, this sets out a different scenario for diazotrophs, which would grow better in the core of warm and nutrient-poor anticyclonic eddies, than in that of cold and nutrient-rich cyclonic eddies.

Church et al. (2009) studied a three-year series of SSHA values at station ALOHA and compared it to  $N_2$  fixation rates and *nifH* gene diversity. These authors observed that pronounced positive SSHA values coincided with enhanced  $N_2$  fixation rates and higher temperatures. However, these authors also pointed out that negative SSHA could promote the upwelling of waters

enriched in PO<sub>4</sub><sup>3-</sup> and SiO<sub>2</sub> relative to fixed N, which would enhance the growth of phosphorus-limited diazotrophs and diatom-diazotroph symbiosis limited by silica. In order to test the hypothesis by Church et al. (2009), we operationally divided our transect into two broad areas: an area dominated by weak positive or nearly neutral SSHA values (termed +SSHA zone, east of ~45°W), and an area where strong negative SSHA values predominate (termed -SSHA zone, west of  $\sim$ 45°W) (Figures 2a, 3). The separation point between both areas roughly coincides with the Mid-Atlantic Ridge. Similarly to Church et al. (2009), N<sub>2</sub> fixation rates were higher where positive SSHA values predominated (+SSHA zone), and lower coinciding with negative SSHA values (-SSHA zone). Indeed, net N<sub>2</sub> fixation rates in >10  $\mu$ m and <10  $\mu$ m organisms were significantly different in the two zones (Wilcoxon test, p = 0.01 and p =0.005, respectively). Net N<sub>2</sub> fixation rates of the <10  $\mu$ m fraction were ~2-fold higher in the +SSHA zone than in the -SSHA zone (Table 1), suggesting that small diazotrophs predominated in the eastern North Atlantic during our sampling. Unfortunately, parallel *nifH* gene analyses are not available to confirm this.

Table 1: Fractionated net  $N_2$  fixation rates, DON release rates, and the percent contribution of DON release to gross  $N_2$  fixation.<sup>a</sup>

	Net N <sub>2</sub> fixation		DON release		% DON release	
Zone	>10 µm	<10 µm	>10 µm	<10 µm	>10 µm	<10 µm
+SSHA	$0.19 \pm 0.10$	$0.14 \pm 0.10$	$0.02 \pm 0.02$	$0.02 \pm 0.03$	10.9 ± 16.1	22.2 ± 21.6
-SSHA	$0.12 \pm 0.07$	$0.06 \pm 0.03$	$0.02 \pm 0.01$	$0.02 \pm 0.01$	17.9 ± 15.9	23.4 ± 16.3

<sup>a</sup>Rates are in nmol L<sup>-1</sup> h<sup>-1</sup>. Values represent the average and standard deviation (average  $\pm$  SD). The +SSHA zone comprises stations 1 to 71, or longitude 13.34 to 44.5°W. The -SSHA zone comprises stations 77 to 139, longitude 44.5 to 76.15°W).

The longitudinal tendency of nutrient concentrations observed is opposite to that expected from nutrient diffusion as a consequence of rising or deepening of the thermocline, i.e. greater concentrations were observed in the +SSHA zone than in the -SSHA zone (Figure 2c), which covers the oligotrophic Sargasso Sea. Differences in the response of diazotrophic activity to mesoscale variability and consequent nutrient inputs to the upper ocean layers between the work of Church et al. (2009) and this study might stem from differential atmospheric inputs in the Pacific and Atlantic Oceans. Voss et al. (2004) also obtained greater  $N_2$  fixation rates towards the west over  $10^{\circ}N$  in the north Atlantic. These authors performed their cruise in autumn, when the intertropical convergence zone (ITCZ) reaches its northernmost position enhancing Saharan dust deposition. Our cruise took place in the late winter (January-March 2011), when dust inputs are also known to be maximum (Torres-Padrón et al., 2002). Voss et al. (2004) found significant correlations between N<sub>2</sub> fixation rates and dissolved iron concentrations. In this study, we found that total net N<sub>2</sub> fixation rates (>10  $\mu$ m plus <10  $\mu$ m) correlated significantly with AOD 550 nm (r<sub>s</sub> = 0.382, p = 0.017), suggesting that nutrients made available through Saharan dust deposition enhance N<sub>2</sub> fixation rates.

#### Conclusions

The results obtained here suggest that the longitudinal variability of  $N_2$  fixation rates in the North Atlantic is affected by atmospheric nutrient inputs, which are maximum in its eastern basin. In contrast to net  $N_2$  fixation rates, the release of recently fixed  $N_2$  as DON did not show any longitudinal trend or variability among size fractions. This suggests that this process is inherent of natural assemblages of diazotrophic organisms and should not be overlooked in order to avoid underestimation of true  $N_2$  fixation rates. Moreover, we have added to the body of evidence that  $N_2$  fixation rates are substantially underestimated when the bubble method is used. Measurements of  $N_2$  fixation rates using the dissolved method combined with measurements of DON release in future samplings will probably diminish global N budget unbalances.

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Pablo Picasso

# **Chapter 4**

Enhancement of nitrogen fixation rates by unicellular diazotrophs vs. *Trichodesmium* after a dust deposition event in the Canary Islands

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# Abstract

We studied the relationship between atmospheric dust deposition, N<sub>2</sub> fixation rates, and the abundance of unicellular diazotrophs and Trichodesmium in weekly or bi-weekly samplings over three months in the Canary Islands. On average, N<sub>2</sub> fixation rates by unicellular diazotrophs and *Trichodesmium* were low (0.2 and 1.66 x 10<sup>-3</sup> nmol N L<sup>-1</sup> h<sup>-1</sup>, respectively). However, N<sub>2</sub> fixation rates associated with unicellular diazotrophs increased by 86% and 92% after a peak of aerosol concentration in samples incubated in both light and dark, while the rates associated with *Trichodesmium* diminished by 34% and 92% in the light and in the dark, respectively. The abundance of unicellular diazotrophs ranged from 4 to 54 cell L<sup>-1</sup>. After the input of aerosols, 66% of the unicellular diazotrophs observed were attached to putatively organic matter particles. Trichodesmium abundance was low (average of 0.5 trichomes L<sup>-1</sup>), mainly in the form of free trichomes, which might hinder the ability of these organisms to take advantage of the Fe dissolved in the water column after dust deposition events. We also highlight the importance of monitoring short-term variability of N2 fixation in order to have a better understanding of the nitrogen cycle in the ocean.

# Introduction

Dinitrogen  $(N_2)$  fixation is an essential gateway of nitrogen into the oligotrophic oceans, where it is thought to fuel up to 50% of the total primary production (Capone et al., 2005). The colonial filamentous cyanobacterium *Trichodesmium* has been long considered as the primary N<sub>2</sub> fixer in the tropical and subtropical oceans. However, the application of molecular tools in oceanography during the past decade has allowed the identification of a wide variety of unicellular cyanobacterial and heterotrophic diazotrophs through detection and quantification of the *nifH* gene (Langlois et al., 2008). Unicellular diazotrophs occupy a wide latitudinal and depth range and their N<sub>2</sub> fixation rates may locally equal or exceed those of *Trichodesmium* (Moisander et al., 2010). In the eastern North Atlantic Ocean, unicellular diazotrophs are believed to dominate over Trichodesmium, which in turn is more abundant and active in its western basin (Montoya et al., 2007). Indeed, diazotrophs <10 µm exhibit higher N<sub>2</sub> fixation rates than >10  $\mu$ m ones in the Canary Current (CC) system (Benavides et al., 2011). Moreover, the presence of non-cyanobacterial *nifH* genes has been reported in the oligotrophic oceans (Langlois et al., 2008), but their contribution to overall N<sub>2</sub> fixation is as yet unknown (Sohm et al., 2011c).

Embedded in the CC, the subtropical waters of the Canary Islands are

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mostly oligotrophic due to the quasi-permanent thermocline caused by a strong surface heating throughout the year. The thermocline is only eroded during January-March 2010, favoring the entrance of cold nutrient-rich waters into the euphotic zone, and hence enhancing primary productivity during the so-called 'late winter bloom' (Hernández-León et al., 2010). On the other hand, the islands are situated in the outer boundary of the inter-tropical convergence zone (ITCZ) and therefore receive frequent inputs of Saharan dust. In previous years, recurrent peaks of dust deposition have been observed from February to March over the island of Gran Canaria (Hernández-León et al., 2010). Dust potentially fertilizes the water column with phosphorus (P) and iron (Fe), among other trace metals (Mills et al., 2004; Moore et al., 2009). P is needed for cell membrane, adenosine triphosphate (ATP) and deoxyribonucleic acid (DNA) synthesis (Dyhrman et al., 2007), while Fe is the major cofactor of the enzyme responsible for N<sub>2</sub> fixation (nitrogenase reductase), other redox enzymes, and an important structural constituent of diazotrophs (Berman-Frank et al., 2007).

Growth of oceanic diazotrophs can be limited by P, Fe, or both (reviewed by Sohm et al., 2011c). *Trichodesmium* is known to fix more  $N_2$  in the presence of dissolved Fe provided through natural aerosol deposition (Moore et al., 2009), or by artificial fertilization (Kustka et al., 2003). This filamentous cyanobacterium is also limited by P availability (Dyhrman et al., 2007). Both *Trichodesmium* and unicellular diazotrophs such as *Crocosphaera watsonii* are capable of up-regulating Fe- and P-specific scavenging systems or to reduce Fe and P cellular quotas when exposed to low concentrations (Webb et al., 2001; Dyhrman et al., 2007).

Fe cellular quotas are especially high in marine diazotrophs (Berman-Frank et al., 2007), which suggests that these organisms are more severely affected by Fe than by P limitation. However, research conducted in the North Atlantic over the last decade points to an stimulation of  $N_2$  fixation by Fe in Saharan dust, which in turn raises N:P ratios above Redfield levels making P the eventual limiting nutrient for diazotrophs in this basin (Sohm et al., 2011c).

In order to address the differential role of unicellular and colonial diazotrophs and their response to Fe inputs, we monitored the  $N_2$  fixation activity and abundance of diazotrophic organisms of the surface waters North off Gran Canaria Island (Canary Islands Archipelago) in weekly or bi-weekly periods from February to May 2010 and investigated its relation with aerosol concentrations.

# **Materials and methods**

#### Sampling and hydrographic measurements

A station ~10 nautical miles to the North of Gran Canaria island (28° 21' N – 15° 23' W) was sampled seven times from 08 February to 13 May 2010 onboard the R/V *Atlantic Explorer*. In each sampling date, temperature, salinity, fluorescence and photosynthetically active radiance (PAR) data were recorded by means of a SBE25 conductivity-temperature-depth (CTD), a Sea-Tech fluorometer and a Li-Cor PAR sensor. Seawater was recovered using Niskin bottles. The mixed layer depth (MLD) was estimated from a water column density ( $\sigma_t$ ) increase of 0.125 kg m<sup>-3</sup> with respect to surface values.

#### Nutrients and chlorophyll a

Samples for nitrate and nitrite ( $NO_3^{-} + NO_2^{-}$ ), phosphate ( $HPO_4^{2-}$ ) and silicate (SiO<sub>2</sub>) analysis were collected from the surface with 15 mL polyethylene tubes (Van Waters and Rogers, VWR) and stored frozen (-20°C) until analysis ashore. The samples were analyzed with a Technicon II segmented-flow autoanalyzer. The detection limits were 0.1 µmol L<sup>-1</sup> for NO<sub>3</sub><sup>-</sup>, 0.03 µmol L<sup>-1</sup> for NO<sub>2</sub><sup>-</sup>, 0.024 µmol L<sup>-1</sup> for HPO<sub>4</sub><sup>2-</sup>, and 0.016 µmol L<sup>-1</sup> for SiO<sub>2</sub>. Chlorophyll a (Chl a) concentrations were estimated from seawater samples (500 mL) filtered through 25 mm Whatman GF/F filters and stored frozen until analysis. Pigments were extracted in cold acetone (90%) for 24 h and analyzed by means of a 10-AU Turner Designs bench fluorometer, previously calibrated with pure Chl a (Sigma Aldrich), according to Holm-Hansen et al., (1965).

#### Aerosol optical depth, dust deposition, and Fe content

Daily moderate resolution imaging spectroradiometer (Aqua-MODIS) aerosol optical depth data at 550 nm (AOD 550 nm) were obtained from the national aeronautics and space administration (NASA) Goddard Earth Sciences Data and Information Services Center Giovanni (NASA GES DISC) online database for our oceanographic station coordinates (see above). Daily concentrations of suspended particles <10  $\mu$ m (PM<sub>10</sub>) and NO<sub>x</sub> (nitrous oxide + nitric monoxide + nitrogen dioxide) were obtained from the Canary Government air quality online database (Red de Control y Vigilancia de la Calidad del Aire de Canarias). For the latter, a station placed in the outskirts of the village of Arucas (North of Gran Canaria island, 28° 6' N – 15° 14' W) was chosen since it was the closest to our sampling site available in the online database.

Atmospheric total particulate matter (TSP), was collected onto Whatman GF/A 20 x 25 cm fiberglass filters using a high volume sample (HVS) pumping system (MCV) at a flow-rate of 50 m<sup>3</sup> h<sup>-1</sup>. Each sampling period lasted 24 h, starting at 08:00 h. The collector was placed 10 m above the ground at 27° 59' N 15° 24' W). For Fe analysis, the filters were treated with nitric and hydrochloric acid, according to the Beyer modified method (López Cancio et al., 2008). Fe was determined by atomic emission spectrophotometry (AES) using an inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin Elmer 3200 DV).

#### N<sub>2</sub> fixation and Trichodesmium abundance

 $N_2$  fixation was measured using the acetylene reduction assay (ARA; Stal, 1988). Triplicate sets of 2 L of surface seawater were filtered through 25 mm GF/F filters. The filters were placed in 10 mL crimp-head vials (Chrompack) and humidified with ~0.5 mL of GF/F-filtered surface seawater. The vials were sealed with rubber stoppers and aluminum caps using a crimper. Then, 2 mL of acetylene were injected with a Hamilton gas-tight syringe. Acetylene was generated from calcium carbide (CaC<sub>2</sub>, Sigma Aldrich) by adding Milli-Q water in a reaction flask (Stal, 1988). The gas was recovered in 1 L tedlar gas bags with polypropylene valves (Support, Knowledge and Choice, SKC). The ethylene contamination of the generated acetylene was <0.014 mg L<sup>-1</sup>. Of each triplicate set of samples, one was incubated in the light and the other in the dark, wrapped in opaque nylon clothes. Incubations were performed on-deck for 3 h. The incubators were covered with mesh to mimic 5 m depth in situ PAR levels.

A Nitex nylon 50  $\mu$ m mesh size net was towed twice vertically from 100 m depth to the surface at a speed of 20 m min<sup>-1</sup>, filtering 6.28 m<sup>3</sup> of seawater. The recovered sample was concentrated to 240 mL of which 60 mL were transferred to 125 mL polypropylene containers (VWR) and fixed with 40 mL of 10% borax-buffered formaldehyde to a final concentration of 4%. These samples were kept at room temperature in the dark until being analyzed for the abundance of *Trichodesmium* using an inverted microscope. The rest of the sample collected with the net was separated into 30 mL subsamples. These subsamples were filtered onto 25 mm GF/F filters and equally processed for the ARA technique as explained above to obtain N<sub>2</sub> fixation rates by the >50  $\mu$ m fraction, ideally corresponding to *Trichodesmium* and/or symbionts in larger organisms such as diatoms or copepods.

The ethylene and acetylene content of samples was measured using a Chrompack CP9001 gas chromatograph equipped with a flame ionization

detector (FID) and a wide-bore silica fused (0.53 mm internal diameter) Porapak U column (Chrompack). The carrier gas was N<sub>2</sub> at 10 mL min<sup>-1</sup> and the flows of hydrogen and air for the FID were 30 and 300 mL min<sup>-1</sup>, respectively. The temperatures for injector, detector and oven were 90°C, 120°C, and 55°C, respectively. Acetylene reduction was converted to N<sub>2</sub> fixation rates by applying a conversion factor of 4:1 and equations in Stal (1988). Blank GF/Fs were incubated with the same volume of filtered seawater and acetylene. A set of 6 blanks was made once in every sampling date (3 incubated in the dark, 3 incubated in the light). Each ARA rate calculated was corrected with its respective blank, therefore we obtained specific detection limits for each sampling time point. The detection limit of the ARA technique ranged between 0.01 and 0.12 nmol ethylene, defined as three times the standard deviation of the difference between the ethylene produced after the incubation period and the ethylene content of the blanks.

# *Tyramide signal amplification – fluorescent in situ hybridization (TSA-FISH)*

Seawater was filtered through 0.2, 3, and 10 µm 47 mm white polycarbonate filters (General Electric Power & Water, GE-Osmonics Poretics) under low vacuum pressure (<100 mm Hg). Volumes ranged from 0.2 to 4 L depending on the saturation of the filters. The filters were firstly fixed with 1% paraformaldehyde buffered with phosphate buffered saline (PBS, Sigma Aldrich) to a pH 8.2 for 15 minutes at room temperature, and then rinsed twice with PBS. Subsequently, the filters were dehydrated with ethanol molecular grade series (50%, 80%, and 100%) for 10 min, allowed to dry for a few seconds, and finally stored at -20°C until analysis. A subsample of the filter (1/8) was used for TSA-FISH. Hybridizations with horse radish peroxidase (HRP)-labeled (Thermo Fisher Scientific GmbH) 16S rDNA Nitro821 probe were done after permeation of the cells with lysozyme (5 mg mL<sup>-1</sup> in lysozyme buffer, Roche) for 30 minutes at 37°C, followed by three rinses of Milli-Q sterile water and ethanol series (50%, 80%, and 100%). The cells were subsequently processed for hybridization steps and TSA reactions using 50% of formamide concentration in the hybridization buffer (Biegala and Raimbault 2008). The filters were finally mounted in microscope slides, applying mounting medium with 4',6-diamidino-2-phenylindole (DAPI, Vectashield).

The slides were examined under a Leica DM 2500 microscope equipped with a 12 V/100W Halogen lamp, objectives HCX FL Plan10x/0.25, N Plan 40x/0.65 Ph2, N Plan 63x/0.80 0.17D and N Plan 100x/1.25 oil Ph3, dicroic filters of  $360 \pm 20$  nm excitation and  $410 \pm 5$  nm emission for the DAPI (blue

fluorescence), and 480 ± 40 nm excitation and 527 ± 30 nm emission for the fluorescein isothiocyanate (FITC) associated with Nitro821 probe (green fluorescence). Due to the low abundance of diazotrophs, we chose to count the filters in a systematic way in order to cover the entire surface of each filter slice. Cells that were present as free-living, symbionts to larger cells or attached to particles were noted and counted as small (<1  $\mu$ m), medium (>1 - <3  $\mu$ m), and large (>3  $\mu$ m).



Fig. 1. (A) Seawater temperature ( $^{\circ}$ C), (B) Chlorophyll *a* (Chl *a*) concentrations (µg L<sup>-1</sup>) at the surface and at 20 m depth, and mixed layer depth (MLD, m), and (C) surface nitrate plus nitrite (NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup>), silicate (SiO<sub>2</sub>), and phosphate (HPO<sub>4</sub><sup>2-</sup>) concentrations (µmol L<sup>-1</sup>) at 28° 21' N – 15° 23' W over the period of study (8 February - 13 May 2010). Note that temperature and MLD data are not available until 17 March 2010.

# Results

# Hydrography, nutrients, and Chl a

The temperatures observed during our sampling period ranged from 18°C to >20°C. The first 100 m presented temperatures >19.5°C throughout this period, which is about 0.5 - 1°C higher than observed for the same area in previous years (Arístegui et al., 2009). These high temperatures translated into a weak superficial water column stratification that intensified and reached higher depths from mid April to the end of May 2010 (Fig. 1a; Note that CTD data was only available from 17 March 2010). Concomitantly, the Chl *a* concentrations were low and ranged from ~0.02 to 0.12  $\mu$ g L<sup>-1</sup> (Fig. 1b). These values are much lower than expected for this time of the year, when the late winter bloom takes place and Chl *a* concentrations can reach values up to 1  $\mu$ g L<sup>-1</sup> (Arístegui et al., 2009).

According to the warm temperatures and the stratification observed, nutrient concentrations were low (Fig. 1C).  $NO_3^- + NO_2^-$  and  $SiO_2$  were <1 µmol L<sup>-1</sup> throughout the sampling period. Minimum values of these nutrients were observed coinciding with the maximum MLD (Fig. 1B, C). Later, the concentrations of  $NO_3^- + NO_2^-$  and  $SiO_2$  increased to 0.6 and 0.3 µmol L<sup>-1</sup>, respectively, when the stratification of the water column extended down to ~100 m depth. Nutrient concentration data from posterior dates are not available, but a continued decrease is expected as they are depleted by phytoplankton while the water column keeps stratified throughout the rest of the year. HPO<sub>4</sub><sup>2-</sup> concentrations showed a peak at the beginning of March 2010 and then kept on decreasing until the end of the sampling period.

#### Aerosols and dust Fe content

AOD 550 nm values increased from February to May 2010. Two maxima were observed in 19 March and 28 April 2010 (1.2 and 1.41, respectively; Fig. 2A). The concentration of PM<sub>10</sub> also increased during the studied period. The maximum PM<sub>10</sub> value observed (247.43  $\mu$ g m<sup>-3</sup>) coincided with a peak in AOD 550 nm on 19 March 2010 (Fig. 2A, B). A peak of the concentration of NO<sub>x</sub> in suspended material was observed from 26 to 28 February 2010 (Fig. 2C). The concentrations then decreased dramatically. Three peaks >15  $\mu$ g m<sup>-3</sup> were observed from mid March through late April 2010.

The Fe concentration of the dust particles collected ranged from 0.17 to 1.04  $\mu$ g m<sup>-3</sup>. The maximum concentrations observed (0.67 to 1.04  $\mu$ g m<sup>-3</sup>) occurred in March 2010 and coincided with the highest AOD 550 nm and PM<sub>10</sub> values (Fig. 2B, D). A secondary peak was observed on 29 April 2010,



associated with much lower  $PM_{10}$  values (~35 µg m<sup>-3</sup>).

Fig. 2. (A) Aerosol optical depth (AOD) at 550 nm at 28° 21' N – 15° 23' W, (B) suspended particles <10  $\mu$ m (PM<sub>10</sub>) ( $\mu$ g m<sup>-3</sup>), (C) nitrous oxide plus nitric oxide and nitrogen dioxide (NO<sub>x</sub>) concentration ( $\mu$ g m<sup>-3</sup>) at 28° 6' N – 15° 14' W, and (D) concentration of iron (Fe) in atmospheric dust collected at 27° 59' N – 15° 24' W ( $\mu$ g m<sup>-3</sup>). Solid lines show linear fits. Dashed lines show the limits of the y-axis breaks.

#### N<sub>2</sub> fixation

 $N_2$  fixation rates in unscreened surface seawater samples ranged from 0.01 to 0.1 nmol N L<sup>-1</sup> h<sup>-1</sup> from February to mid-March 2010 (both in the light and in the dark; Fig. 3A). With the extension of stratification throughout the first 100 m of the water column (Fig. 1A), these  $N_2$  fixation rates increased up to seven-fold, reaching maximum values of 0.21 and 0.71 nmol N L<sup>-1</sup> h<sup>-1</sup> for samples incubated in the dark and in the light, respectively (Fig. 3A). Replicates incubated in the light showed higher values than replicates incubated in the dark, with the exception of the two first samplings.

In an opposite pattern, >50  $\mu$ m samples presented higher values from February to mid-March 2010, presenting rates >2 x10<sup>-3</sup> nmol N L<sup>-1</sup> h<sup>-1</sup>. These

rates dropped by an order of magnitude with the onset of the water column stratification down to 100 m depth. N<sub>2</sub> fixation values remained to be <2 x 10<sup>-3</sup> nmol N L<sup>-1</sup> h<sup>-1</sup> throughout the rest of the sampling period (Fig. 3B). Replicates incubated in the dark had higher rates than those incubated in the light, with the only exception of 23 March 2010, when both rates were almost equal. Both light and dark >50  $\mu$ m N<sub>2</sub> fixation rates were significantly correlated with *Trichodesmium* abundance (r<sup>2</sup> = 0.86 and 0.69, respectively, n=7 and 6, respectively, both p<0.05). Since the net was towed from 100 m deep to the surface, N<sub>2</sub> fixation rates in samples >50  $\mu$ m incubated in the dark could be associated with *Trichodesmium* living in deeper layers of the water column, or either just be a carryover from energy acquired during carbon and N<sub>2</sub> fixation in the light. Diazotrophic activity in the dark would have probably ceased if the incubation had lasted longer than the 3 h used here.



Fig. 3. (A) Surface total N<sub>2</sub> fixation (unscreened water, nmol N L<sup>-1</sup> h<sup>-1</sup>) in the light and in the dark, and (B) >50 µm N<sub>2</sub> fixation (sample recovered from 50 µm net towed twice from 100 m depth to the surface, nmol N L<sup>-1</sup> h<sup>-1</sup>) in the light and in the dark. Error bars represent standard deviation. The first data point in (B) is not available.

# Abundance of diazotrophic organisms

*Trichodesmium* appeared majorly as free trichomes. Only two tuftshaped colonies -with less than 20 trichomes each- were found during the inspection of the seven samples presented in this study (data not shown). *Trichodesmium* abundance ranged from ~0.2 to 1.2 trichomes L<sup>-1</sup> (Fig. 4A).



Fig. 4. (A) *Trichodesmium* abundance (trichomes L<sup>-1</sup>), (B) abundance of unicellular diazotrophs with size <1  $\mu$ m, >1 - <3  $\mu$ m, and >3  $\mu$ m, and (C) abundance of unicellular diazotrophs found as free-living organisms, attached to particles or in symbiosis with larger planktonic organisms (cell L<sup>-1</sup>).

A ten-fold increase was observed from the first to the second sampling time point, which decreased two-fold coinciding with stronger mixing of the water column and a deeper MLD (mid-March 2010; Fig. 1B). The abundances in April and May 2010 were the lowest found in this study. Unicellular diazotroph abundance was studied by hybridization with probe Nitro821. Organisms were classified under three size classes (small <1  $\mu$ m, medium >1 - <3  $\mu$ m, and large  $>3 \mu$ m), and also as free-living, attached to particles or symbionts. The total number of unicellular diazotrophs increased steadily from February to a maximum on 23 March 2010, coinciding with a peak of AOD 550 nm and  $PM_{10}$ (Fig. 2A, B; 4B) and the highest concentrations of Fe in dust observed during the study period (Fig. 2D). Then, their abundance dropped dramatically to values <10 cell L<sup>-1</sup>, coinciding with a second peak of AOD 550 nm observed in early May, which was not reflected in PM<sub>10</sub> (Fig. 3A; 4B), and coincided with relatively lower Fe concentrations (Fig. 2D). Unicellular diazotrophs <1 µm typically represented a low percentage of the total (<10%; Fig. 4B), but reached 48% coinciding with an aerosol concentration peak. Medium-sized cells (>1  $\mu$ m - <3  $\mu$ m) were the most abundant throughout the study period (43% on average), except on 23 March 2010 when they only represented  $\sim$ 13% of the total. The larger organisms (>3 µm) typically represented a third of the total and only increased slightly on 23 March 2010.

The great majority of unicellular diazotrophs were found as free-living cells (Fig. 4C). On 23 March 2010, 35% of them appeared attached to putatively organic particles. Symbionts were only found in low densities in March 2010. These were symbionts of copepods (data not shown), although we cannot guarantee that these were true symbionts and not just cells superimposed on copepods during the filtration of samples.

#### Discussion

Biological activity rates such as net primary production are enhanced when aerosols rich in P, Fe, and other trace metals are made available through the deposition of desert dust (Duarte et al., 2006). Likewise, diazotrophic  $N_2$ fixation has been reported to increase as much as two-fold when Saharan dust is added to seawater samples (Mills et al., 2004). In this study, we observed a transition from a slight superficial stratification to a water column stratified down to 100 m depth (Fig. 1A). This transition coincided with a peak of AOD 550 nm,  $PM_{10}$ , a secondary peak of  $NO_x$  concentration, and the maximum values of Fe in dust observed throughout the study period (Fig. 2A, D). This transition marked a clear difference in the  $N_2$  fixation rates and the abundance of unicellular diazotrophs vs. those of *Trichodesmium* (Fig. 3A, B).

PART II: Results

Rates measured on unscreened seawater likely correspond to unicellular diazotrophic cyanobacteria and heterotrophic diazotrophs due to the low abundance of *Trichodesmium* in the CC waters (Benavides et al., 2011; this study), the low probability of catching colonies when using Niskin bottles (Chang, 2000), and the practical absence of symbionts in our samples.  $N_2$ fixation rates measured on unscreened surface seawater increased by 86% and 92% in samples incubated in the light and in the dark (respectively) after the peak of AOD 550 nm, PM<sub>10</sub>, and Fe concentration observed in 19 March 2010. Nevertheless, no response was observed to the secondary peak of dust deposition and Fe concentration that occurred by the end of May (Fig. 2A, B, D; 3A). The values of all these parameters were, however, considerably lower in the May deposition event than in the March 2010 deposition event.

On average, 43% of the unicellular diazotrophs observed belonged to the medium size class (>1  $\mu$ m - <3  $\mu$ m), while ~33% belonged to the >3  $\mu$ m size class (Fig. 4B). Using probe Nitro821, Biegala and Raimbault (2008) found high abundances of yet unidentified diazotrophic picocyanobacteria with sizes ranging between 0.2  $\mu$ m and 3  $\mu$ m. Since *Crocosphaera* are known to be >3  $\mu$ m and unicellular cyanobacteria of group A (UCYN-A) are <1  $\mu$ m (Zehr et al., 2008; Moisander et al., 2010), the unicellular diazotrophs in the medium size range (>1  $\mu$ m - < 3  $\mu$ m) found in this study likely belong to these unidentified picocyanobacteria (Biegala and Raimbault, 2008). Further *nifH* gene analyses are needed to attain an accurate phylogenetic classification, which unfortunately are not available in this case. According to the literature, the organisms in the larger size class (>3  $\mu$ m) could be unicellular cyanobacteria of the groups B and C, such as *Crocosphaera* and *Cyanothece*, respectively. These diazotrophs have been previously found in the North Atlantic (Langlois et al., 2005, 2008).

The abundance of free-living unicellular diazotrophs dropped from 92% to 31% after the aerosol input peak (Fig. 4C), coinciding with an increase from 5% to 66% of diazotrophs attached to presumably organic matter particles. Unicellular diazotrophs associated with particles have been observed before using the TSA-FISH technique (Le Moal et al., 2011). The size of the cells shown in Fig. 5A, B, and D ranges from 0.6 to ~1  $\mu$ m. According to this size range, these could be either heterotrophic diazotrophs or UCYN-A (Le Moal et al., 2011). Nevertheless, the probe Nitro821 shows several mismatches with heterotrophic bacteria (Biegala and Raimbault, 2008). Thus, it is unlikely that the observed cells were heterotrophic, albeit not impossible.

Heterotrophic diazotrophs do not have a self-sufficient means to obtain carbon (non photosynthetic organisms), which is needed to fix  $N_2$ , a process high in energy cost. Hence, it is plausible that organic matter particle-

heterotrophic diazotroph associations occur (Le Moal et al., 2011). However, this would be also the case for UCYN-A, which lack the oxygen-evolving photosystem II and are unable to fix carbon (Zehr et al., 2008). The small unicellular diazotrophs observed in Fig. 5A, B, and D likely belong to UCYN-A. This group is numerous and occurs in a wide range of temperatures in the ocean (Moisander et al., 2010), and is the most abundant unicellular diazotroph in the CC region (N.S.R. Agawin, unpublished results). UCYN-A are thought to rely on the organic matter produced by other phytoplankton (Tripp et al., 2010). Accordingly, the observed increase of <1  $\mu$ m organisms would be expected to be accompanied by an enhancement of Chl *a* concentrations, as a response to Saharan dust inputs (Duarte et al., 2006). However, such an enhancement was not observed (Fig. 1B).



Fig. 5. Examples of unicellular diazotrophs attached to particles. Cells were hybridized with probe Nitro821, stained with green fluorescence (FITC) using the TSA-FISH technique.
PART II: Results

Recently, cultured and field populations of *Crocosphaera* have been observed embedded in extracellular polysaccharides (EPS) (Sohm et al., 2011a). The cells in Fig. 5C are somewhat larger ( $\sim 1 - 2 \mu m$ ), but could not belong to the small size class of *Crocosphaera* which sizes  $\sim 3 \mu m$ . Recently, in a cruise over the subtropical North Atlantic we observed that <10  $\mu m$  diazotrophs released  $\sim 23\%$  of their recently fixed N<sub>2</sub> as DON (M. Benavides, unpublished results). Therefore, although more experimental evidence is needed to prove it, the particles observed could have been organic matter released by the unicellular diazotrophs themselves.

Being attached to organic particles confers a number of advantages to microbes. Microorganisms are attracted by the chemical gradients produced by the organic and inorganic nutrients of these particles, where intense microbial activity and carbon cycling takes place (Azam and Long, 2001). Likewise, the attachment of unicellular diazotrophs to organic particles would provide them with a lower oxygen and nutrient-rich environment where fixing  $N_2$  is less energy demanding (Riemann et al., 2010), and Fe is made bioavailable through binding with organic molecules (Biegala and Raimbault, 2008).

The stratification of the water column observed after the first dust input maximum (Fig. 1A; 2A, B) suggests that these associated particles would gain weight and thus could sink out of the mixed layer fairly quickly. However, these biota-particle associations are known to leave a 'plume' rich in dissolved organic matter and bioavailable Fe as they sink (Azam and Long, 2001). This plume could have supported the unicellular diazotrophic activity observed after the dust deposition event (Fig. 4C). Conclusively, just as organic particles colonized by microorganisms are important 'hotspots' of carbon cycling, their potential role in nitrogen cycling could be important too and requires further study.

The late winter bloom is a well-studied recurrent feature in the Canary Islands waters (Hernández-León et al., 2010). The progressive heating observed in the CC waters in the last decade (Arístegui et al., 2009) seems to have prevented the occurrence of this phenomenon in 2010. In the present study, we noted a superficial stratification (first ~10 m) that extended down to ~100 m from early April 2010 (Fig. 1A). The enhancement of the water column stratification coincided with the increase of N<sub>2</sub> fixation rates by unicellular diazotrophs.

Unicellular diazotrophs occupy a wider latitudinal and depth range than *Trichodesmium* in the oceans (Moisander et al., 2010). In habitats such as upwelling ecosystems, these organisms tolerate higher nutrient concentrations and lower temperatures than it was thought to be admissible for  $N_2$  fixers (Benavides et al., 2011). Unicellular diazotroph abundance decreased

dramatically in the last two sampling time points (Fig. 4B, C) when the upper water column was fully stratified and the surface temperature >20°C. In the northwest African upwelling system, the concentration of these organisms has been found to be two- to four-fold higher than in the open ocean waters of the CC (N.S.R. Agawin, unpublished results). The extent to which a cold and nutrient-rich environment is a prerequisite for the proliferation of unicellular diazotrophs in the CC waters is unknown.

Trichodesmium abundances and >50  $\mu$ m N<sub>2</sub> fixation rates were significantly correlated, indicating that these rates are majorly attributable to *Trichodesmium* and not to cyanobionts in diatoms (not found in the 50  $\mu$ m net samples, nor in TSA-FISH analysis) or in copepods (only found in low abundances in the month of March 2010, Fig. 4C). Trichodesmium-associated N<sub>2</sub> fixation rates decreased by 92% and 34% after the aerosol deposition peak observed in samples incubated in the light and in the dark, respectively (Fig. 3B). Correlations between dissolved Fe concentrations and Trichodesmium abundance have been previously reported in the north Atlantic (Moore et al., 2009). A recent study provided experimental evidence of how *Trichodesmium* puff-shaped colonies trap dust particles and pull them towards the core of the colony (Rubin et al., 2011). This unique strategy allows Trichodesmium colonies to process and solubilize dust particles to take advantage of the Fe and any other nutrients they may contain. Rubin et al., (2011) however reported that free trichomes do not present this ability. The predominance of free trichomes over colonies in CC waters (Benavides et al., 2011), suggests that in this form, they are not able to take advantage of the Fe present in dust particles and might explain the absence of a response in their N<sub>2</sub> fixation rates to dust inputs. Trichodesmium is known to grow efficiently in warm, oligotrophic and stratified waters (Capone et al., 2005) and to have very high Fe cellular quotas (Kustka et al., 2003; Berman-Frank et al., 2007). Therefore, an increase in  $N_2$ fixation and abundance was rather expected after the warming and consequent stabilization of the water column, which coincided with the input of aerosols.

The second aerosol concentration peak observed in May (Fig. 2A) did not incite a response in *Trichodesmium* populations and their diazotrophic activity either. However, if we consider specific N<sub>2</sub> fixation rates (i.e., nmol N trichome<sup>-1</sup> h<sup>-1</sup>), the values obtained in the three last sampling points (when *Trichodesmium* abundance is low) are in the range of those observed before (ranging from 1.09 x 10<sup>-4</sup> and 2.56 x 10<sup>-3</sup> nmol N trichome<sup>-1</sup> h<sup>-1</sup>). Thus, it seems that *Trichodesmium* was unaffected by dust deposition and the Fe concentration of dust, maintaining fairly constant N<sub>2</sub> fixation rates throughout the study period. Another possible explanation to the low *Trichodesmium* N<sub>2</sub> fixation rates are the peaks of NO<sub>x</sub> concentration in aerosols, which could have enhanced the availability of fixed nitrogen in the water column and inhibit Trichodesmium nitrogenase activity.

In this study we observed a high variability in  $N_2$  fixation rates and abundance of diazotrophs within a short period of time. We underscore the importance of seasonal variability and the potential bias of usual snapshot sampling programs in global estimates of diazotrophic activity. Short-term variability may be even more important in areas affected by sudden changes, such as dust inputs or mesoscale events, like in the Canary Islands waters.

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"Pedras no meu caminho? Guardo-as todas, vou construir um castelo..."

Fernando Pessoa

# **Chapter 5**

Dominance of unicellular cyanobacteria (UCYN<sub>2</sub>-fix lineage) in the diazotrophic community in the subtropical Northeast Atlantic Ocean

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# Abstract

The horizontal and vertical distribution of cells in the unicellular cyanobacteria dinitrogen (N<sub>2</sub>)-fixing (UCYN<sub>2</sub>-fix) lineage were studied in the subtropical Northeast Atlantic Ocean (28.8 - 42°N/ 9 - 20°W), in stations encompassing different water conditions (from oceanic oligotrophic waters to upwelling areas and temperature range of 13.1 to 24.2 °C). These were investigated through size fractionated *nifH* gene nested polymerase chain reaction (PCR) analysis and tyramide signal amplification fluorescent in situ hybridization (TSA-FISH) using probe Nitro821. Results showed that in samples from the surface, the mixed layer depth (MLD) and the deep chlorophyll maximum (DCM), >50% of the *nifH* genes recovered belonged to the <3  $\mu$ m fraction, consistent with TSA-FISH results where the <3  $\mu$ m Nitro821-positive cells were more abundant in most cases. Phylogenetic analysis of representative samples revealed that most of the sequences belonged to UCYN-A.  $N_2$  fixation rates in the <3  $\mu$ m fraction (presumably representing UCYN-A) contributed dominantly to total N<sub>2</sub> fixation (>50%) in the stations studied. Correlation analyses suggest positive relationships of the abundance and activity of UCYN-A with temperature, and inverse correlations with dissolved oxygen  $(O_2)$  levels. Their dominance in the nitrate  $(NO_3)$ -rich upwelling regions studied demonstrate that their activities may not be as strongly regulated by fixed nitrogen as it was previously thought.

# Introduction

Increased sensitivity of modern techniques has pointed the potential important role of unicellular cyanobacteria (of the UCYN<sub>2</sub>-fix lineage, <10  $\mu$ m) in significantly fixing N<sub>2</sub>, comparable with or exceeding the rates by the larger (>200  $\mu$ m) filamentous cyanobacterium *Trichodesmium* (Montoya et al., 2004; Langlois et al., 2008; Benavides et al., 2011), which was believed to be the most abundant and active diazotrophic N<sub>2</sub>-fixing microorganism in the oceans (Capone et al., 1997). The recent discovery of cells of the UCYN<sub>2</sub>-fix lineage has been made possible using modern molecular techniques. These showed the presence of fragments of the unicellular cyanobacterial *nifH* genes, which encode the Fe-protein component (dinitrogenase reductase) of nitrogenase, the enzyme responsible for N<sub>2</sub> fixation (Zehr et al., 1998; Zehr et al., 2001). Another approach was made possible by the development of oligonucleotide primer Nitro821, which targets the 16S ribosomal ribonucleic acid (rRNA) gene of UCYN<sub>2</sub>-fix cells (Mazard et al., 2004) using the TSA-FISH technique (Biegala and Raimbault, 2008). This technique can be used to estimate whole

cell in situ abundance of different sizes of cells within the UCYN<sub>2</sub>-fix lineage.

To date, three groups have been phylogenetically described within the UCYN<sub>2</sub>-fix lineage based on the *nifH* cyanobacterial sequences: Group A (UCYN-A), Group B (UCYN-B, e.g. Crocosphaera watsonii WH8501), and Group C (UCYN-C) closely related to Cyanothece sp. ATCC5114) (Needoba et al., 2007). Of these groups, UCYN-A has received the most attention in very recent research on diazotrophs (Zehr et al., 2008; Goebel et al., 2010; Tripp et al., 2010; Moisander et al., 2010) because of its peculiar characteristics. Although it remains uncultivated, it has a reduced genome (1.44 megabases) compared to other cells in the UCYN<sub>2</sub>-fix lineage such C. watsonii WH8501 (5.46 megabases) and Cyanothece sp. ATCC5114 (6.24 megabases) (Tripp et al., 2010). The UCYN-A cells are the smallest among the three groups. UCYN-A are spherical cells ≤1 µm in diameter (Goebel et al., 2008). UCYN-B are also spherical cells with a reported diameter range of 3-5 µm in laboratory isolates (Goebel et al., 2008), and 3-8 µm in cultures and natural samples (Moisander et al., 2010). Finally, UCYN-C cells have been recently described as having 2.5-3 µm width and 4-6 µm length (Taniuchi et al., 2012). UCYN-A have been recently found to have a novel metabolism lacking the O<sub>2</sub>-producing photosystem II complex of the photosynthetic apparatus (Zehr et al., 2008), which enables them to fix N<sub>2</sub> during the day. In contrast, other UCYN fix N<sub>2</sub> during the night for temporal segregation of O<sub>2</sub>-generating photosynthesis from the  $N_2$  fixation processes (e.g. Church et al., 2005; Zehr et al., 2007). The latter process being affected with  $O_2$  because the nitrogenase enzyme is irreversibly inhibited by O<sub>2</sub> (Burgess and Lowe, 1996). Moreover, UCYN-A has been found to lack major metabolic pathways such as tricarboxylic acid cycle (TCA), and has a photofermentative metabolism which may render them dependent or in close association with other organisms (Tripp et al., 2010). Indeed, in a very recent paper Thompson et al. (2012) have reported the symbiosis of UCYN-A cells with a eukaryotic alga.

Unlike the larger N<sub>2</sub> fixer *Trichodesmium*, less is known about the distribution and growth requirements of cells in UCYN<sub>2</sub>-fix lineage groups, but recent research has indicated they have spatial distributions different from those of *Trichodesmium* (Moisander et al., 2010; Goebel et al., 2010). Warm ( $\geq$  25 °C) stratified waters and oligotrophic conditions are generally required for proliferation of *Trichodesmium* (Capone et al., 1997), while N<sub>2</sub>-fixing cells in UCYN<sub>2</sub>-fix lineage groups have been found in various locations including regions where the temperature is lower than the range where *Trichodesmium* is usually found (Langlois et al., 2005; Moisander et al., 2010), and in deeper and nutrient-rich areas (Rees et al., 2009; Church et al., 2009; Moisander et al., 2010). Most of the reports on the distribution of cells in UCYN<sub>2</sub>-fix lineage (particularly UCYN-A) were studies done in the Pacific Ocean (Zehr et al., 2001).

2008), and only a few in the Atlantic Ocean, focused at tropical and subtropical latitudes (<30 °N) (Langlois et al., 2005, 2008; Goebel et al., 2010). UCYN<sub>2</sub>-fix lineage studies are virtually non existent in areas further north (>30 °N) of the Atlantic Ocean, particularly in upwelling regions (Luo et al., 2012). There is a need to better characterize the spatial distribution of the cells in the UCYN<sub>2</sub>-fix lineage in the oceans to be able to assess comprehensively their impact on the global scale in N<sub>2</sub> fixation.

Here we investigate the horizontal and vertical distribution of cells in the UCYN<sub>2</sub>-fix lineage in the subtropical Northeast Atlantic Ocean (28.87 to 42.00°N and 9.01 to 20.02°W) through size fractionated *nifH* gene nested PCR analysis (Langlois et al., 2005) and TSA-FISH using probe Nitro821 (Biegala and Raimbault, 2008), which also allows to classify the UCYN<sub>2</sub>-fix cells detected as free-living, occurring as symbionts or complexed to particles. Furthermore, size fractionated measurements of N<sub>2</sub> fixation rates were done using both acetylene reduction assay (ARA) (Stal, 1988; Capone, 1993), and by the <sup>15</sup>N<sub>2</sub> stable isotope technique (Montoya et al., 1996). These are published elsewhere (Benavides et al., 2011), but will be used here for correlation analysis purposes. The study was carried out in stations encompassing water conditions (from oceanic oligotrophic waters to upwelling areas), and depths and temperature range of 13.1 to 24.2 °C allowing further analysis on how the distribution and activity of UCYN<sub>2</sub>-fix cells may be affected by different growth conditions.

# Materials and methods

# Study sites, hydrography and nutrient analyses

The study was carried out on board the R/V Sarmiento de Gamboa during the three legs of the CAIBEX project cruise ('Shelf-Ocean Exchanges in the Canaries-Iberian Large Marine Ecosystem') during the summer of 2009. The CAIBEX project was planned to carry interdisciplinary studies of two active upwelling filament systems that export water from the coastal zone into the oligotrophic subtropical gyre of the North Atlantic Ocean. The two upwelling filament systems studied were Cape Silleiro (Northwest Iberia, from 6-24 July, leg 1) and Cape Ghir (Northwest Africa, from 16 August - September 5, leg 3) (Fig. 1). A large-scale survey was also conducted around a box between 20°W and the Canary Islands, enclosing the two upwelling filament appears intermittently but recurrently every summer while the Cape Ghir filament is a strong and quasi-permanent feature (Arístegui et al., 2009).

During the time of the cruise, the Cape Silleiro filament did not appear and the stations sampled were coastal upwelling (stations S05 to S08) and open ocean stations (stations S01 to S03) during leg 1 (Fig. 1). During leg 2, 17 stations were sampled during the large-scale survey, and finally during leg 3, stations sampled were along the path of the Cape Ghir filament (G12, G17, G22, G40, G44, G48) and stations outside the filament (GM2 and GT2 located inside and outside the upwelling area, respectively) (Fig. 1).

At each station during the three legs of the cruise, temperature, salinity, and chlorophyll fluorescence were recorded by means of a CTD SeaBird 911 Plus conductivity-temperature-depth (CTD) system, mounted on a General Oceanics 24 bottle rosette sampler equipped with 12-L Niskin Bottles. Dissolved  $O_2$  and chlorophyll fluorescence were also measured with auxiliary sensors attached to the CTD system. Samples for inorganic nutrient analyses were collected at each station. Details on these analyses may be found in Benavides et al. (2011).

# Primary production and fractionated N<sub>2</sub> fixation

Primary production was measured by transferring surface (~5 m) seawater to 1 L transparent polycarbonate bottles (Nalgene). 1 mL of NaH<sup>13</sup>CO<sub>3</sub> (0.28 M; 99 at.%; Sigma-Aldrich) was added to a final enrichment of ~10%. After 24 h of incubation on light-attenuated on-deck incubators cooled with surface seawater, the samples were filtered onto pre-combusted (5h, 650°C) GF/F filters (Whatman), and analyzed onshore for particulate organic carbon (POC) and <sup>13</sup>C atom % enrichment using a Thermo Flash EA 1112 elemental analyzer interfaced by a Conflo III with a Thermo Delta V Advantage isotope ratio mass spectrometer (IRMS). Carbon uptake rates were calculated with the equations in Hama et al. (1983).

Samples for the measurement of N<sub>2</sub> fixation rates were collected from the surface (~5 m) with 12-L Niskin bottles. N<sub>2</sub> fixation rates were measured using two techniques: the ARA (Stal, 1988; Capone, 1993), and the <sup>15</sup>N<sub>2</sub> stable isotope technique (Montoya et al., 1996). In leg 2 (CAIBOX) fractionated rates (<10  $\mu$ m and <3  $\mu$ m) were measured, while in legs 1 and 3 (Cape Silleiro and Cape Ghir), only whole seawater and <10  $\mu$ m N<sub>2</sub> fixation rates were measured due to logistical constraints. Further details on the application of the two N<sub>2</sub> fixation techniques used may be found in Benavides et al. (2011).

# *Size fractionated* nifH *gene nested PCR analysis, cloning and sequencing of clones*

During all three legs of the cruise, 10 L of water from each of three depths (surface, MLD and DCM) were filtered in line using 47 mm Fluoropore (pore size 10 μm), 47 mm MF-Millipore (pore size 3 μm), and 47 mm Durapore (pore size 0.22 µm) to collect deoxyribonucleic acid (DNA) material from the following size fractions: >10  $\mu$ m, 3-10  $\mu$ m, and <3  $\mu$ m. The filters were frozen and stored at -80°C until further processing for nucleic acid extraction (Nogales et al., 2007) using half of each filter. The nested PCR amplification technique used here is a modification of the method in Langlois et al. (2005). The first round of PCR [10x buffer II, MgCl<sub>2</sub> (final concentration, 4 mM), 10 mM deoxynucleoside triphosphates, 80 pmol of each primer: nifH4 (A. vinelandii positions 546 to 562; 5'-TTY TAY GGN AAR GGN GG-3') and nifH3 (A. vinelandii positions 1018 to 1002; 5'-ATR TTR TTN GCN GCR TA-3'), (Zehr and McReynolds, 1989; Zani et al., 2000)], consisted of: 10 min at 95°C; 35 cycles, each of 1 min at 95°C, 1 min at 45°C, and 1 min at 72°C; and finally 10 min at 72°C. The second round of PCR used 10x buffer II, MgCl<sub>2</sub> (final concentration, 6 mM), 10 mM deoxynucleoside triphosphates and 80 pmol of four primer sets, using a similar thermocycler program but with an annealing temperature of 54°C and only 28 cycles. The mixture of four primers amplifying nifH fragments for UCYN Groups A, B and C (Needoba et al., 2007) and Trichodesmium used in the second PCR were designed by selecting 52 sequences of cyanobacterial *nifH* genes (Table 1).

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Group	Forward primer	Reverse primer
UCYN-A	5'-CGTYTAATGTTGCAYTGTAAAGC-3'	5'-CCTAATACATCATAAGATACG-3'
UCYN-B	5'-CGTTTAATC CTCAACTGTAAAGC-3'	5'-CCTAATACGTCGTAGGATACG-3'
UCYN-C	5'-CGT TTG ATG CTA CAC ACT AAA GC-3'	5'-CCTAATACGTCATAGGATACG-3'
Trichodesmium	5'-CGTTTAATCTTAAACGCTAAAGC-3'	5'-CCTAGTACGTCATAGCTTACG-3'

The relative quantity of DNA from the PCR products was determined using Image Lab Software (Bio-Rad) by measuring the intensity of the bands from agarose gel electrophoresis. The quantities were normalized with the bands from the 100 Base-Pair Ladder (GE-Healthcare). Amplicons of ~300-bp size as revealed by agarose gel electrophoresis in 15 representative samples which showed relative high quantity of DNA (Figs. 4, 5, 6) were cloned using the TOPO TA cloning kit (Invitrogen), according to the manufacturer's instructions. Ten clones from each cloned library of the samples were randomly picked and sequenced in the forward direction using the M13F primer with a BigDye vs. 3.1. The phylogenetic analyses of  $\sim 150$  nifH obtained were analyzed sequences using BLASTn (http://www.ncbi.nlm.nih.gov). related Closest known and unknown from published nifH sequences were retrieved GenBank (http://www.ncbi.nlm.nih.gov) and aligned with sequences obtained during the study using CLC Sequence Viewer 6 software. Alignments based on a 199 bp DNA sequence were used to construct a neighbor-joining phylogenetic tree rooted to the Methylomonas rubra clone 23-4 nitrogenase iron protein (nifH) gene (AF484673) and relationships were bootstrapped 1000 times using CLC Sequence Viewer 6.

#### Size fractionated TSA-FISH using probe Nitro821

In situ whole-cell detection of probe Nitro821-targetted unicellular N<sub>2</sub>fixing cyanobacteria was done following the methods in Zwirglmaier (2005) and Biegala and Raimbault (2008). Depending on the degree of saturation of the filters, volumes between 250 mL and 4 L of seawater samples were filtered from the three depths (surface, MLD and DCM) on 47 mm Isopore<sup>™</sup> PC membrane filters of three different pore sizes (10  $\mu$ m, 3  $\mu$ m, 0.22  $\mu$ m) and fixed with 1% paraformaldehyde (pH 8.2, buffered with phosphate buffered saline -PBS-) for 15 minutes at room temperature, and clarified with PBS by filtration. Filters were air-dried for approximately 5 min and cells were subsequently dehydrated with ethanol (EtOH) molecular grade series (50%, 80% and 100%). each for 10 minutes at room temperature, and stored at -20°C until further analysis. Filtered samples which gave *nifH* positive results from PCR analyses described above were analyzed for TSA-FISH. A subsample of the filter (1/8) was used for TSA-FISH. Hybridizations with HRP (Horse Radish Peroxidase)labeled (Thermo Fisher Scientific GmbH) 16S rRNA Nitro821 probe were done after permeabilization of the cells with Lysozyme (5 mg mL<sup>-1</sup> in lysozyme buffer, Roche) for 30 minutes at 37°C, followed by three rinses of Milli-Q sterile water, and EtOH molecular grade series (50%, 80% and 100%). The cells were subsequently processed for hybridization steps and TSA reactions using 50% of formamide concentration in the hybridization buffer (Biegala and Raimbault, 2008). The filters finally were mounted in a microscope slide, applying mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vectashield).

The slides were inspected under an epifluorescence microscope [Leica DM 2500 equipped with 12 V/100W Halogen lamp, objectives HCX FL Plan10x/0.25, N Plan 40x/0.65 Ph2, N Plan 63x/0.80 0.17D and N Plan 100x/1.25 oil PH3, filters ( $360 \pm 20$  excitation (ex.),  $410 \pm 5$  emission (em.) for the DAPI (blue fluorescence); L5 ( $480 \pm 40$  ex.,  $527 \pm 30$  em.) for the fluorescein isothiocyanate (FITC) associated with Nitro821 probe (green

fluorescence); L3(450-490 ex., 515 long pass em.) for chlorophyll fluorescence (orange fluorescence); equipped with a digital camera (Leica DFC420C with software for control of time of exposure, zoom and color. The exposure time was adjusted for improved visualization of FITC-labeled cells (to reduce the interference of the chlorophyll autofluorescence). The filters were counted in a systematic way to cover the entire surface of each filter slice. Nitro821 positive cells were counted under objective 100x. Cells that were present as (1) free-living, (2) as symbionts to larger cells, and (3) complexed to putatively organic particles were noted and counted as small (<1  $\mu$ m), medium (1-3  $\mu$ m) and large (>3  $\mu$ m) cells.

### Data and statistical analyses

Size fractionated N<sub>2</sub> fixation rates for the >10  $\mu$ m and 3-10  $\mu$ m fractions were calculated as the difference between N<sub>2</sub> fixation rate of whole water sample and <10  $\mu$ m fraction, and the difference between N<sub>2</sub> fixation rate of <10  $\mu$ m and <3  $\mu$ m fractions, respectively. The normal distribution of data was checked with Shapiro-Wilk W test and, if necessary, data were logarithmically transformed prior to analyses. Pearson correlation and regression analyses were used to determine the relationships between N<sub>2</sub> fixation rates, size fractionated *nifH* gene nested PCR analysis, TSA-FISH counts, and the various physico-chemical factors measured (temperature, salinity, chlorophyll fluorescence, dissolved O<sub>2</sub>, total primary production, PO<sub>4</sub><sup>3-</sup>, NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup>). Statistical analyses were performed using SPSS software.

# Results

# Hydrography, primary production and nutrient analyses

Table 2 summarizes the MLD and DCM selected physico-chemical parameters and primary production in surface waters during the three legs of the cruise. In the CAIBOX grid (leg 2), the MLD and DCM were generally deeper and surface waters exhibited warmer temperatures, lower concentration of inorganic nutrients ( $PO_4^{3-}$ ,  $NO_3^{-} + NO_2^{-}$ ) and lower primary production than Cape Silleiro (leg 1) and Cape Ghir (leg 3) (Table 2). The surface waters in Cape Ghir exhibited the highest concentration of inorganic nutrients ( $PO_4^{3-}$ ,  $NO_3^{-} + NO_2^{-}$ ) and primary production among the three legs of the cruise, while surface chlorophyll fluorescence was highest in Cape Silleiro (Table 2). Detailed hydrography studies of the region are published elsewhere (Carracedo et al., 2012; Troupin et al., 2012).

### Size-fractionated N<sub>2</sub> fixation rates

Hourly N<sub>2</sub> fixation rates using the ARA technique in the Cape Silleiro filament system (leg 1) of the <10  $\mu$ m fraction ranged from 0.14 – 4.57 and 0.64 – 4.03 nmol N m<sup>-3</sup> h<sup>-1</sup> during light and dark incubations respectively, and net daily rates of 16.9 – 186.1 nmol N m<sup>-3</sup> d<sup>-1</sup> using the <sup>15</sup>N<sub>2</sub> technique. In the other filament system in Cape Ghir (leg 3), hourly N<sub>2</sub> fixation rates using the ARA technique of the <10 µm fraction ranged from 0.72 – 39.86 and 0.37 – 4.99 nmol N m<sup>-3</sup> h<sup>-1</sup> during light and dark incubations respectively, and <sup>15</sup>N<sub>2</sub> daily N<sub>2</sub> fixation rates ranged between 7.9 and 174.3 nmol N m<sup>-3</sup> d<sup>-1</sup>. These N<sub>2</sub> fixation rates for  $<10 \ \mu m$  fraction in both filaments systems corresponded to approximately >50% of total N<sub>2</sub> fixation (Benavides et al., 2011; Figs. 1, 2). In the finer resolution of size fractionated N<sub>2</sub> fixation rates determined in the large-scale survey (leg 2, CAIBOX, grid box), hourly N<sub>2</sub> fixation rates using the ARA technique in the <3  $\mu$ m fraction ranged from 0.55 – 4.61 and 0.46– 2.76 nmol N m<sup>-3</sup> h<sup>-1</sup> during light and dark incubations respectively, and <sup>15</sup>N<sub>2</sub> fixation rates ranged from 12.0 to 90.0 nmol N m<sup>-3</sup> d<sup>-1</sup>. N<sub>2</sub> fixation rates for the <3  $\mu$ m fraction measured during the CAIBOX grid (leg 2) corresponded to approximately >50% of total N<sub>2</sub> fixation, while those of the 3–10 µm fraction corresponded 0 – 37 % of total N<sub>2</sub> fixation (Figs. 1, 2). The N<sub>2</sub> fixation rates of the  $<3 \mu m$  fraction, measured in the dark, were inversely linearly correlated with primary production and dissolved O<sub>2</sub>, but positively linearly correlated with temperature and salinity during the CAIBOX (leg 2; Table 3).

# *Size fractionated* nifH *gene nested PCR analysis, cloning and sequencing of clones*

Results of *nifH* gene amplification in samples from the surface, MLD and DCM during the three legs revealed that most (>50%) of the *nifH* genes recovered were from the <3 µm fraction, ranging from 34 - 406 ng, 10 - 421 ng, and 48 - 810 ng (per 5-L sample filtered) in Cape Silleiro (leg 1), CAIBOX (leg 2) and Cape Ghir (leg 3), respectively (Figs. 3, 4, 5). The *nifH* genes were also recovered from the 3-10 µm and >10 µm fractions in the surface and MLD, but not in the DCM, unlike in the <3 µm fraction which was recovered in all three depths (Figs. 3, 4, 5). During the CAIBOX (leg 2), the relative quantity of *nifH* genes recovered in the <3 µm fraction in all three depths was significantly positively correlated with temperature and salinity, but significantly negatively correlated with dissolved  $O_2$  and chlorophyll fluorescence (Table 3). The relative quantity of *nifH* genes recovered in the >10 µm fraction during the CAIBOX (leg 2) and 3-10 µm fraction in all three legs were also significantly positively correlated with temperature (Pearson r = 0.28, p < 0.05, n = 51 and (Pearson r = 0.27, p < 0.05, n = 102, respectively, data not shown).



Fig. 1: Relative contribution of the different size fractions to overall N<sub>2</sub> fixation rates (nmol N h<sup>-1</sup> m<sup>-3</sup>) based on Acetylene Reduction Assays in surface waters in the three legs of the cruise (Cape Silleiro, CAIBOX grid and Cape Ghir) measured in (A) light and (B) dark incubations. The sizes of the circles are proportional to total N<sub>2</sub> fixation rate as indicated in the legend.

Leg	MLD (m)	DCM (m)	Temperature (ºC)	Salinity	Fluorescence (arbitrary units)	Dissolved O <sub>2</sub> (mg L <sup>-1</sup> )	NO3 <sup>-</sup> + NO2 <sup>-</sup> (μmol L <sup>-1</sup> )	PO4 <sup>3.</sup> (μmol L <sup>-1</sup> )	Primary production (mmol C m <sup>-3</sup> h <sup>-1</sup> )
Cape Silleiro	26 ± 10	38 ± 23	16.88 ± 1.48	35.65 ± 0.06	$0.48 \pm 0.34$	7.5 ± 0.4	0.67 ± 0.79	0.05 ± 0.06	$0.15 \pm 0.07$
(Leg 1, n=8)	(15-40)	(12-80)	(15.05-18.50)	(35.57-35.75)	(0.10-1.00)	(7.1-8.0)	(0.00-2.11)	(0.00-0.18)	(0.03-0.24)
CAIBOX grid	42 ± 7	82 ± 20	21.84 ± 2.15	36.46 ± 0.53	$0.11 \pm 0.07$	6.5 ± 0.4	0.12 ± 0.13	0.03 ± 0.02	$0.02 \pm 0.01$
(Leg 2, n=17)	(25-55)	(53-115)	(18.58 -24.18)	(35.70 -37.11)	(0.03-0.28)	(6.0-7.6)	(0.03-0.61)	(0.02-0.05)	(0.00-0.04)
Cape Ghir	23 ± 6	40 ± 9	19.67 ± 1.34	36.37 ± 0.05	$0.06 \pm 0.04$	6.7 ± 0.2	2.22 ± 3.41	0.68 ± 0.31	$0.12 \pm 0.14$
(Leg 3, n=8)	(15-30)	(25-50)	(16.65-21.15)	(36.28-36.45)	(0.04-0.15)	(6.2-6.9)	(0.06-10.45)	(0.36-1.36)	(0.04-0.45)

Table 2: Average (± SD) and range (minimum-maximum) of surface values (~5 m depth) of selected physicochemical parameters, hydrography and primary production in the different stations in each leg of the CAIBEX cruise.

Phylogenetic analysis of the ~150 *nifH* gene sequences obtained from the 10 clones of amplicons from 15 samples (Figs. 3, 4, 5) revealed that the majority (95%) of the sequences had similarities of 98-100% to UCYN-A (Fig. 6). Only 8 of the sequences (mostly from station X17 in the CAIBOX grid) were not classified as UCYN-A (Fig. 6), but were 100% similar to *nifH* of uncultured clones from hypoxic basins within the California Bight (www.ncbi.nlm.nih.gov/nucleotide/337733918?report=genbank&log\$=nuclto p&blast rank=1&RID=6BG42Z5B01R).

### Size fractionated TSA-FISH using probe Nitro821

Results of size fractionated TSA-FISH using probe Nitro821 revealed a community of N<sub>2</sub> fixers in different size ranges, <1  $\mu$ m, 1-3  $\mu$ m and >3  $\mu$ m. In most cases, the <1 µm and 1-3 µm were more abundant than >3 µm Nitro821positive cells. Generally, total Nitro821-positive cells reached the highest abundance at all depths in Cape Ghir, reaching up to 38 x 10<sup>3</sup> cells m<sup>-3</sup>, 32 x 10<sup>3</sup> cells m<sup>-3</sup>, and 19 x 10<sup>3</sup> cells m<sup>-3</sup> for the surface, MLD and DCM, respectively (Fig. 7). The Nitro821-positive cells in different size classes (<1  $\mu$ m, 1-3  $\mu$ m and >3  $\mu$ m) can occur as free-living, complexed to putatively organic particles and within larger cells (Benavides et al., in press). In general terms, the proportion of cells occurring as free-living were the most abundant in all legs (could reach up to 100% of all Nitro821-positive cells), followed by cells complexed to organic matter, and lastly by cells within other larger cells. However, in Cape Silleiro the proportion of >3 µm Nitro821-positive cells occurring as symbionts within larger cells were more significant than those being complexed to organic matter (Table 4). The total number of free-living small (<1  $\mu$ m) Nitro821-positive cells in all three legs and three depths was significantly positively correlated with salinity but significantly negatively correlated with dissolved O<sub>2</sub> and chlorophyll fluorescence (Table 3).

#### Discussion

Nitrogen fixation activities of the <10  $\mu$ m fraction in surface waters, albeit low (<200 nmol N m<sup>-3</sup> d<sup>-1</sup>) were detected throughout the study area in the subtropical Northeast Atlantic Ocean from 28.87 °N to 42.00 °N (Benavides et al., 2011). The N<sub>2</sub> fixation rates of the <10  $\mu$ m presumably correspond to activities of smaller UCYN<sub>2</sub>-fix cells and/or heterotrophic diazotrophs (Benavides et al., 2011), as larger *Trichodesmium* trichomes measured 10-20  $\mu$ m in length in our study sites (M. Benavides, unpublished results). In other sites of the tropical North Atlantic, *Trichodesmium* can be found in low abundances (<10% total abundance) in the <10  $\mu$ m fraction (Goebel et al.,

2010). Moreover, other diazotrophs such as *Richelia intracellularis* -which is the typical endosymbiont of the diatoms *Rhizosolenia* spp. or *Hemiaulus* spp.-have size ranges of 15 to 100  $\mu$ m and 15 to 35  $\mu$ m, respectively, and thus it is unlikely



Fig. 2.: Relative contribution of the different size fractions to overall N<sub>2</sub> fixation rates (nmol N d<sup>-1</sup> m<sup>-3</sup>) based on <sup>15</sup>N<sub>2</sub> technique in surface waters in the three legs of the cruise (Cape Silleiro, CAIBOX grid and Cape Ghir). The sizes of the circles are proportional to total N<sub>2</sub> fixation rate as indicated in the legend

that they were present in the <10  $\mu$ m fraction. The predominance of N<sub>2</sub> fixation by the smaller size fraction (<10  $\mu$ m) in all our study sites is consistent with previous results in the eastern Atlantic Ocean at lower latitudes than our study sites (Voss et al., 2004; Montoya et al., 2007; Staal et al., 2007; Goebel et al., 2010). Finer resolution of the size-fractionated measurements of N<sub>2</sub> fixation during the CAIBOX leg cruise revealed that the >10  $\mu$ m and <3  $\mu$ m size fractions contributed dominantly (>50% of total N<sub>2</sub> fixation), more than the 3-10  $\mu$ m fraction (0-37% of total N<sub>2</sub> fixation) (Figs. 1, 2). The N<sub>2</sub> fixation in the <3 µm fraction presumably represents predominantly the activities of UCYN-A cells. These are the smallest ( $\leq 1 \mu m$  in diameter; Goebel et al., 2008) among the other cells in the UCYN<sub>2</sub>-fix lineage ( $\geq$  3 µm; Goebel et al., 2008; Taniuchi et al., 2012). Hence, UCYN-A likely can pass through the 3 µm filter better than cells from the other UCYN groups. However, the UCYN-A cells can also contribute to the  $N_2$  fixation in the 3-10  $\mu$ m fraction, as these cells can be associated with larger phytoplankton cells and particulate material of >3  $\mu$ m (Sohm et al., 2011b; Thompson et al., 2012).

The dominance of the  $N_2$  fixation activities of the <3  $\mu$ m fraction measured in surface waters during the CAIBOX (leg 2) is consistent with the results of the *nifH* gene amplification, where most (>50%) of the *nifH* genes recovered were from the  $<3 \mu m$  fraction in samples from the surface waters in almost all stations (Fig. 3). The predominance of the  $<3 \mu m$  fraction extended to the MLD (15 -55 m, Table 2) and DCM depths (12-115 m, Table 2), based on the amount of *nifH* genes recovered (Fig. 4, 5). The larger (3-10  $\mu$ m and >10 μm) *nifH* containing cells were more restricted in the surface and MLD (Fig. 4, 5). The identity of the cells in the UCYN<sub>2</sub>-fix lineage present in the <3  $\mu$ m fraction points to be UCYN-A (sequence similarities of 98-100%), as revealed by cloning and sequencing of representative samples (Fig. 6) in the three depths sampled. Moreover, the presence of UCYN-A in the 3-10  $\mu$ m fractions (based by cloning and sequencing) indicates they can occur either as free-living, complexed to organic matter, or associated within larger cells (Zehr et al., 2008; Tripp et al., 2010). A minor fraction (5%) of the clones sequenced were identical (100% sequence similarity) to *nifH* gene containing uncultured bacterium clone from hypoxic basins within the California Bight, which remains to be further investigated on their physiology, distribution and ecological significance.

Consistent with results from *nifH* gene analysis that most (> 50%) of the *nifH* genes recovered were from the <3  $\mu$ m fraction, results from size fractionated TSA-FISH using probe Nitro821 revealed that, in most cases, the <1  $\mu$ m and 1-3  $\mu$ m are more abundant than the >3  $\mu$ m Nitro821-positive cells, with a generally higher proportion of cells occurring as free-living than complexed to organic matter or associated within larger cells. The identity of



Fig. 3: Relative contribution of the different size fractions to overall quantity of *nifH* (ng) recovered in surface waters in the three legs of the cruise (Cape Silleiro, CAIBOX grid and Cape Ghir). The sizes of the circles are proportional to the total quantity of *nifH* recovered as indicated in the legend. Samples chosen for cloning and sequencing are indicated as a (<3  $\mu$ m), b (3-10  $\mu$ m) and c (>10  $\mu$ m).

Table 3: Significant Pearson correlations (r, p < 0.05) between N<sub>2</sub> fixation rates, size fractionated *nifH* gene nested-PCR analysis and TSA-FISH counts of the smallest size fractions (y-variables) and the various physico-chemical factors (x-variables) measured. These significant relationships were then fitted into significant equations as indicated.

Cruise Leg	Biological parameter	Temperature (ºC)	Salinity	Fluorescence (arbitrary units)	Dissolved $O_2$ (mg L <sup>-1</sup> )	Pirmary production
CAIBOX grid (Leg 2)	N2 fixation rate (nmol.N m <sup>-3</sup> h <sup>-1</sup> ) <3 μm fraction	r = 0.62 y = -2.69 + 0.18x (r <sup>2</sup> =0.37, p <0.05, n=15)	r = 0.58 y = -22.93 + 0.66x (r <sup>2</sup> =0.30, p <0.05, n=15)		r = -0.58 y = 6.82 - 0.84x (r²=0.38, p <0.05, n=15)	r = -0.56 y = 1.66 – 22.75x (r²=0.32, p <0.05, n=15)
CAIBOX grid (Leg 2)	<i>nifH</i> (ng) <3 μm fraction	r = 0.49 y = -113.7 + 10.5x (r <sup>2</sup> =0.09, p <0.05, n=51)	r = 0.52 y = -4338 + 122x (r <sup>2</sup> =0.28, p <0.05, n=51)	r =48 y = 116.7e <sup>-0.74x</sup> (r <sup>2</sup> =0.09, p <0.05, n=51)	r = -0.49 y = 513.4 - 61.4x (r <sup>2</sup> =0.12, p <0.05, n=51)	
All legs	FISH counts, (number of free cells m <sup>-3</sup> ) <1 μm fraction		r = 0.47 y = -49533 + 1394x (r²=0.22, p <0.05, n=44)	r = -0.29 y = 1859e <sup>-01.88x</sup> (r <sup>2</sup> =0.17, p < 0.05, n=44)	r = -0.35 y = 5736 - 660x (r²=0.08, p <0.05, n=44)	

	<1 µm free	< 1 µm complexed to particles	< 1 µm inside larger cells	1-3 µm free	1-3 μm complexed	1-3 μm inside larger cells	> 3 µm free	> 3 µm complexed to particles	> 3 µm inside larger cells
Leg 1 Surface (n=3)	103-617	0-51	0-51	103-1780	0	0	0-257	0	0-10
	(312 ± 181)	(17 ± 30)	(17 ± 30)	(6257 ± 10006)			(141 ± 130)		3 ± 6
	60-100%	0-10%	0-10%	30-100%	0%	0%	0-100%	0%	0-6%
	(80 ± 20%)	(3 ± 6%)	(3 ± 6%)	(58 ± 52%)			(65 ± 56%)		(2 ± 3%)
Log 1	0-103	0-515	0-25	0-103	0-51	0-51	0-257	0	0-103
MLD	(51 ± 51)	(171 ± 297)	(8 ± 14)	(50 ± 51)	(31 ± 27)	(31 ± 27)	(93 ± 143)		(34 ± 59)
(n=3)	0-100%	0-95%	0-5%)	0-67%	0-33%	0-33%	0-100%	0%	0-29%
( 0)	(67 ± 58%)	(32 ± 55%)	(2 ± 3%)	(30 ± 34%)	(18 ± 17%)	(18 ± 17%)	(57 ± 52%)		(10 ± 16%)
Log 1	103-617	0-3910	0	0-60	0	0	0-154	0	0-51
DCM	(360 ± 364)	(1955 ± 2765)		(37 ± 32)			(64 ± 77)		(13 ± 26)
(n=4)	3-100%	0-97%	0%	0-100%	0%	0%	0-100%	0%	0-25%
(11-1)	(51 ± 69%)	(49 ± 69%)		(67 ± 58%)			(44 ± 51%)		(6 ± 12%)
Log 2	2-4650	0-8	0-3	2-6119	0-468	0-34	2-966	0-393	0-57
Surface	(1399 ± 1667)	(1 ± 3)	$(0.3 \pm 0.9)$	(1821 ± 1891)	(81 ± 156)	(5 ± 11)	(321 ± 264)	(105 ± 153)	(8 ± 18)
	98-100%	0-2%	0-0.20%	14-100%	0-76%	0-10%	52-100%	0-49%	0-12%
(11-10)	(99 ± 1%)	$(0.2 \pm .6\%)$	(0.02 ± 0.07%)	(87 ± 27%)	(11 ± 24%)	(2 ± 4%)	(83 ± 20%)	(14 ± 18%)	(2 ± 4%)
Leg 2	116-7551	0	0-8	240-4104	0-3718	0-9896	42-1066	0-597	0-68
MLD (n=9)	(2179 ± 2412)		(1 ± 3)	(2220 ± 1708)	(561 ± 1292)	(1129 ± 3486)	(475 ± 390)	(176 ± 245)	(12 ± 27)
	0-100%	0%	0-1%	0-100%	0-49%	0-68%	8-100%	0-85%	0-18%
	(89 ± 35%)		$(0.1 \pm 0.3\%)$	(71 ± 39%)	(9 ± 17%)	(8 ± 24%)	(75±31%)	$(22 \pm 30\%)$	(3 ± 7%)
Leg 2 DCM (n=9)	0-2670	0-412	0	0-4102	0-5539	0-1780	0-2475	0-338	0-733
	$(1087 \pm 1025)$	(46 ± 137)		$(1602 \pm 1279)$	$1183 \pm 2300$	(198 ± 593)	(852 ± 968	(61 ± 125)	(84 ± 243)
	0-100%	0-39%	0%	0-100%	0-74%	0-45%	33-100%	0-67%	0-42%
. ,	(85 ± 34%)	(4 ± 13%)	0.55	(66 ± 39%)	(17±31%)	(5 ± 15%)	(75±37%)	(9 ± 22%)	(5±14%)
Leg 3	13-3365	0-102	0-55	204-310/1	0-825	0-110	250-3627	(122 + 240)	0-62
Surface	$(1407 \pm 1389)$ 75 10004	$13 \pm 30$ 0.2504	/ ± 19 0 204	$(7212 \pm 10463)$	$(110 \pm 289)$ 0.1204	$(14 \pm 39)$ 0.204	$1328 \pm 1100$	$(132 \pm 249)$	$(13 \pm 22)$
(n=8)	(97 + 906)	$(3 \pm 90\%)$	$(0.2 \pm 0.70\%)$	$(98 \pm 40\%)$	(2 + 406)	$(0.2 \pm 0.5\%)$	(76 + 35%)	(0-44.0) (10 + 17%)	(1 + 1506)
	313-7539	0	0	271-15112	0-2709	0-651	0-9151	0-147	0.70
Leg 3	(2006 + 2394)	0	0	(5261 + 5820)	(390 + 948)	(130 + 247)	(1895 + 3033)	(27 + 51)	(18 + 27)
MLD (n=8)	100%	0%	0%	64-100%	0-36%	0-8%	0-100%	0-37%	0-5%
	$(100 \pm 0\%)$	0,0	0,0	$(92 \pm 13\%)$	$(7 \pm 13\%)$	$(1 \pm 3\%)$	$(80 \pm 35\%)$	$(6 \pm 13\%)$	$(1 \pm 2\%)$
	0-2041	0	0	0-5651	0-11161	0	698-1378	0	0-827
Leg 3	(1293 ± 845)			2619 ± 2263	(2232 ± 4991)		(990 ± 292)		(165 ± 370)
DCM (n=5)	100%	0%	0%	0-100%	0-73%	0%	59-100%	0%	0-41%
	(80 ± 45%)			(65 ± 48%)	(15 ± 33%)		(92 ± 18%)		(8 ± 18%)

Table 4: Range (and average ± SD) of cell numbers (cells m<sup>-3</sup>) and percentage (% of cells to total of each size class) of Nitro821-positive cells found as free-living, complexed to particles and inside larger cells.

Nitro821-positive spherical cells <1 µm presumably represents UCYN-A (Goebel et al., 2008). The Nitro821-positive cells classified from 1-3 µm may contain UCYN-A, since Nitro821-positive cells up to 1.5 µm have recently been affiliated to UCYN-A based on 16S and *nifH* phylogenies (Le Moal et al., 2011). Additionally, the 1-3  $\mu$ m fraction may contain UCYN-B (whose sizes can be  $\geq$ 3 µm, Goebel et al., 2008), and other non-identified N<sub>2</sub> fixers (Biegala and Raimbault, 2008). Nitro821-positive cells of >3 µm may contain UCYN-B, UCYN-C and other non-identified N<sub>2</sub> fixers. However, N<sub>2</sub> fixing cells closely affiliated to UCYN-B and UCYN-C have not been detected by cloning and sequencing of representative samples, implying that either they were not present or their abundances were too low to be detected by PCR amplification. This suggests that the Nitro821-positive cells from 1-3 µm may be also UCYN-A, with a possible minor contribution of the identified cluster of N<sub>2</sub> fixers (Fig. 6). The presumably UCYN-A cells were found in all depths (from surface down to the DCM) as free-living cells, but when complexed to organic matter and associated to larger cells, they were found mainly in the surface and MLD. The presumed abundances of these UCYN-A cells (<1 plus 1-3 µm Nitro821positive free-living cells) ranged from  $1 \times 10^2$  to  $8.6 \times 10^3$  cells L<sup>-1</sup> with no clear abundance pattern along the depth of the water column. To date, these are first estimates of UCYN-A cells detected in this region of the Atlantic (>30 °N) using whole-cell TSA-FISH technique using a 16S rDNA probe specific for the UCYN<sub>2</sub>fix lineage. Our estimated abundance of UCYN-A cells in our study sites compared well to estimates in the Mediterranean, where UCYN-A cells (Nitro821-positive free-living cells of  $\sim 0.8$  to 1.5 µm) ranged from 1 to 6 x 10<sup>3</sup> cells L-1 (Le Moal et al., 2011). However, they were present at lower concentrations in the Mediterranean in the DCM depths (Le Moal et al., 2011). In the Atlantic region, published abundances of UCYN-A are based on quantitative PCR (qPCR), which allows the quantification of *nifH* copy numbers per unit volume.

In the tropical eastern North Atlantic (near Cape Verde Islands, <27°N), UCYN-A were found at 6.8 x  $10^2 - 0.13 \times 10^6$  *nifH* copies L<sup>-1</sup> (Turk et al., 2011). Across the tropical Atlantic Ocean UCYN-A were present at 4.8 x  $10^2 - 0.17 \times 10^6$  *nifH* copies L<sup>-1</sup> on which maximum abundances were found at deeper waters (~ 41 m), and dominating the western part of the eastern Atlantic near the Cape Verde Islands (Goebel et al., 2010). Other studies in the North Atlantic Ocean (17 - 25 °N) reported maximum abundances of 1 x  $10^6$  *nifH* copies L<sup>-1</sup> (Langlois et al., 2008). Although the abundances of UCYN-A found here are generally within the ranges found in previous studies in other sites in the Atlantic at lower latitudes than our study stations, the maximum abundances were below the maxima found in these previous studies in the Atlantic and other studies in the Pacific, where they can reach abundances of  $10^6$  *nifH* 



Fig. 4: Relative contribution of the different size fractions to overall quantity of *nifH* (ng) recovered at the bottom of the mixed layer depths (MLD) in the three legs of the cruise (Cape Silleiro, CAIBOX grid and Cape Ghir). The sizes of the circles are proportional to the total quantity of *nifH* recovered as indicated in the legend. Samples chosen for cloning and sequencing are indicated as a (<3  $\mu$ m), b (3-10  $\mu$ m) and c (>10  $\mu$ m).



Fig. 5: Relative contribution of the different size fractions to overall quantity of *nifH* (ng) recovered in the deep chlorophyll maximum (DCM) depths in the three legs of the cruise (Cape Silleiro, CAIBOX grid and Cape Ghir). The sizes of the circles are proportional to the total quantity of *nifH* recovered as indicated in the legend. Samples chosen for cloning and sequencing are indicated as a (<3  $\mu$ m), b (3-10  $\mu$ m) and c (>10  $\mu$ m).



Fig. 6: Phylogenetic tree of *nifH* sequences. Sequences obtained in this study are referred to (i) by the oceanographic cruise CAIBEX, (ii) the stations at which they are were sampled, (iii) the depth thy were sampled (A=surface, B= MLD, C= DCM), and (iv) clone number. Bootstrap values >50% are indicated at the nodes. Scale bar=0.05 substitution per nucleotides.

copies L<sup>-1</sup>, dominating diazotroph abundances (Church et al., 2009; Moisander et al., 2010).

The distributional pattern of cells in the UCYN<sub>2</sub>-fix lineage and their rates of N<sub>2</sub> fixation are apparently driven by different environmental requirements. Of the environmental factors reported in the literature, temperature seems to be one of the major drivers in shaping the distribution of the different groups of diazotrophic cyanobacteria. Distributions of Trichodesmium are often constrained by 20 - 25 °C isotherms (Capone et al., 1997), whereas diazotrophs in the UCYN<sub>2</sub>-fix lineages are found in cooler temperatures, in larger latitudinal geographic ranges (Moisander et al., 2010). Within the UCYN<sub>2</sub>-fix lineage, UCYN-A and UCYN-B are reported to have different optimum temperatures of 24 °C and 29 °C, respectively. Also, they have different spatial distributions, with UCYN-A occupying deeper cooler waters than UCYN-B (Moisander et al., 2010). Although they have a lower optimum temperature, the abundance of UCYN-A which can be represented by the relative quantity of *nifH* genes recovered in the  $<3 \mu m$  fraction (presumably dominated by UCYN-A based on the molecular techniques employed here) is positively linearly correlated with temperature (Table 3). Within the temperature range of our data set (Table 2), this correlation fits with the increasing part of the non-linear relationship (polynomial) between UCYN-A abundance and temperature reported in Moisander et al. (2010). A non-linear relationship (polynomial) between UCYN-A abundance and temperature has been described where increases in temperature initially correspond to increases in of UCYN-A abundance, but only up to 24 ºC. Subsequent increases in temperature correspond to a decline in UCYN-A abundances (Moisander et al., 2010). The physiological basis for this relationship needs further investigation.

We also found here that UCYN-A activity ( $N_2$  fixation rates of the <3 µm fraction) correlates positively with temperature (Table 3). There may be unknown diazotrophs contributing but not as predominant as that of UCYN-A, which appeared in 5% of the clones sequenced in of representative samples. UCYN-A lack the O<sub>2</sub>-producing photosystem II complex of the photosynthetic apparatus (Zehr et al., 2008), which enables them to fix N<sub>2</sub> during the day. It is also consistent that they can fix N<sub>2</sub> in darkness, independent of light availability and dependent on organic carbon for their metabolism (Tripp et al., 2010). However, the circadian rhythm of N<sub>2</sub> fixation of UCYN-A remains unclear because, based on *nifH* expression, they exhibit different diel patterns at different areas (Turk et al., 2011). Salinity, like temperature, also showed positive correlations with N<sub>2</sub> fixation rates and abundance of UCYN-A cells



Fig. 7: Relative contribution of the different size fractions to overall Nitro821-positive cells from TSA-FISH technique in samples from the surface, at the bottom of the mixed layer depths (MLD) and in the deep chlorophyll maximum (DCM) depths in the three legs of the cruise (A) Cape Silleiro, (B) CAIBOX grid and (C) Cape Ghir. The heights of the bars are the total abundance of Nitro821-positive cells.

(Table 3). However, these correlations may be due to correlations between temperature and salinity variations in North Atlantic waters (Postnov et al., 2007) or alternatively, there is indeed a physiological basis for these correlations which needs to be investigated further.

Oligotrophic conditions are generally required for the proliferation of *Trichodesmium* (Capone et al., 1997), and availability of fixed nitrogen (ammonium,  $NO_{3}$ ) has long been recognized as an inhibitor of  $N_2$  fixation (Ramos et al., 1985; Martín-Nieto et al., 1991; Sanz-Alférez and Campo, 1994). However, the dominant contribution of the <10 µm fraction in the  $NO_{3}$ -rich upwelling regions studied (and in particularly in Cape Ghir) (Benavides et al., 2011) is consistent with reports in other nutrient-enriched estuarine and coastal waters (Short and Zehr 2007; Raimbault and Garcia, 2008; Sohm et al., 2011b). This suggests that  $N_2$  fixation may not be as strongly regulated by fixed nitrogen as previously presumed.

Alternatively, different diazotrophic groups may have differential inhibition responses to fixed nitrogen availability (Martín-Nieto et al., 1991; Sanz-Alférez et al. 1994). Here, we also found evidences of inverse relationships between N<sub>2</sub> fixation rates and abundance of cells (presumably UCYN-A) with chlorophyll fluorescence and total primary production particularly in the CAIBOX grid oligotrophic open ocean stations (Tables 2, 3). These correlations are contrary to the suggested link between high phytoplankton biomass and high UCYN-A abundance (Moisander et al. 2010; Tripp et al., 2010). Otherwise, UCYN-A may depend on other sources of dissolved organic matter (DOM), which is the case of heterotrophic bacteria. It is well established that in pelagic waters dissolved carbohydrate inputs from sources other than the phytoplankton compartment can support bacterial growth (Cherrier and Bauer, 2004; Young et al., 2005). This hypothesis can explain the observation of the lack of a positive correlation with the phytoplankton production and associated chlorophyll fluorescence and explain why they can predominantly exist as free-living cells. Here we also found them in lesser proportion to be associated with larger cells and particulate material, as previously reported based on size fractionated TSA-FISH techniques (Le Moal and Biegala 2009). Alternatively, the UCYN-A cells and other larger phytoplankton cells can be competing for limiting nutrients (e.g. phosphorus and iron), and the UCYN-A cells -smaller in size and thus more efficient in nutrient uptake- render the growth and production of larger phytoplankton cells limited by these factors. Clearly, more studies on the nutrition kinetics and lifestyle of UCYN-A cells are needed.

Dissolved  $O_2$  levels showed up here to be negatively correlated with  $N_2$  fixation rates and abundance of UCYN-A cells (Table 3). This observation is

consistent with the genetic characteristic implied by UCYN-A genome sequence of the presence of  $O_2$ -sensitive nitrogenases (Tripp et al. 2010). Other studies have not detected a significant correlation between UCYN-A cells and environmental dissolved  $O_2$  levels (Moisander et al., 2010), and to our knowledge, this is the first study reporting this correlation. The role of these diazotrophs in supplying fixed  $N_2$  in regions characterized by presence of permanent  $O_2$  minimum zones (OMZ) should therefore be investigated.

In summary, we have provided evidence that in the subtropical Northeast Atlantic ocean region studied (>30 °N), <3  $\mu$ m cells diazotrophs, with most of the sequences belonging to UCYN-A, dominate the N<sub>2</sub> fixing community. Their wider vertical and horizontal distribution than the larger cells indicates that are able to grow in different environmental conditions. Based on the range of data here, their activities and abundance seemed to be regulated by temperature and O<sub>2</sub> levels, but not as strongly regulated by fixed nitrogen as demonstrated by their dominance in NO<sub>3</sub>-rich upwelling regions. Further studies on the factors regulating their abundance and activity and how these in turn relates with their lifestyles (and vice-versa) are needed to understand better the functioning of UCYN-A cells which is emerging to be a cosmopolitan diazotroph species in the world's oceans.

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"¿Para qué escribe uno, si no es para juntar sus pedazos? Desde que entramos en la escuela o la iglesia la educación nos descuartiza: nos enseña a divorciar el alma del cuerpo y la razón del corazón. Sabios doctores de Ética y Moral han de ser los pescadores de la costa colombiana, que inventaron la palabra sentipensante para definir al lenguaje que dice la verdad".

Celebración de las bodas de la razón y el corazón Eduardo Galeano

# Chapter 6

Contribution of N<sub>2</sub> fixation to new production and excess nitrogen in the subtropical northeast Atlantic margin

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# Abstract

We used <sup>15</sup>N-labeled substrates to measure dinitrogen (N<sub>2</sub>) fixation, nitrate  $(NO_3)$  and ammonium  $(NH_4)$  uptake, regeneration and associated dissolved organic nitrogen (DON) release in a coastal upwelling system (Cape Ghir,  $\sim 31^{\circ}$ N) and an open ocean grid (bounded between  $25^{\circ}$ –  $42^{\circ}$ N and  $20^{\circ}$ W) along the Canary Current region, during the summer of 2009. New production (Pnew =  $NO_3^{-1}$  uptake +  $N_2$  fixation + DON released from  $NO_3^{-1}$  uptake -  $NO_3^{-1}$ regeneration) was higher in the upwelling than in the open ocean zone (0.126 and 0.014  $\mu$ mol N L<sup>-1</sup> h<sup>-1</sup>, respectively), while regenerated production (Preg =  $NH_{4^+}$  uptake + DON released from  $NH_{4^+}$  uptake +  $NH_{4^+}$  regeneration) was similar in both zones (0.157 and 0.133 µmol N L<sup>-1</sup> h<sup>-1</sup>, respectively). The resulting f-ratio (Pnew/Pnew+Preg) for the open ocean and upwelling zones was 0.08 and 0.48, respectively. The availability of nitrogen in excess of that expected from Redfield stoichiometry is generally attributed to N<sub>2</sub> fixation. A previous study indicated that our open ocean grid zone had an excess nitrogen production rate of  $40 \pm 22 \times 10^{10}$  mol N y<sup>-1</sup>. We revisited this budget including new dissolved organic matter and NO<sub>3</sub>- fluxes through the Strait of Gibraltar and estimated a new nitrogen excess rate of  $22 \pm 19 \times 10^{10}$  mol N y<sup>-1</sup>. The average volumetric rate of N<sub>2</sub> fixation for this zone was only to  $1.3 \times 10^{-3}$  nmol N L<sup>-1</sup> d<sup>-1</sup>, indicating that its influence in Pnew and nitrogen excess production in this part of the Atlantic is negligible.

# Introduction

One of the greatest current challenges for oceanographers is to balance the oceanic nitrogen budget. At present, it is estimated that nitrogen losses exceed global nitrogen fixation in the upper water column by  $\sim 14 \times 10^{12}$  mol N y<sup>-1</sup> (Codispoti, 2007). Massive combined nitrogen losses through denitrification and anammox in sediments and oxygen minimum zones of the eastern tropical Pacific Ocean push the global budget towards net loss (Deutsch et al., 2001), although the opposite may be found in other basins. In the North Atlantic Ocean, where pelagic denitrification is thought to be negligible (Hansell et al., 2007), excess fixed nitrogen -calculated under the assumption of a Redfieldian nitrogen to phosphorus (N:P) molar ratio of 16has been repeatedly observed with varying rates: 0.15 – 0.46 × 10<sup>12</sup> mol N y<sup>-1</sup>, 0.8 × 10<sup>12</sup> mol N y<sup>-1</sup>, 2 × 10<sup>12</sup> mol N y<sup>-1</sup>, and 2 – 9 × 10<sup>12</sup> mol N y<sup>-1</sup> by Hansell et al. (2004; 2007), Gruber and Sarmiento (1997), and Capone et al. (2005), respectively. Dinitrogen (N<sub>2</sub>) fixation is a biologically mediated process that reduces N<sub>2</sub> to ammonium via the nitrogenase enzyme complex, carried by several species of cyanobacteria, bacteria and some Archaea (Riemann et al., 2010). N<sub>2</sub> fixation uses phosphate (PO<sub>4</sub><sup>3-</sup>), but not combined inorganic nitrogen, resulting in a non–Redfieldian process that consumes inorganic nutrient salts with a N:P <16, leaving a signal of excess nitrogen mineralization (a 'nitrogen anomaly') in the 70–700 m layer of the North Atlantic Ocean (Mahaffey et al., 2005).

The excess nitrogen production rates are generally attributed to N<sub>2</sub> fixation, and are usually calculated using geochemical approaches. These involve the use of nitrate (NO<sub>3</sub><sup>-</sup>) and PO<sub>4</sub><sup>3-</sup> concentrations to calculate the excess nitrogen concentration over phosphorus as compared with the expected Redfield ratio of N:P = 16:1 (the N\* parameter; Gruber and Sarmiento, 1997; Hansell et al. 2004; 2007). This parameter measures net N<sub>2</sub> fixation, i.e. the resulting excess or deficit of NO<sub>3</sub><sup>-</sup> resulting from the difference between denitrification and N<sub>2</sub> fixation.

Excess nitrogen production can also be estimated using biological approaches, which include the direct measurement of biological N<sub>2</sub> fixation rates using the acetylene reduction assay (ARA; Stal, 1988) or the stable isotope <sup>15</sup>N (Montoya et al., 1996; Mohr et al., 2010). Geochemically estimated rates usually exceed biologically estimated rates by an order of magnitude (Mahaffey et al., 2005). This discrepancy among methods impedes global oceanic nitrogen balancing based on the available data. There are a number of reasons why such differences exist between methods. While geochemical methods attempt to measure N<sub>2</sub> fixation rates in large oceanic areas (e.g. basin scale) and large periods of time (e.g. years), biological methods are based on bioassays performed on small volumes (usually from a few mL to 5 L), and short periods of time (up to 24 h). There is also a lag of time and space between the biological process and its geochemical signature. The high N:P organic matter produced by N<sub>2</sub> fixation in the epipelagic layer sinks to the mesopelagic layer where it is remineralized. Nitrogen anomalies are maximum in the thermocline and travel away from the area where N<sub>2</sub> fixation occurs along isopycnal surfaces, making gradients in nitrogen excess the only way to localize the production areas (Mahaffey et al., 2005). Moreover, Redfield stoichiometry is generally assumed in these geochemical calculations (C:N:P = 106:16:1), while in practice particulate organic matter (POM), and especially dissolved organic matter (DOM), can often have C:N:P ratios well over Redfield (e.g. Vidal et al., 1999; Doval et al., 2001; Hopkinson and Vallino, 2005). Although new methodological improvements may bring geochemical and biological rates closer in the future (Mohr et al., 2010; Großkopf et al., 2012), the nitrogen anomaly found in the North Atlantic Ocean remains unexplained to date.

In the eastern subtropical North Atlantic, Álvarez and Álvarez-Salgado (2007) calculated an excess of  $40 \pm 22 \times 10^{10}$  mol N y<sup>-1</sup> for the region bounded by  $25^{\circ}$ – $42^{\circ}$ N and  $20^{\circ}$ W. These authors tentatively attributed this excess to N<sub>2</sub> fixation, but the lack of in situ measurements in the area could not confirm it by that time. The aim of this paper is to verify if the quantified excess nitrogen could be supported by N<sub>2</sub> fixation and to assess the role of N<sub>2</sub> fixation in nitrogen cycling in this area during the summer, when N<sub>2</sub> fixation rates are presumably maxima due to the higher temperatures. With this purpose, we measured NO<sub>3</sub><sup>-</sup> and ammonium (NH<sub>4</sub><sup>+</sup>) uptake and regeneration, and the associated dissolved organic nitrogen (DON) release in the upper water column of this study area, and compared it to the N<sub>2</sub> fixation measurements reported in Benavides et al. (2011) for the same cruise.

# **Materials and methods**

### Sampling and hydrographic measurements

Sampling was performed from 25 July to 5 September 2009 during the 'Shelf-Ocean Exchanges in the Canaries-Iberian Large Marine Ecosystem' (CAIBEX) cruises, onboard R/V Sarmiento de Gamboa. A total of 73 stations were sampled in an open-ocean grid which covered the area between the northwest (NW) Iberian Peninsula and the Canary Islands, drawing a box enclosed by the 20<sup>o</sup>N meridian (the 'CAIBOX' grid; Fig. 1a). In 17 of the stations (white dots in Figure 1a), samples for inorganic nutrients, dissolved organic carbon (DOC), and total dissolved nitrogen (TDN) analyses were taken at 15 levels, from surface to bottom. In these 17 stations, samples for the determination of N<sub>2</sub> fixation rates were only collected at the surface (5 m). At even stations of the open-ocean grid (Fig. 1a, stations X2, X4, X6, X8, X10, X12 and X16) samples for the determination of nitrogen uptake and regeneration and DON release rates were taken a two selected depths: surface (5 m) and the deep chlorophyll maximum (DCM). In addition, inorganic nutrients, DOC, TDN and nitrogen metabolic rates were also measured in another 8 stations in the upwelling region off Cape Ghir, in the NW coast of Africa (Fig. 1b). Hereafter, these two areas will be operationally referred to as 'non upwelling' and 'upwelling'-affected, respectively.

At each station, temperature, salinity and photosynthetically active radiance (PAR) data were recorded by means of a SBE 911 plus CTD, equipped with a Sea-Tech fluorometer and Li-Cor PAR sensor. Temperature and salinity were also obtained by means of a Seasoar towed undulating system (Chelsea

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Technologies Group), fitted with a SBE 911 CTD. The Seasoar was towed over the study area performing six transects parallel to the coast, and undulating between 10 and 400 m depth. The near-surface ( $\sim$ 5 m) temperature and salinity data were obtained from the flow-through system of the ship by means of a SBE 21 Seacat Thermosalinograph.

## Dissolved inorganic and organic nitrogen and carbon analyses

Seawater was collected using 12 L Niskin bottles mounted on a General Oceanics rosette sampler. In the non upwelling-affected stations, samples for inorganic nutrient analyses were drawn into 50 mL polyethylene containers (VWR) and immediately analyzed on-board using a Prescop Alpkem autoanalyzer with detection limits of 0.05  $\mu$ M, for NO<sub>3</sub><sup>-</sup> and Si(OH)<sub>4</sub>, and 0.02  $\mu$ M for NO<sub>2</sub><sup>-</sup> and HPO<sub>4</sub><sup>2-</sup>. In the upwelling-affected stations, samples were recovered in 15 mL polyethylene tubes (VWR) and stored frozen at  $-20^{\circ}$ C. These samples were analyzed in the land-based laboratory using an AA3 Bran+Luebbe autoanalyzer with detection limits of 0.01 and 0.03  $\mu$ M for NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>, respectively. Samples for the determination of the NH<sub>4</sub><sup>+</sup> concentration in the surface (5 m) and DCM depth were directly sampled from the rosette into 100 mL borosilicate bottles (Duran Schott) and measured on board with a Perkin-Elmer LS55 spectrofluorometer following the method by Holmes et al. (1999). The detection limit for this method is 5 nM.

Samples for DOC and TDN analyses in the upper 150 m were filtered through Whatman GF/F filters in an acid-cleaned all-glass filtration system under positive pressure of high purity N<sub>2</sub>. The filtrate was collected in 10 mL precombusted (450°C, 6 h.) glass ampoules. Samples from below 150 m were directly collected in the glass ampoules. After acidification to pH< 2 with phosphoric acid, the ampoules were flame-sealed and stored in the dark at 4°C until analysis. They were measured with a nitrogen-specific Antek 7020 nitric oxide chemiluminescence detector coupled in series with the carbon-specific infrared gas analyzer of a Shimadzu TOC–5000 organic carbon analyzer (Álvarez–Salgado and Miller, 1998). The measurement error was ± 0.7  $\mu$ M for carbon and ± 0.3  $\mu$ M for nitrogen. Their respective accuracies were tested with the reference materials provided by Prof. D.A. Hansell (Univ. of Miami), which were run once per day just after calibration with a mixed standard of potassium hydrogen phthalate and glycine. DON was obtained by subtracting NO<sub>3</sub>-, NO<sub>2</sub>- and NH<sub>4</sub>+ (when available) from TDN.



Fig. 1: Maps of stations sampled. (a) The non upwelling-affected area, sampled from 25 July to 14 August 2009. The dashed line indicates the approximate position of the Azores Front (AF) during the cruise. White dots indicate stations where dissolved inorganic and organic nitrogen concentrations were measured. (b) The upwelling-affected area, sampled from 16 August to 5 September 2009.

# Nitrogen uptake, nitrogen regeneration and DON release

 $NO_{3}$  and  $NH_{4}$  uptake, regeneration and associated release as DON was measured at the surface (5 m) and DCM depth of stations X2, X4, X6, X8, X10, X12, X14 and X16 in the non upwelling-affected area (Fig. 1a), and at all stations of the upwelling-affected area (Fig. 1b). For <sup>15</sup>N experiments, water was directly transferred from the Niskin bottles to 2 L transparent polycarbonate bottles (Nalgene) in duplicate. <sup>15</sup>N-labeled substrate was added
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to the bottles as 1 mL of <sup>15</sup>KNO<sub>3</sub> (200  $\mu$ M; 99 at.%), or 1 mL of <sup>15</sup>NH<sub>4</sub>Cl (20  $\mu$ M; 99 at.%) (Sigma-Aldrich). For the non upwelling-affected stations, isotope enrichments were on average 22 and 27% for NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>, respectively. In the case of the upwelling-affected stations, average enrichments were 5 and 56% for NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>, respectively.

Replicate bottles were immediately filtered after inoculation of the labeled substrates to obtain the initial <sup>15</sup>N enrichment of samples. The other set of replicate bottles were equally inoculated and placed in on-deck incident light-calibrated containers cooled with surface seawater for 3–4 h. A hand light-meter (Biospherical Instruments) and neutral density filters (Lee filters) were used to mimic the light levels measured on PAR profiles at each station. After the incubation period, the samples were gently filtered through precombusted GF/F filters (vacuum pressure <100 mm Hg), wrapped in precombusted aluminium foil and stored at  $-20^{\circ}$ C. The uptake of NO<sub>3</sub><sup>-</sup> was calculated according to the equations in Dugdale and Wilkerson (1986). NH<sub>4</sub><sup>+</sup> uptake rates were corrected for isotopic dilution as indicated by Glibert et al. (1982). Wherever under detection limit NH<sub>4</sub><sup>+</sup> concentrations were observed, values were substituted by the detection limit of the method (i.e. 5 nM), resulting in 50% enrichments, which can be considered as the threshold between real and potential uptake rates (Fernández et al., 2009).

The filtrates were kept to analyze final NO<sub>3</sub>-, NO<sub>2</sub>-, NH<sub>4</sub>+ and DON concentrations (see above), and the <sup>15</sup>N-enrichment of the final NO<sub>3</sub>-, NH<sub>4</sub>+ and DON pools using the protocol proposed by Slawyk and Raimbault (1995). For the latter, 80 mL of filtrate were stored in 100 mL borosilicate flasks (Duran Schott) and poisoned with 1% HgCl<sub>2</sub> (Sigma-Aldrich) to interrupt any microbial activity. The extraction protocol consists of three steps: (1)  $NO_3^-$  and NO<sub>2</sub>- are reduced and stripped off together with initial NH<sub>4</sub>+ as ammonium sulfate, (2) oxidation of the remaining dissolved nitrogen (i.e. the DON pool) to  $NO_{3}$ - by persulfate oxidation (Valderrama, 1981), and (3) repetition of step (1) to strip off DON as ammonium sulfate. From the samples incubated with <sup>15</sup>NO<sub>3</sub><sup>-</sup>, rates of NO<sub>3</sub><sup>-</sup> uptake and DON release from NO<sub>3</sub><sup>-</sup> are obtained. From the samples incubated with <sup>15</sup>NH<sub>4</sub><sup>+</sup>, rates of NH<sub>4</sub><sup>+</sup> uptake, NO<sub>3</sub><sup>-</sup> regeneration, NH<sub>4</sub><sup>+</sup> regeneration and DON release from NH<sub>4</sub><sup>+</sup> are obtained. Nitrogen regeneration rates (NO<sub>3<sup>-</sup></sub> and NH<sub>4<sup>+</sup></sub>) were calculated by applying the equations in Fernández and Raimbault (2007). DON release rates from  $NO_3^-$  and  $NH_4^+$  uptaken by microorganisms were calculated with the equations in Slawyk et al. (1998).

 $N_2$  fixation was assayed only in the surface (~5 m) depth as outlined in Benavides et al. (2011), at stations X1 to X17 in the non upwelling-affected area, and at all stations in the upwelling-affected area. Briefly, seawater was transferred to 1 L transparent polycarbonate bottles (Nalgene). The bottles were filled to overflow using silicone tubing and preventing the introduction of air bubbles. Then they were sealed with septum screw caps before 2 mL of  $^{15}N_2$ -gas (99 at.%; Tracer Tec) were injected through the septum using a gastight syringe (Hamilton). The pressure across the septum was equilibrated by allowing the excess air to escape through a sterile syringe tip piercing the septum at the same time as the  $^{15}N_2$ -gas was being injected. Finally, the bottles were placed in the on-deck incubator for 24 h. After the incubation, samples were filtered through precombusted GF/Fs (vacuum pressure <100 mm Hg), wrapped in precombusted aluminium foil and stored at  $-20^{\circ}$ C until analysis.  $N_2$ fixation rates were calculated after Montoya et al. (1996).

The concentration of particulate organic nitrogen (PON) and the isotopic ratio  $({}^{15}N/{}^{14}N)$  of samples was obtained by means of a Thermo Flash 1112 elemental analyzer interfaced by a Conflo III with a Thermo Delta V Advantage isotope ratio mass spectrometer (IRMS).

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#### Hydrography

Sections of temperature and salinity in the upper 200 m of the open ocean transect (non upwelling-affected area, Fig. 1a) are shown in Fig. 2. Temperature increased southwards along the transect (Fig. 2a). A sharp thermocline was observed in the northern part of the transect (stations X2 to X6, Fig. 1a), where temperatures of 19–20°C were measured in the upper 40 m, then decreasing to 15–17°C at 50–60 m depth. The Azores Front was located slightly north of station X8 and can be clearly identified by the tilting in the isotherms. South of the Azores Front, the entire water column warmed up and temperatures >  $19^{\circ}$ C were observed down to ~80 m depth. This pattern was only disrupted after station X16, when reaching the coastal transition zone (CTZ) between the NW African upwelling ecosystem and the Canary Islands (Fig. 1b). Salinity mimics the structure of temperature in the upper 200 m of the open ocean transect (Fig. 2b). In the northern part of this transect, salinity decreased sharply from 38 to  $\sim$ 36.5 in the upper  $\sim$ 60 m depth. The Azores Front was also easily depicted by the tilting isohalines observed near to station X8. South of the Azores Front, the isohalines deepened (38 at  $\sim$ 100 m depth) and a stratified area was observed from stations X8 to X16. Stratification disruption associated with the CTZ was also observed in the isohalines (Fig. 2b).



Fig.2: (a) Temperature and (b) salinity sections from the non upwelling-affected area. The position of the stations sampled is displayed over the upper x-axis of (a).

Surface and 50 m temperature and salinity distributions from the upwellingaffected area are shown in Fig. 3. We chose to represent temperature and salinity at 50 m because it is representative of the DCM at most stations sampled in this area (Table 1). From the distribution of temperature and salinity at 5 m we see that an upwelling filament extended up to  $\sim 11.5^{\circ}$ N, which is >150 km offshore. A tongue of cold upwelled water (16-18°C) and associated low salinity ( $\sim$ 36.2) extended over the shelf from the northern part of the NW African coast and the sampled stations. The coldest SST values (<17°C) were detected at ~31°N (Fig. 3a). An area of warmer (20-21°C) and saltier water (36.5-36.6) was observed leeward of the upwelling-affected stations, over the bay of Agadir (Morocco).



Figure 3: Temperature and salinity at the surface ( $\sim 5$  m) and 50 m from the upwelling-affected area. Black dots represent stations where <sup>15</sup>N experiments were preformed. Surface temperature (a), and salinity (b) were obtained from thermosalinograph data (white line). Temperature and salinity at 50 m (figures c and d, respectively) were obtained by the combination of CTD casts (white circles) and Seasoar measurements (white squares).

At 50 m depth, the filament decreased in width. The coldest temperatures (15°C) were observed near the coast leeward of the upwelling-affected stations. Stations G12, G17 and G22 were clearly situated over the path of the filament, were temperatures at 50 m depth ranged from 17 to 17.5°C. Station GT2 was situated at the edge of the filament, and stations G40, G44 and G48 at its tipend, where higher temperatures (17.5 to 18°C) were measured.

### NO<sub>3</sub>-, NH<sub>4</sub>+, DON and DOC distributions

In situ surface  $NO_3^-$  concentrations were generally <1  $\mu$ M at all the stations sampled in both study areas with few exceptions (Table 1). These surface concentrations were fairly stable along the non upwelling-affected stations (at ~0.1  $\mu$ M), being somewhat more variable at the DCM. Peaks of  $NO_3^-$  were measured in the upwelling-affected area associated with the coastal upwelling (station GM2), and stations over the upwelling filament (G17 and G44). In the non upwelling-affected area, DCM  $NO_3^-$  concentrations equaled those of the surface at most stations, while in the upwelling-affected area

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increases of up to 1  $\mu$ M were observed with respect to surface values. NH<sub>4</sub><sup>+</sup> concentrations were under the detection limit at several stations (indicated as n/d in Table 1). Where detectable, NH<sub>4</sub><sup>+</sup> concentrations were <50 nM in most cases, being somewhat higher north of the Azores Front (stations X2 to X8). NH<sub>4</sub><sup>+</sup> concentrations at the DCM were generally higher than at the surface.

DON concentrations were very stable along the non upwelling-affected stations' transect, generally ranging from 5.0 to 6.5 µM, except at station X4, where anomalously high concentrations of  $7-9 \mu M$  were recorded. Generally, DON concentrations were higher at the surface than at the DCM, although stations X14 and X16 were an exception. DOC concentrations ranged from  $\sim$ 50 to 80 µM. The influence of the Azores Front was again noted in surface DOC concentrations. The largest values were found in the area affected by the front (stations X8 - X10) and then decreased towards the NW African coast. This effect was not observed at the DCM. DOC concentrations at the DCM remained stable along the non upwelling-affected area with the exception of station X14. In the upwelling-affected area, DON concentrations were much more variable. Surface concentrations averaged  $\sim 7 \mu$ M, but a peak > 9  $\mu$ M was observed at the most offshore station (G48). At the DCM, the concentrations were also variable and a peak  $>10 \mu$ M was observed at station G40. DOC concentrations were lower in the upwelling-affected area than in the non upwelling-affected area, ranging from ~50 to 60 µM. Surface DOC concentrations tended to increase from the coast to the open ocean, while DCM values remained  $\sim$  54  $\mu$ M, with the exception of two peaks of  $\sim$ 63  $\mu$ M at stations GM2 and G17.

#### Nitrogen uptake, regeneration and release

Surface and DCM  $NO_3^-$  uptake,  $NH_4^+$  uptake,  $NO_3^-$  regeneration,  $NH_4^+$  regeneration, DON release from uptaken  $NO_3^-$  and  $NH_4^+$ ,  $N_2$  fixation, and the associated new and regenerated production rates (Pnew and Preg, respectively) were averaged for the non upwelling-affected and upwelling-affected areas (Table 2). Note that  $N_2$  fixation rates are only available for the surface.  $NO_3^-$  uptake was two orders of magnitude lower than  $NH_4^+$  uptake in the non upwelling-affected area, while these were similar in the upwelling-affected area (Table 2). The regeneration of  $NO_3^-$  was low in the non upwelling-affected area and not detectable in the upwelling-affected area.  $NH_4^+$  regeneration was one order of magnitude lower than  $NH_4^+$  uptake in the non upwelling-affected area, and two orders of magnitude lower in the upwelling-affected area. DON release from  $NO_3^-$  was similar in both areas, while DON release from  $NH_4^+$  was one order of magnitude greater in the upwelling-affected area than in the non upwelling-affected area.  $N_2$  fixation was very low in both areas, being somewhat higher in the upwelling-affected area.

Pnew was corrected by adding N<sub>2</sub> fixation -as another source of new nitrogen-, and by subtracting NO<sub>3</sub><sup>-</sup> regeneration -a source of regenerated nitrogen- (Raimbault and Garcia, 2008). Preg was corrected by adding NH<sub>4</sub>+ regeneration to NH<sub>4</sub><sup>+</sup> uptake. DON released from NO<sub>3</sub><sup>-</sup> or NH<sub>4</sub><sup>+</sup> uptake was added to Pnew and Preg, respectively (considering the 'loss' of inorganic nitrogen to the DON pool; Raimbault and Garcia, 2008). Thus, the f-ratio was calculated as follows: f-ratio =  $(NO_3^- \text{ uptake} + \text{DON release from } NO_3^- + N_2)$ fixation –  $NO_3^-$  regeneration) / ( $NO_3^-$  uptake +  $N_2$  fixation –  $NO_3^-$  regeneration +  $NH_{4^+}$  uptake + DON release from  $NH_{4^+}$  +  $NH_{4^+}$  regeneration). The  $NO_{3^-}$ regeneration rates measured were low or undetectable in the two sampled areas, so its effect in reducing Pnew was minimal. Similarly, N<sub>2</sub> fixation rates were several orders of magnitude lower than NO<sub>3</sub><sup>-</sup> uptake, therefore N<sub>2</sub> fixation usually contributed <1% to Pnew (Table 2). Pnew was much greater than Preg in the upwelling affected area, while they were similar in the non upwellingaffected area. Accordingly, the f-ratio was very low in the non upwellingaffected oligotrophic waters (0.08), and higher (0.48) in the upwelling area (Table 2).

If we consider gross  $NO_{3^-}$  and gross  $NH_{4^+}$  uptake as  $NO_{3^-}$  uptake plus DON release from  $NO_{3^-}$ , and  $NH_{4^+}$  uptake released from  $NH_{4^+}$  (Bronk et al., 1994), we find that DON release from  $NO_{3^-}$  is more important in the non upwelling-affected area, while DON release from  $NH_{4^+}$  is more important in the upwelling-affected area (Table 3). Nevertheless, the variability was very high (note high standard deviation values in Table 3).

### Discussion

# Uptake and regeneration of $NO_{3^{-}}$ and $NH_{4^{+}}$ and associated DON release in open ocean and upwelling zones

It is generally assumed that primary production in upwelling systems is mainly supported by  $NO_{3}$ - ('new nitrogen'), which is regenerated at the base of the euphotic zone and reaches the photic layer through upward diffusive fluxes. Instead, productivity in open ocean oligotrophic ecosystems is thought to rely on  $NH_{4}$ + ('regenerated nitrogen') due to the year-round persistence of a strong thermocline that prevents nutrient-rich deep waters to reach the photic layer (Dugdale and Goering, 1967). The f-ratio quantifies the contribution of Pnew to total production (Pnew + Preg) (Eppley and Peterson, 1979).

Area	Station	Latitude (ºN)	Longitude (ºN)	MLD (m)	DCM (m)	Surface NO₃⁻ (µM)	DCM NO <sub>3</sub> - (μM)	Surface NH₄⁺ (μM)	DCM NH₄⁺ (µM)	Surface DON (µM)	DCM DON (µM)	Surface DOC (µM)	DCM DOC (µM)
	X2	41.50	10.58	40	60	0.13	0.12	-	0.117	6.5	5.8	68.8	63.0
ted	X4	41.50	15.29	30	67	0.09	0.09	0.064	0.070	8.6	6.9	73.6	65.4
affec	X6	41.44	19.26	38	55	0.15	0.05	0.157	0.179	6.1	5.3	71.4	63.4
ing-s	X8	37.98	20.01	25	80	0.14	0.14	0.013	0.087	-	_	78.7	63.0
well	X10	34.99	19.97	40	80	0.08	0.08	n/d	0.028	6.6	5.6	79.1	67.3
dn u	X12	31.50	20.00	45	105	0.13	0.11	0.020	0.015	6.2	6.1	70.2	61.2
No	X14	29.85	19.19	45	90	0.17	0.18	n/d	0.019	5.6	6.2	64.9	74.5
	X16	29.25	15.93	50	110	0.05	0.05	n/d	0.012	5.0	5.8	68.4	61.4
	GM2	31.00	10.01	15	25	3.00	2.77	n/d	n/d	-	-	62.56	59.34
ъ	G12	30.74	10.65	15	30	0.61	1.95	n/d	0.009	6.88	3.73	_	_
ecte	G17	30.90	10.80	20	40	1.35	2.20	0.002	0.072	6.91	6.53	54.14	62.75
g-aff	G22	31.00	10.91	25	40	0.38	1.56	0.015	0.305	6.74	-	49.18	53.08
elling	GT2	31.11	10.60	25	45	0.82	0.93	n/d	n/d	5.47	-	56.99	62.68
Jpwe	G40	31.01	11.31	30	48	0.55	1.65	n/d	n/d	7.22	10.44	55.08	53.91
	G44	30.95	11.40	28	43	1.50	0.38	n/d	0.021	6.18	7.44	59.32	53.51
	G48	30.89	11.45	27	50	0.76	1.46	0.007	n/d	9.46	3.45	60.05	53.95

Table 1: Position of the mixed layer depth (MLD) and the deep chlorophyll maximum (DCM) at the stations sampled in the non upwelling-affected and upwelling-affected areas. In situ concentrations (previous to incubations with added  $^{15}NH_{4^+}$  or  $^{15}NO_3^-$ ) of nitrate (NO<sub>3</sub><sup>-</sup>), ammonium (NH<sub>4^+</sub>), dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) (all in  $\mu$ M) at the surface and at the DCM of all the stations sampled. Under detection limit NH<sub>4<sup>+</sup></sub> concentrations are depicted as n/d (not detectable).

Table 2: Average and standard deviation ( $\pm$  SD) values of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> uptake and regeneration, and dissolved organic nitrogen (DON) release derived from NO<sub>3</sub><sup>-</sup> uptake (DON-NO<sub>3</sub><sup>-</sup>) and from NH<sub>4</sub><sup>+</sup> uptake (DON-NH<sub>4</sub><sup>+</sup>), and N<sub>2</sub> fixation, all in µmol L<sup>-1</sup> h<sup>-1</sup>. All values are average measurements made at the surface (5 m) and at the deep chlorophyll maximum (DCM), except for N<sub>2</sub> fixation, which was only measured at the surface. NO<sub>3</sub><sup>-</sup> regeneration was not detectable (n/d) in the upwelling-affected stations.

	NO3 <sup>-</sup> uptake	NH₄⁺ uptake	NO3 <sup>-</sup> regeneration	NH4 <sup>+</sup> regeneration	DON-NO3 <sup>-</sup> release	DON-NH4 <sup>+</sup> release	N <sub>2</sub> fixation	Pnew	Preg	f-ratio
Non upwelling- affected	$0.008 \pm 0.007$	0.143 ±0.187	$0.002 \pm 0.002$	0.012 ± 0.013	$0.008 \pm 0.486$	$0.001 \pm 0.002$	1.3 ± 0.7 x10 <sup>-6</sup>	0.014	0.157	0.084
Upwelling affected	0.122 ± 0.086	0.105 ± 0.078	n/d	0.008 ± 0.023	0.004 ± 0.006	0.020 ± 0.033	5 ± 5 x10-6	0.126	0.133	0.487

Area	DON release from NO3 <sup>-</sup> contribution to gross NO3 <sup>-</sup> uptake (%)	DON release from NH4* contribution to gross NH4* uptake (%)
Non upwelling-affected	26.48 ± 30.41	2.21 ± 3.18
Upwelling-affected	2.59 ± 2.67	14.16 ± 13

Table 3: Percentage DON release contribution to gross  $NO_{3^-}$  and  $NH_{4^+}$  uptake in non upwelling-affected and upwelling-affected areas.

Upwelling systems are expected to have high f-ratios, while oligotrophic systems usually present low f-ratios. Nevertheless, the recent inclusion of  $N_2$  fixation,  $NH_4^+$  and  $NO_3^-$  regeneration and DON release rates in the computation of f-ratios has shown that Pnew is generally overestimated when nitrification is not taken into account, while Preg is generally underestimated when  $NH_4^+$  regeneration is not taken into account (e.g. Fernández and Raimbault, 2007), while gross  $NO_3^-$  and  $NH_4^+$  uptake rates are underestimated if the associated DON released is not accounted for (e.g. Bronk et al., 1994). In this study, all the above-mentioned fluxes have been included in the calculation of Pnew, Preg and f-ratios, enhancing the robustness of our results.

The NO<sub>3</sub><sup>-</sup> uptake rates in the upwelling-affected zone were in the range of those observed in the upwelling systems off California (0.1 - 0.55 µmol N L<sup>-1</sup> h<sup>-1</sup>; Dugdale et al., 2006), and off Peru (0.11 - 0.14 µmol N L<sup>-1</sup> h<sup>-1</sup>; Blasco et al., 1984), although more recently, rates one to two orders of magnitude lower (0.003 - 0.02 µmol N L<sup>-1</sup> h<sup>-1</sup>) have been reported for the Peruvian and Chilean upwelling systems (Raimbault and Garcia, 2008; Fernández et al., 2009). While these authors observed considerably high NO<sub>3</sub><sup>-</sup> regeneration (nitrification) rates in the surface waters of the South Pacific Ocean upwelling systems (0.125 × 10<sup>-3</sup> - 0.001 µmol N L<sup>-1</sup> h<sup>-1</sup>), this flux was not detectable during our study in the coastal upwelling off Cape Ghir. In the non-upwelling affected zone (CAIBOX), NO<sub>3</sub><sup>-</sup> uptake and regeneration rates were similar to that observed in a study in the same longitudinal range, but slightly to the north (between ~38 and 45°N) by Fernández and Raimbault (2007), but one to two orders of magnitude higher than previously observed off the coast of Portugal in an area included in our CAIBOX (Slawyk et al., 1997).

Generally, we observed that our NH<sub>4</sub><sup>+</sup> uptake rates were considerably higher than reported in other studies. For example, Fernández et al. (2009) measured NH<sub>4</sub><sup>+</sup> uptake rates ranging from 0.59 × 10<sup>-3</sup> to 0.006 µmol N L<sup>-1</sup> h<sup>-1</sup> off the Peruvian upwelling system, and Raimbault and Garcia (2008) measured a maximum of maximum 0.02 µmol N L<sup>-1</sup> h<sup>-1</sup> off the Chilean upwelling system. In an open ocean area of the North Atlantic, Fernández and Raimbault (2007) measured NH<sub>4</sub><sup>+</sup> uptake rates up to ~0.01 µmol N L<sup>-1</sup> h<sup>-1</sup>. All these rates are two to three orders of magnitude lower than our NH<sub>4</sub><sup>+</sup> uptake rates measured in the upwelling-affected and non upwelling-affected areas (Table 2). However, it should be noted that the standard deviation values of our NH<sub>4</sub><sup>+</sup> uptake rates were considerably high (Table 2), and that non-detectable NH<sub>4</sub><sup>+</sup> concentrations where substituted by 5 nM (the detection limit of the method used; Holmes et al., 1999), and therefore those rates can be considered as 'potential' NH<sub>4</sub><sup>+</sup> uptake rates (Fernández et al., 2009), potentially overestimating the Preg rates obtained here.

The NH<sub>4</sub><sup>+</sup> regeneration rates measured in this study fall within the range observed in other upwelling and open ocean areas. For example, Fernández et al. (2009) measured NH<sub>4</sub><sup>+</sup> regeneration rates ranging from 0.006 to 0.02  $\mu$ mol N L<sup>-1</sup> h<sup>-1</sup> off the coast of Peru, and our rates are also in the range of those observed in an upwelling-affected area off the Galician coast (maximum 0.03  $\mu$ mol N L<sup>-1</sup> h<sup>-1</sup>; Varela et al., 2003). Similarly, the rates of NH<sub>4</sub><sup>+</sup> regeneration observed in the non upwelling-affected area are in the range of those observed in the North Atlantic by Fernández and Raimbault (2007) (up to ~0.02  $\mu$ mol N L<sup>-1</sup> h<sup>-1</sup>).

In our study, Preg rates were similar in the upwelling-affected and non upwelling-affected zones, corroborating the importance of nitrogen regeneration in sustaining primary production (e.g. Fernández and Raimbault, 2007; Clark et al., 2011; Fernández and Farías, 2012). As previously mentioned, open ocean oligotrophic zones are expected to have higher Preg rates than coastal upwelling zones, a pattern typically related to nutrient availability and the related planktonic community structure: picoplankton dominates in oligotrophic areas (e.g. Zubkov et al., 2000), while autotrophic microplankton such as diatoms usually dominate in active upwelling systems (e.g. Hutchings et al., 1995). In contrast, pico- and nanoplanktonic cells predominate the NW Iberian and NW African upwelling systems, whereas diatoms only thrive during upwelling pulses (Espinoza-González et al., 2012; Anabalón et al., in prep.). In a study performed in the same time of the year, Baltar et al. (2009b) found high abundances of heterotrophic prokaryotes in an area where the upwelling filament stretching off Cape Jubi (south of Cape Ghir, off the NW African coast) interacts with the oligotrophic open ocean waters. Similarly, Raimbault and Garcia (2008) observed high NH<sub>4</sub><sup>+</sup> regeneration rates in the upwelling off Chile, associated with the high abundance of heterotrophic plankton. Another piece of evidence is the increasing surface DOC concentrations observed as we move offshore (Table 1), which indicate an enhancement of microbial loop processes, as previously seen in the coastal

upwelling zone off Cape Ghir García-Muñoz et al. (2005).

The DON release rates observed in our study were in the order of those previously measured by Varela et al. (2003) off the Spanish Atlantic coast, but usually an order of magnitude higher than those observed in other oceanic zones (see Table 8.9. in Bronk and Steinberg, 2008). If we consider gross nitrogen uptake as net nitrogen uptake (nitrogen incorporated into biomass) plus nitrogen 'lost' as DON, we can calculate the percentage contribution of DON release to gross nitrogen uptake (Bronk et al., 1994). The percentage contributions of DON release to gross NO<sub>3</sub><sup>-</sup> and gross NH<sub>4</sub><sup>+</sup> uptake observed (Table 3) were in the range of those previously measured in the Atlantic (Varela et al., 2005). DON release derived from NO<sub>3</sub><sup>-</sup> predominated in non upwelling-affected zone, while DON released from NH<sub>4</sub><sup>+</sup> predominated in the upwelling-affected zone (Table 2). This discrepancy between open ocean and coastal zones has been observed in a range of studies (reviewed by Bronk and Steinberg, 2008).

#### Contribution of N<sub>2</sub> fixation to Pnew and nitrogen excess

The exclusion of N<sub>2</sub> fixation rates as a source of Pnew can significantly underestimate the f-ratio (Raimbault and Garcia, 2008). N<sub>2</sub> fixation has been estimated to fuel up to 50% of primary production in oligotrophic oceanic areas (Capone et al., 2005). This was not the case in this study, where the contribution of  $N_2$  fixation to Pnew was negligible (<1% in all cases). Similarly, Raimbault and Garcia (2008) did not find a significant contribution of N2 fixation to Pnew in the upwelling system off Chile. Benavides et al. (2011) measured gross and net N<sub>2</sub> fixation as derived from the acetylene reduction assay and the uptake of <sup>15</sup>N<sub>2</sub>, respectively. The difference between these two measurements is a good proxy for dissolved nitrogen release (Gallon et al., 2002). In the CAIBEX cruises, an average of  $\sim 60\%$  of the N<sub>2</sub> fixed was 'lost' as dissolved nitrogen (Benavides et al., 2011). Even if a 60% increase is applied to all N<sub>2</sub> fixation rates, their contribution to Pnew is still <1%. Mouriño-Carballido et al. (2011) compared N<sub>2</sub> fixation and NO<sub>3</sub><sup>-</sup> diffusion from 16<sup>o</sup>N to 31<sup>o</sup>S over central longitudes of the Atlantic. They found that, in the subtropical North Atlantic ( $\sim 16^{\circ}N-9^{\circ}N$ ), N<sub>2</sub> fixation only contributed 2% to Pnew.

 $N_2$  fixation rates in our study area are likely affected by wind and coastal upwelling intensity, which are more intense in the summer off NW Iberia, but remain more constant throughout the year off NW Africa (Arístegui et al., 2009). Coastal upwelling provides high  $NO_3^-$  concentrations, which raise Pnew rates (see the average  $NO_3^-$  uptake and Pnew rates for the upwelling-affected area in Table 2). We must note that the measurements presented here

correspond to the summer, when the upwelling intensity is maximum off Cape Ghir (Arístegui et al., 2009).

It should be also noted that the N<sub>2</sub> fixation rates presented are likely underestimated according to recent improvements of the  ${}^{15}N_2$  method. N<sub>2</sub> fixation rates measured using  ${}^{15}N_2$  bubble injections gives rates 50 to 570% times lower than when  ${}^{15}N_2$  is added dissolved in sample seawater (Mohr et al., 2010; Großkopf et al. 2012), so a general increase of basin-scale N<sub>2</sub> fixation rates in the North Atlantic is expectable when the new method is applied in different locations.

As previously mentioned, Álvarez and Álvarez-Salgado (2007) estimated a net nitrogen excess accumulation rate of 40  $\pm$  22  $\times$  10<sup>10</sup> mol N y<sup>-1</sup> or 658  $\pm$ 354  $\mu$ mol N m<sup>-2</sup> d<sup>-1</sup> with a geochemical box model, which they hypothesized should be due to  $N_2$  fixation after discounting other possible sources. This contrasts with the low average N<sub>2</sub> fixation rate measured in this study. Considering an average mixed layer depth of 100 m, the resulting volumetric rate of 6.6  $\pm$  3.5 nmol N L<sup>-1</sup> d<sup>-1</sup> estimated from Álvarez and Álvarez-Salgado (2007) is still three orders of magnitude higher the average rate measured experimentally in the same area  $(1.3 \times 10^{-3} \text{ nmol N L}^{-1} \text{ d}^{-1};$  Benavides et al., 2011). If the average N<sub>2</sub> fixation rate for the CAIBOX area was 50% higher, the resulting average N<sub>2</sub> fixation rate of  $2.4 \times 10^{-3}$  nmol N L<sup>-1</sup> d<sup>-1</sup> would be still three orders of magnitude lower than estimated by Álvarez and Álvarez-Salgado (2007). Even if the highest increment observed between methods is applied (570% by Großkopf et al., 2012), the nitrogen excess rate observed would still be two orders of magnitude higher. Nevertheless, we must note that surface N<sub>2</sub> fixation rates are not necessarily representative of the whole water column. For example, the widespread unicellular diazotrophic cyanobacteria of group A (UCYN-A) are known to inhabit deeper layers of the world oceans (Moisander et al., 2010), and are the most abundant oceanic diazotrophs (Luo et al., 2012). Moreover, non-cyanobacterial diazotrophs such as  $\alpha$ - or  $\beta$ proteobacteria and Archaea also fix N<sub>2</sub> in deeper layers (Riemann et al., 2010). However, deep profiles of N<sub>2</sub> fixation rates in this area of the North Atlantic are not available in the literature to date. In summary, a better assessment of depth-integrated N<sub>2</sub> fixation rates in this area are needed in order to estimate its true contribution to Pnew.

## Other sources of excess nitrogen accumulation in the water column

Nitrogen anomalies are caused by processes that happen in non-Redfieldian proportions, that is, processes that selectively accumulate nitrogen or selectively consume phosphorus (Landolfi et al., 2008). The uptake and regeneration of  $NO_{3^-}$  and  $NH_{4^+}$  are expected to occur in Redfieldian proportions; therefore, the nitrogen excess computed by Álvarez and Álvarez-Salgado (2007) in our study area must be caused by other processes. Besides  $N_2$  fixation, Landolfi et al. (2008) considered the following reasons behind nitrogen anomalies: (1) deposition of atmospheric combined nitrogen, which usually has high N:P ratios (Chen et al., 2007; Baker et al., 2010; Kanakidou et al., 2012); (2) advection and subsequent remineralization of phosphorusdepleted DOM (Hopkinson and Vallino, 2005; Mather et al., 2008); and (3) preferential uptake and/or remineralization of phosphorus over nitrogen (Clark et al., 1998). Below, we include a brief discussion of the role of each of these possibilities in the CAIBOX area (non upwelling-affected stations, Fig. 1a). Nitrogen excess estimations are not available for the upwelling-affected area (Cape Ghir, Fig. 1b), and therefore will not be further discussed here.

(1) The contribution of atmospheric combined nitrogen deposition to the nitrogen anomaly in the North Atlantic has been previously argued (e.g. Landolfi et al., 2008; Zamora et al., 2010). However, as discussed in Álvarez and Álvarez-Salgado (2007), present modeled rates of atmospheric combined nitrogen deposition to the North Atlantic (compiled in Table 4) do not explain the nitrogen excess observed in the CAIBOX area.

(2) A second factor behind nitrogen anomalies would be the injection of dissolved organic matter (DOM) with an N:P ratio > 16 into the mesopelagic layer before it is fully remineralized (Mahaffey et al., 2005; Hansell et al., 2007). It is important to note that the CAIBOX area is bounded to the east by the coastal upwelling system of Iberia – NW Africa. In this sense, García-Muñoz et al. (2005), observed that ~60% of the net primary production near Cape Ghir (~30–31°N) was exported as DOM via upwelling filaments. These are recurrent features of the Iberian-NW African upwelling system and may extend hundreds of kilometers offshore, impacting the thermocline of the North Atlantic subtropical gyre (Álvarez-Salgado et al., 2007). In addition, the release of DON derived from NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> uptake represented considerable percentages of gross uptake (Table 3). This suggests that, adding to atmospheric combined nitrogen deposition, DON advected from the coastal upwelling, and DON produced in situ through NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> uptake may contribute substantially to the observed nitrogen excess in this area.

(3) In contrast with the DON pool, which contains semilabile and refractory fractions (Bronk et al., 2007), the dissolved organic phosphorus (DOP) pool is mostly biologically available thanks to the widespread alkaline phosphatase enzyme (Dyhrman et al., 2007). Low inorganic phosphorus availability in the North Atlantic triggers the uptake of DOP, which is selectively remineralized over DON (Kolowith et al., 2001). The preferential remineralization of

Table 4: Modeled rates of atmospheric nitrogen deposition to the North Atlantic compiled in Baker et al. (2010). Deposition rates were recalculated for the CAIBOX area considering a surface equal to  $1.1 \times 10^{12} \text{ m}^2$ .

Reference	North Atlantic atmospheric nitrogen deposition (Gmol N y-1)	CAIBOX atmospheric nitrogen deposition (mol N y <sup>-1</sup> )		
Duce et al., 1991	619	$1.27 \ge 10^{10}$		
Prospero et al., 1996	409	$0.84 \ge 10^{10}$		
Dentener et al., 2006	492	$1.01 \ge 10^{10}$		
Luo et al., 2007	462	0.95 x 10 <sup>10</sup>		
Baker et al., 2010	483	0.99 x 10 <sup>10</sup>		
Baker et al., 2010	456-710	$a1.12-1.46 \ge 10^{10*}$		

<sup>a</sup>Considering a 13–47% increase in nitrogen deposition rates when soluble organic nitrogen is added.

phosphorus over nitrogen should be assessed by the observation of profiles of DON:DOP ratios (Clark et al., 1998), but unfortunately, DOP data are not available for this study.

#### Nitrogen budgets in the CAIBOX area

Revisiting the calculations by Álvarez and Álvarez-Salgado (2007) (Fig. 4a), they assumed that the organic nitrogen concentrations measured by Doval et al. (2001) in the eastern North Atlantic-Azores Front region were representative for the whole study area to calculate the organic nitrogen flux from the adjacent ocean into the CAIBOX area. Within the framework of the CAIBEX project, full-depth organic nitrogen profiles were measured in the CAIBOX area in such a way that organic nitrogen fluxes can be recalculated more precisely (Lønborg et al, in prep.). Following Álvarez and Álvarez-Salgado (2007), the total transport of organic nitrogen from the open ocean to the CAIBOX area can be ascribed to the Ekman (0.25  $\times$  10<sup>6</sup> m<sup>3</sup> s<sup>-1</sup>) and overturning  $(3 \times 10^6 \text{ m}^3 \text{ s}^{-1})$  transports. The average total organic nitrogen concentration in the surface layer of the CAIBOX area was 6.7  $\pm$  1.0  $\mu$ M, which translates into an organic nitrogen flux into the CAIBOX of 5.4  $\pm$  0.8  $\times$  10<sup>10</sup> mol N y<sup>-1</sup> due to Ekman transport. The average total organic nitrogen concentration in the upper 200 m and from 500 m to the sea-bottom of the CAIBOX area were 6.4  $\pm$  1.0 and 3.9  $\pm$  1.0  $\mu$ M. The vertical gradient, estimated as the difference between the mean concentration below 500 m and that in the upper 200 m, multiplied by the overturning transport, yielded an organic nitrogen flux into the CAIBOX of 26  $\pm$  13  $\times$  10<sup>10</sup> mol N y<sup>-1</sup>. Therefore, the

recalculated organic nitrogen flux into the box is  $32 \pm 13 \times 10^{10}$  mol N y<sup>-1</sup> (Fig. 4c). The previous estimate of the flux of NO<sub>3</sub><sup>-</sup> ito the Atlantic Ocean was  $27 \pm 13$  10<sup>10</sup> mol N y<sup>-1</sup> (Fig. 4b), which is considerably reduced considering the more recent estimate of 13.9 ± 0.3 × 10<sup>10</sup> mol N y<sup>-1</sup> (Fig. 4d), provided by Huertas et al. (2012).



Fig. 4: Schemes showing the (a) organic nitrogen (NOrg) and (b) nitrate (NO<sub>3</sub>) budgets in the CAIBOX region as presented in the study of Álvarez and Álvarez-Salgado (2007), compared to the revisited estimates of budgets of (c) NOrg and (d) NO<sub>3</sub> presented in this study.

Furthermore, Álvarez and Álvarez-Salgado (2007) did not account for the previously referred export of  $19 \times 10^{10}$  mol y<sup>-1</sup> of organic nitrogen from the coastal upwelling of Iberia–NW Africa because they considered that this coastal upwelling system was within the limits of their box model. Therefore, they assumed that the organic nitrogen produced in the coast, exported to the open ocean, and accumulated within the box, would be part of the recycled production of the system and would not contribute to the net production budget. However, it has been suggested (Hansell and Carlson, 2002; Hansell et al., 2009) that the fate of the material exported from the coast is to accumulate in the adjacent ocean; in this case, the steady-state assumption of Álvarez and Álvarez-Salgado et al. (2007) would not be accurate, implying that part (if not all) of the 19 × 10<sup>10</sup> mol N y<sup>-1</sup> of the nitrogen excess could be explained by accumulation within the limits of the box (Fig. 4c). In summary, if the original budget by Álvarez and Álvarez-Salgado et al. (2007) is revisited incorporating new flux estimates of the import of NO<sub>3</sub><sup>-</sup> form the Mediterranean (Huertas et al., 2012), the organic nitrogen from the surrounding ocean and the accumulation of organic nitrogen from the adjacent coastal upwelling system, the nitrogen excess would reduce to  $22 \pm 19 \times 10^{10}$  mol N y<sup>-1</sup> or  $353 \pm 306 \mu$ mol N m<sup>-2</sup> d<sup>-1</sup>. This nitrogen excess rate is about half of the previous estimate by Álvarez and Álvarez-Salgado (2007). In conclusion, although the revisited rate is lower than previously estimated, this excess is still not explained by in situ measurements of N<sub>2</sub> fixation and estimates of atmospheric fixed nitrogen inputs and outputs in this area of the eastern North Atlantic.

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PART III: Synthesis and further research

## **General discussion**

# Magnitude and distribution of $N_2$ fixation rates in the subtropical Northeast Atlantic

Recently, Luo et al. (2012) gathered all the N<sub>2</sub> fixation and diazotrophic organisms' abundance data available to date and composed a freely available online database (http://doi.pangaea.de/10.1594/PANGAEA.774851). The comparison of N<sub>2</sub> fixation rates among the different ocean basins (Table 1) shows that the lowest areal N<sub>2</sub> fixation rates have been measured in the South Atlantic, while the areal rates measured in the North Atlantic are roughly half of those observed in the other basins.

Table 1: Global weight-averaged annual  $N_2$  fixation rates. Modified from Table S3 in the Supplementary Information of Großkopf et al. (2012).

Ocean basin	North/South limit (ºN)	Areal rate (µmol N m <sup>-2</sup> d <sup>-1</sup> )	Areal rate (mol N m <sup>-2</sup> y <sup>-1</sup> )	Area (10º km²)	Basin rate (Tg N y <sup>-1</sup> )
N Atlantic	50/-5	64	0.023	39.2	12.7
S Atlantic	-5/-30	19	0.07	15.9	1.5
N Pacific	30/0	115	0.042	56.3	33.0
S Pacific	0/-35	103	0.038	56.1	29.5
N Indian	25/0	115	0.042	15.8	9.3
S Indian	0/-35	103	0.038	31.8	16.7

Despite that the North Atlantic comprises the greatest amount of  $N_2$  fixation measurements available worldwide (Luo et al., 2012), most of this research has focused in the tropical Northwest Atlantic, where high  $N_2$  fixation rates are mostly attributable to the ubiquitous colonial cyanobacterium *Trichodesmium* (e.g. Carpenter and Romans 1991; Capone et al., 1997, 2005). Fewer  $N_2$  fixation rates have been measured in the subtropical Northeast Atlantic. Here, the whole seawater  $N_2$  fixation rates available stem from a limited numbers of publications, which are basically the works of Moore et al. (2009), Fernández et al. (2010), Turk et al. (2011) and Großkopf et al. (2012). The estimates provided in this thesis increase considerably the database of  $N_2$  fixation rates in this region (see **Annex II**).

Table 2 shows the average volumetric  $N_2$  fixation rates in the tropical, subtropical and temperate latitudinal bands of the North Atlantic Ocean based on the Luo et al. (2012) database and the data collected in this thesis.

Atlantic Ocean areas	Average $N_2$ fixation (nmol L <sup>-1</sup> d <sup>-1</sup> )
Tropical (0 - 23.5ºN)	3.59 (17.87)
Subtropical (23.5 - 40ºN)	1.60 (4.56)
*Temperate (>40ºN)	2.28 (4.34)
All NE	3.10 (15.16)
Subtropical NE	1.16 (1.93)
Subtropical NE % contribution to all the subtropical latitudinal band	72.45%

Table 2: Average whole seawater  $N_2\,fixation$  rates in the North Atlantic Ocean. Standard deviation is given in brackets.

\*the northernmost  $N_2$  fixation rate available in the literature is at  ${\sim}56^{\varrho}N.$ 

As mentioned above, the highest  $N_2$  fixation rates of the North Atlantic can be found in the tropical latitudinal band, being more than 2-fold higher than those measured in the subtropical latitudinal band. Surprisingly, the average rate of  $N_2$  fixation in the temperate latitudinal band is quite high. However, it must be noted that measurements in this area are rather scarce and therefore this average is based on very few data (see Figure 3b in Luo et al., 2012).

Overall, we estimate that the subtropical Northeast Atlantic provides >70% of the N<sub>2</sub> fixed in the entire subtropical North Atlantic latitudinal band. This geographical trend is in part explained by the greater amount of iron-rich Saharan dust received by this side of the subtropical North Atlantic (Prospero, 1981). Indeed, N<sub>2</sub> fixation rates in the Northeast Atlantic are in good agreement with dissolved iron concentrations (Moore et al., 2009), and aerosol optical depth at 550 nm (AOD 550 nm) (Fernández et al., 2010; Chapter 3). However, the cold seawater temperatures and high dissolved inorganic nitrogen (DIN) concentrations associated with the coastal upwelling off Northwest Africa may preclude diazotrophy to some extent (Gruber and Sarmiento, 1997). Such conditions seem to select unicellular diazotrophic cyanobacteria (UCYN) over *Trichodesmium* in these waters. In **Chapter 1**, we estimated that the <10 μm fraction contributes 60-90% to total gross N<sub>2</sub> fixation. In **Chapter 3**, we observed that east of  $45^{\circ}$ W over  $24.5^{\circ}$ N this fraction contributed  $\sim 39\%$  $(\pm 20\%)$  to total N<sub>2</sub> fixation. In **Chapter 4**, rates associated with unicellular diazotrophs were 3- to >100-fold higher than those associated with Trichodesmium. The predominance of  $N_2$  fixation by the <10  $\mu$ m fraction is consistent with previous results in the eastern Atlantic Ocean at lower latitudes (Voss et al., 2004; Montoya et al., 2007; Goebel et al., 2010).

Nonetheless, the importance of  $N_2$  fixation rates by the <10  $\mu$ m fraction

could be underestimated since most of the data available correspond to the surface ocean layer (generally <200 m, where >10  $\mu$ m diazotrophs thrive - mainly *Trichodesmium* and diazotrophs in symbiosis with diatoms-), while UCYN and specially non-cyanobacterial diazotrophs rather dominate in deeper layers of the ocean (Hewson et al., 2007; Riemann et al., 2010; Hamersley et al., 2011). Recent evidence suggests that the non-cyanobacterial *nifH* is more abundant than the UCYN and *Trichodesmium nifH* abundance together in the world's oceans (Farnelid et al., 2011), indicating that the global distribution of diazotrophic activity needs to be reassessed including deeper water column measurements.

#### Diversity of diazotrophic organisms

Here we have assessed the abundance and diversity of diazotrophic organisms through *Trichodesmium* counts and molecular biology techniques to detect *nifH* genes (TSA-FISH and nested-PCR). In **Chapter 1** we measured the abundance of Trichodesmium in the summer of 2009 between ~42°N to ~28°N along 20°W. These were up to 0.43 trichomes L-1, while the abundance of Trichodesmium in the upwelling systems of Cape Silleiro and Cape Ghir was generally <0.1 trichomes L-1. Trichodesmium is largely affected by the availability of fixed nitrogen (Mulholland et al., 2001; Holl and Montoya, 2005). Thus, it can be expected that the high DIN concentrations usually observed in these upwelling regions (Arístegui et al., 2006), which ranged from  $\sim 0.7$  to > 2 $\mu$ M during our cruise in the summer of 2009, limited its proliferation. In **Chapter 4** we measured the abundance of *Trichodesmium* in a station  $\sim 10$ nautical miles to the north of the Canary Islands from February to May 2010. In this case, abundances were higher than those observed in **Chapter 1** (0.2 - 1.2)trichomes L<sup>-1</sup>), and DIN concentrations lower ( $\sim 0.5 \mu$ M). The majority of the Trichodesmium individuals observed in these studies were present as free trichomes, instead of as tuft- or puff-shaped colonies, as they are commonly found in the tropical western Atlantic (Capone et al., 1997). The few published studies conducted in the subtropical eastern Atlantic also report that Trichodesmium appears mainly as free trichomes (Fernández et al., 2010; González-Taboada et al., 2010).

*Trichodesmium* thrives in warm (>20°C) and stratified waters where it often appears forming dense blooms, which accumulate in the surface thanks to the positive buoyancy provided by their air vesicles (Villareal and Carpenter, 2003). The cold temperatures and turbulence -inherent to subtropical eastern Atlantic areas such as the Canary Current- likely prevent the proliferation of *Trichodesmium* (Carpenter and Price, 1976). However, there are some specific

areas where *Trichodesmium* can grow rapidly and form blooms. For example, the flow of the prevailing trade winds is interrupted when it encounters the Canary archipelago, creating a leeward zone of stratified and warm waters south of the islands (Basterretxea et al., 2002). Enhanced iron deposition propitiated by the proximity of the Sahara desert combined with a strong rise in surface water temperature at these leeward zones may have been the cause of recurrent blooms of *Trichodesmium* observed in the past years (e.g. Ramos et al., 2005), calling the attention of the local press (Fig. 1).



Fig. 1. Coastal concentrations of *Trichodesmium* published in the local press of the Canary Islands. Extracted from <u>www.isladelanzarote.org/?p=19290</u> and <u>www.universocanario.com/siete-islas/microalgas-/menos-toxicas/trichodesmium/poco-agresivas/5061</u>. Both webpages were accessed on 29<sup>th</sup> October 2012.

UCYN abundance data is also presented in the Luo et al. (2012) database mentioned above. As shown on Table 3, most of the UCYN of the North Atlantic belong to group A, which are especially abundant in the tropical North Atlantic. In the subtropical latitudinal band however, UCYN-B are more abundant. UCYN-C have only been detected in the tropical North Atlantic. Representatives of groups UCYN-B (e.g. Webb et al., 2009) and UCYN-C (Taniuchi et al., 2012) have been isolated and are available in culture. Attempts to maintain UCYN-A in cultures have been unsuccessful (Zehr et al., 2008).

Table 3: Average *nifH* copies in the North Atlantic. All values are given in  $x10^6$  *nifH* copies m<sup>-3</sup>. Data was extracted from Luo et al. (2012) online database.

	UCYN-A	UCYN-B	UCYN-C
Tropical (0 - 23.5ºN)	53747	9.87	6.83
Subtropical (23.5 - 40ºN)	963	19230	0.00
*Temperate (>40ºN)	271	0.00	0.00

\*the northernmost *nifH* copy number available in the literature is at  $\sim$ 42<sup>o</sup>N.

PART III: Synthesis and future research

UCYN-A are small (<1  $\mu$ m) photoheterotrophic cyanobacteria which lack the oxygen-evolving photosystem II and are not capable of fixing carbon (Zehr et al., 2008). Unlike UCYN-B and -C, this type of metabolism allows UCYN-A to fix N<sub>2</sub> during the day (Church et al., 2005), while their inability to fix carbon makes them dependent on essential compounds produced by other organisms for their nutrition (Tripp et al., 2010).

UCYN-A are the most abundant diazotrophic cyanobacteria of the global ocean (Luo et al., 2012). In **Chapters 4 and 5** we measured the abundance of UCYN by the TSA-FISH technique using probe Nitro821. Although this technique does not allow the phylogenetic identification of diazotrophic cells, it gives another useful information such as their size, shape and their associations with particles or other organisms. About 20% of the cells counted in **Chapter 4** were <1  $\mu$ m, likely belonging to UCYN-A. In **Chapter 5** we also studied the diversity of the *nifH* gene by nested-PCR analysis in an open ocean area and two active coastal upwelling zones of the Canary Current. Phylogenetic analysis of amplicon clones from 15 samples collected during these cruises (**Chapter 5**) revealed that 95% of the sequences had similarities of 98-100% to UCYN-A (see Fig. 2).

An interesting result of the TSA-FISH analyses performed in **Chapters 4** and 5 is the relatively high abundance of UCYN cells that appeared attached to or embedded in particles (Table 4). This 'particle-attached mode of life' provides a number of advantages to microorganisms. Bacteria colonize sinking or suspended organic particles (Azam and Long, 2001), promoting enhanced respiration as a result of organic matter oxidation. This activity may provide the low oxygen conditions needed for N<sub>2</sub> fixation (Riemann et al., 2010). Moreover, the organic molecules of the particle (probably solubilized from particulate organic matter by the colonizing bacteria) may help binding trace nutrients such as iron (**Chapter 4**), which is known to limit N<sub>2</sub> fixation (e.g. Kustka et al., 2002).

Cruise	Latitude/Longitude	Date	% attached to particles	% free living	% symbionts to other cells
CAIBOX	~42-28ºN/~9-20ºW	Jul-Aug 09	14.50	81.65	3.86
Ghir	~30-32ºN/~9-12ºW	Aug-Sep 09	7.63	90.10	2.27
Silleiro	~41-42ºN/~9ºW	Jul 09	13.92	65.71	20.38
Lucifer	28º21'N/15º23'W	Feb-May 10	24.71	73.97	1.36

Table 4: Percentage UCYN found either attached to particles, free living or in symbiosis with other cells using TSA-FISH with probe Nitro821 in the different cruises performed throughout this thesis.



Fig. 2. Phylogenetic tree of *nifH* sequences. Sequences obtained in this study are referred to (i) by the oceanographic cruise CAIBEX, (ii) the stations at which they were sampled, (iii) the depth they were sampled (A=surface, B= mixed layer depth -MLD-, C= deep chlorophyll maximum -DCM-), and (iv) clone number. Bootstrap values >50% are indicated at the nodes. Scale bar=0.05 substitution per nucleotides.

In general, diazotroph-particle associations have been described for noncyanobacterial diazotrophs of Cluster III (see review by Riemann et al., 2010), although these likely occur in some UCYN too.

It has been suggested that the metabolism and small genome of UCYN-A (1.44 Mb; Tripp et al., 2010) indicates it likely lives in symbiosis with other organisms (Pérez-Brocal et al., 2006), or depends on recently produced or standing-stock oceanic organic matter (Tripp et al., 2010). In the work of Tripp et al. (2010), virtually all the UCYN-A *nifH* found was recovered from the <1 μm fraction, suggesting these cells were not in symbiosis with larger organisms. In the Benguela upwelling system, Sohm et al. (2011b) found high abundances of UCYN-A at stations where <10  $\mu$ m N<sub>2</sub> fixation rates represented only ~30% of total  $N_2$  fixation. In their study, the only other detectable diazotroph was Crocosphaera, which was present at much lower abundances. The only explanation for this incongruence is that UCYN-A were also responsible for >10  $\mu$ m N<sub>2</sub> fixation activity (i.e. UCYN-A in symbiosis with larger cells or attached to particles would not pass through a 10 µm filter pore). In **Chapter 4**, most of the cells that appeared attached to particles (see Fig. 5 from Chapter 4) ranged from 0.6 to  $\sim 1 \,\mu m$  in size. According to this size range it is probable that these cells were UCYN-A. Furthermore, other picoplanktonic diazotrophs have also been observed in association with particles in the Southwest (Biegala and Raimbault, 2008) and Equatorial Pacific (Bonnet et al., 2009), and in the Mediterranean Sea (Le Moal and Biegala, 2009; Le Moal et al., 2011). More recently, Thompson et al. (2012) found UCYN-A cells in symbiosis with prymnesiophytes at station ALOHA. These authors attributed the previous absence of UCYN-A cells in larger size fractions to sample fractionation and manipulation, which often can detach cells from their hosts hampering the study of these associations, which often happens with organism-particle associations too (Simon et al., 2002).

All this evidence suggests that, just as certain heterotrophic diazotrophs, UCYN-A may also present a particle-attached mode of life under particular circumstances. In general, it seems that these associations occur in environments where DIN and particulate organic matter concentrations are high, such as coastal upwelling ecosystems (Sohm et al., 2011b; **Chapter 5**), and other eutrophic coastal areas (Biegala and Raimbault, 2008). Cyanobacteria-particle associations may also occur in other UCYN groups such as *Crocosphaera*, which has been shown to produce extracellular polysaccharides (EPS) in which they embed forming particles (Sohm et al., 2011a).

Finally, other diazotroph groups such as filamentous cyanobacteria in

symbiosis with diatoms or copepods were rarely observed during the cruises performed throughout this thesis. These generally represented <5% of the cells counted in TSA-FISH samples, while diatom-cyanobacteria symbioses were never observed under the microscope during the inspection of samples for *Trichodesmium* abundance.

## Release of recently fixed N<sub>2</sub>

Most of the N<sub>2</sub> fixation rates available in the literature are net N<sub>2</sub> fixation rates (N<sub>2</sub> incorporation into biomass). For the measurement of N<sub>2</sub> fixation rates seawater samples are generally filtered through glass fiber filters (usually 0.7  $\mu$ m nominal pore size). The filtrates are typically discarded, implying that any N<sub>2</sub> that is fixed and released extracellularly is not accounted for. If this flux is substantial, real N<sub>2</sub> fixation rates can be significantly underestimated (Bronk et al., 1994).

Given the present disequilibrium between fixed global nitrogen 'gain' and 'loss' rates (denitrification and anammox exceed N<sub>2</sub> fixation by ~200 Tg N y<sup>-1</sup>; Mahaffey et al., 2005; Codispoti, 2007), reliable global N<sub>2</sub> fixation rates are needed in order to balance the oceanic nitrogen cycle. The recent discovery that earlier published N<sub>2</sub> fixation rates were largely underestimated (Mohr et al., 2010; Großkopf et al., 2012; Wilson et al., 2012; **Chapter 3**) promises that the application of the improved method (dissolved <sup>15</sup>N<sub>2</sub>; Mohr et al., 2010) could bring loss and gain rates closer to each other. Although there have been few dissolved nitrogen release studies so far, results suggest that the contribution of this flux to the correction of N<sub>2</sub> fixation rates could help reaching equilibrium in the fixed nitrogen global budget estimates.

However, we must note that some authors have argued that the low variability of oceanic dissolved organic nitrogen (DON) pools and their poor correlations with  $N_2$  fixation rates indicate that fixed  $N_2$  'loss' to the DON pool is insignificant (Knapp et al., 2011).

Glibert and Bronk (1994) estimated that field populations of *Trichodesmium* released ~50% of their recently fixed N<sub>2</sub> as DON, while cultured *Trichodesmium* IMS101 only released ~8% (Mulholland et al., 2004). Similarly, in **Chapter 2** we observed that cultures of the unicellular diazotroph *Cyanothece* sp. Miami BG 043511 released only ~1% of their recently fixed N<sub>2</sub> as DON, while in **Chapter 3** the <10  $\mu$ m fraction sampled in surface waters of the North Atlantic released ~23%. These differences in DON release dynamics are likely caused by all the factors inherent to natural oceanic waters which are hardly mimicked by lab cultures: light intensity variability, turbulence, nutrient

and temperature stress, bacterivory, viral lysis and grazing.

The release of DON can be measured indirectly as the difference between gross  $N_2$  fixation (measured by the acetylene reduction assay -ARA-) and net  $N_2$  fixation ( $^{15}N_2$  tracer method). The increase of  $N_2$  fixation rates observed when dissolved  $^{15}N_2$  is added instead of the  $^{15}N_2$  bubble suggest that previous differences found between ARA-derived and  $^{15}N_2$  bubble-derived rates (e.g. Gallon et al., 2002; Mulholland et al., 2004) may be in fact negligible (Mohr et al., 2010). However, the measurement of  $^{15}N$ -labeled extracellular DON after a period of incubation with  $^{15}N_2$  does measure actual DON release rates (Glibert and Bronk, 1994; Mulholland et al., 2004; **Chapter 3**).

The measurement of <sup>15</sup>N-labeled DON is labor-intensive and entails some methodological issues (previously discussed in the General Introduction of this thesis). A more straightforward method is needed in order for DON release measurements to be widely practiced.

# The contribution of $N_2$ fixation to new production in the subtropical Northeast Atlantic

The subtropical gyres of the oceans are nutrient impoverished due to the persistent water column stratification, which hinders the entrance of nutrient-rich deep waters to the surface layer (Falkowski, 1997). In these oligotrophic systems, fixed N<sub>2</sub> provided by diazotrophy is thought to support primary production largely (Karl et al. 2002; Capone et al., 2005). Essentially, this idea emerged from the elevated basin-scale N<sub>2</sub> fixation rates derived from geochemical-approach estimates (Table 4). However, the rates obtained with geochemical approaches exceed those obtained with biological approaches typically by an order of magnitude (Table 1). This could either mean that geochemical-approach methods overestimate N<sub>2</sub> fixation rates, or alternatively biological-approach methods underestimate them.

occan. Mounicu nom Manan	cy ct al. (2003).			
Reference	Method	Areal rate (μmol N m <sup>-2</sup> d <sup>-1</sup> )	Study area (10º km²)	Basin-scale areal rat (39.2 x 10 <sup>6</sup> km²)
Michaels et al. (1996)	N*	500-2500	7-19	100.2-500.8
Gruber and Sarmiento (1997)	N*	197	278	395

70-208

45-259

850

6.8

16

17.8

Modified N\*

Modified N\*

 $\delta^{15}N$ 

Hansell et al. (2004)

Capone et al. (2005)

Bates and Hansell (2004)

Table 4: Geochemical-approach estimates of  $N_2$  fixation of different areas of the North Atlantic Ocean. Modified from Mahaffey et al. (2005).

14-41.7

9-52

170.3

In **Chapter 6** we measured new production rates over an open ocean and a coastal upwelling zone, and compared them with N<sub>2</sub> fixation rates measured at the same stations. The contribution of N<sub>2</sub> fixation to new production was <1% in both zones, suggesting that the contribution of diazotrophy to the fixed nitrogen reservoir of the eastern subtropical Atlantic is rather negligible (at least during the summer, when these measurements were done). Similarly, Mouriño-Carballido et al. (2011) measured NO<sub>3</sub>- inputs to the euphotic zone parallel to N<sub>2</sub> fixation measurements in a north-south transect along the central Atlantic Ocean. They estimated that N<sub>2</sub> fixation represented ~2% of the total new nitrogen input to the euphotic zone.

The low in situ N<sub>2</sub> fixation rates measured in the subtropical Northeast Atlantic (see **Annex II**) are at odds with the chemical signatures of N<sub>2</sub> fixation found by the application of the N\* and  $\delta^{15}$ N parameters in this region (e.g. Mahaffey et al., 2003; Reynolds et al., 2007; Bourbonnais et al., 2009). All these authors concluded that N<sub>2</sub> fixation contributed importantly to new production in the eastern North Atlantic. However, the parameters they used are easily masked by other processes such as atmospheric nitrogen deposition and isotopic fractionation (see the Introduction section of this thesis). Indeed, recent evidence indicates that the atmospheric deposition of anthropogenic nitrogen is rapidly approaching the same order of magnitude as ocean N<sub>2</sub> fixation (Duce et al., 2008). According to the deposition rates showed in Table 5, atmospheric nitrogen deposition could represent ~2-100% of the basin-scale N<sub>2</sub> fixation rates estimated by geochemical methods (Table 4).

Reference	Basin-scale areal rate (Tg N y-1)
Duce et al., 1991	8.7
Prospero et al., 1996	5.7
Dentener et al., 2006	6.9
Luo et al., 2007	6.5
Baker et al., 2010	6.4-10

Table 5: North Atlantic basin-scale estimates of atmospheric nitrogen deposition.

However, the excess of fixed nitrogen observed by Álvarez and Álvarez-Salgado (2007) and in **Chapter 6**, is still not explained by these fluxes.

In summary, differences among geochemical- and biological-approach methods likely result from: (1) the different spatial and temporal scales involved, i.e.: geochemical methods use whole basins and years, while biological methods use only a few liters and up to 24 h incubations, (2) overestimations caused by geochemical methods due to masked chemical signals, e.g. deposition of nitrogenous atmospheric materials, or (3) underestimations caused by biological methods due to methodological issues (Mohr et al., 2010).

In the future, geochemical method-based global  $N_2$  fixation rates should be adjusted to discern which percentage is provided by atmospheric nitrogen deposition, and which is truly attributable to diazotrophy. The resulting rate would probably be low enough to be outweighed by new  $N_2$  fixation rates measured using the improved dissolved  ${}^{15}N_2$  method (Mohr et al., 2010), balancing the oceanic fixed nitrogen budget.

## Conclusions

The main conclusions that arise from this thesis are:

- N<sub>2</sub> fixation rates in the subtropical Northeast Atlantic are low compared to the other areas of this basin. In the tropical latitudinal band, the highest N<sub>2</sub> fixation rates are found in its western half. However, in the subtropical latitudinal band, the eastern half provides >70% of the N<sub>2</sub> fixed. The <10  $\mu$ m fraction contributed 60-90% of total gross N<sub>2</sub> fixation, indicating that unicellular diazotrophs predominate in the eastern subtropical North Atlantic Ocean. Differences between gross and net N<sub>2</sub> fixation rates indicated that N<sub>2</sub> fixing cells in the >10  $\mu$ m fraction potentially released between 15 and ~90% of their recently fixed N<sub>2</sub>, while the <10  $\mu$ m fraction could release 25-90%. Potential recently fixed N<sub>2</sub> release by the >10  $\mu$ m fraction was higher in open-ocean areas, while for the <10  $\mu$ m it was higher in coastal upwelling areas.
- Marine diazotrophic unicellular cyanobacteria may need to be under some sort of stress (light, nutrients, predation, bacterivory, turbulence) to release DON extracellularly, as suggested by cultures of the marine diazotrophic unicellular cyanobacterium *Cyanothece* sp. Miami BG 043511, only releasing 1% of their recently fixed N<sub>2</sub> as DON when grown at optimum conditions of light and temperature.
- Oceanic unicellular diazotrophs (<10  $\mu$ m fraction) released ~23% of their recently fixed N<sub>2</sub> as DON, while the >10  $\mu$ m fraction released ~14%, confirming that natural populations of diazotrophs exposed to different environmental factors such as turbulence, excess light or nutrient stress may release a considerable percentage of their recently fixed N<sub>2</sub> extracellularly. However, DON release rates were constant among size fractions, did not follow any geographical trend, and did not correlate with any of the various variables measured (temperature, salinity, nutrients and aerosol optical depth at 550 nm).
- The deposition of iron-rich Saharan dust over the Canary Islands had a differential effect in *Trichodesmium* versus unicellular diazotrophs. After a dust deposition peak, *Trichodesmium* seemed to be unaffected by the

enhanced iron availability, and maintained similar specific N<sub>2</sub> fixation rates. Conversely, N<sub>2</sub> fixation rates by unicellular diazotrophs increased by ~90%. After the dust deposition peak, ~35% of the unicellular diazotrophs appeared attached to putatively organic matter particles. This association may help them bind the iron contained in the dust. In contrast, *Trichodesmium* appeared mainly as free trichomes, which does not allow them to 'trap' and take advantage of the materials contained in dust particles.

- The molecular biology analyses performed (nested-PCR) indicated that most of the <10  $\mu m$  organisms sampled belonged to the UCYN-A group. This was confirmed by the high abundance of <1  $\mu m$  organisms in TSA-FISH analysis.
- The low N<sub>2</sub> fixation rates measured indicate that its contribution to new production in the subtropical Northeast Atlantic is very low (<1%). It is unlikely that this diazotrophic activity produces the fixed nitrogen excess signal recurrently observed over this area of the Atlantic Ocean. Alternatively, the deposition of atmospheric nitrogen and/or advection of dissolved organic matter from the coastal upwelling off Northwest Africa could contribute significantly to the excess nitrogen observed in this region, although present estimates of this fluxes are not enough to explain it.

## **Future lines of research**

"There is an urgent need for oceanographers to embark on a bold exploration of the oceans – this time at the millimeter scale."

Azam and Long, 2001 (Nature 414:495-498)

The early thought that the principal N<sub>2</sub> fixers of the ocean were few (mainly *Trichodesmium* and symbionts of diatoms), and that diazotrophy was restricted to the tropical band of the oceans, where well-lit, warm, oligotrophic and stratified waters prevail, has changed drastically. After almost two decades of intense research, we now know that N<sub>2</sub> fixation can take place at latitudes up to ~50°N (Luo et al., 2012), at depths >800 m (e.g. Hamersley et al., 2011), at temperatures <16°C (e.g. Sohm et al., 2011b), and in waters far from being stratified (e.g. Benavides et al., 2011).

Very recent research has shown that UCYN-A (which have a photoheterotrophic metabolism) are the most numerous diazotrophic cyanobacteria in the world's oceans (Luo et al., 2012), and depend on a external source of organic matter for their nutrition, either from the oceanic organic matter standing stock (Tripp et al., 2010), or as newly reported, from a symbiotic relationship with other plankton (Thompson et al., 2012). Other recent studies suggest that non-cyanobacterial *nifH* predominates over cyanobacterial nifH on a global basis (Farnelid et al., 2011), and that heterotrophs dominate N<sub>2</sub> fixation in open oligotrophic waters like in the South Pacific subtropical gyre (Halm et al., 2011). Altogether, the study of heterotrophic diazotrophy promises the dawning of a new era in oceanic nitrogen cycling. Because heterotrophs depend on organic matter for a nutritious source, the next logical step is to study organic matterheterotrophic diazotrophs interactions in the ocean, like how does the composition of organic matter favor or work against different diazotrophic organism assemblages, or if they can present a 'particle-attached mode of life' under certain circumstances. Previous studies indicated that only 10-15% of the heterotrophs inhabiting the water column live associated with particles (Turley and Mackie, 1994), but more recent research suggests that this mode of life is preferred by microheterotrophs (e.g. Baltar et al., 2009a). Particles in pelagic environments usually have a high concentration of nutrients and organic matter (see Table 5 in Simon et al., 2002), which promotes an intense microbial activity. For example, bacterial colonization may stimulate organic matter oxidation, fostering suboxic conditions within and around the particle, which makes these a perfect loci for  $N_2$  fixers (Riemann et al., 2010).

Another question yet to be resolved is to be able to ascertain 'who is doing what'. In general, studies on oceanic diazotrophy have separated the activity from the phylogeny, i.e.  $N_2$  fixation rates are measured and the diversity of the *nifH* gene identified. Present available techniques include qPCR - which quantifies the amount of *nifH* present in the sample (but not necessarily active)-, and qRT-PCR -which quantifies the actual activity (expression) of the *nifH* gene-. However, the presence and/or expression of the *nifH* gene does not tell us how much  $N_2$  was actually being fixed by that organism. Efforts need to be made to elucidate how the expression of the *nifH* gene can be used as proxy for  $N_2$  fixation rates. In the future, the application of techniques like stable-isotope probing of nucleic acids and nanoSIMS should help ascribing each group of  $N_2$  fixers to their relative diazotrophic activity.

Moreover, it should not be forgotten that **previously published** N<sub>2</sub> **fixation rates need to be corrected according to the improved** <sup>15</sup>N<sub>2</sub> **method** (Mohr et al., 2012). As Großkopf et al. (2012) pointed out, this might be a troublesome -if not impossible- venture. Notwithstanding, the idea of **modeling the correction of the rates** according to the various factors involved (i.e. incubation time, volume of <sup>15</sup>N<sub>2</sub> injected, sample temperature, etc) should not be abandoned. Alternatively, the scientific community should re-sample the oceans applying the new method. The improved <sup>15</sup>N<sub>2</sub> method itself still needs to be further developed and standardized to facilitate its application in different laboratories around the world. In that sense, the N<sub>2</sub> fixation research community recently requested the establishment of a SCOR working group (<u>www.scor-int.org/2012GM/N2%20fixation.pdf</u>), whereby intercalibration exercises will be performed, and a detailed protocol of the method will be redacted for publication.

Based on the activity of autotrophic diazotrophs, most of the previous research on marine diazotrophy focused on the upper layer of the ocean (generally <200 m). However, given the recently extended spatial limits of oceanic N<sub>2</sub> fixation, oceanographers should start sampling deeper layers to reassess the **global diversity and distribution of diazotrophs and N<sub>2</sub> fixation rates**, studying the vertical and horizontal gradients of diazotrophic species and N<sub>2</sub> fixation rates up to the poles, and down to the seafloor. In special, there are large spatial gaps waiting to be more extensively sampled, like the southern Atlantic and the Indian Oceans. Finally, we expect that the application of the improved <sup>15</sup>N<sub>2</sub> method throughout the world's oceans, extending our latitudinal and depth limits, will increase global N<sub>2</sub> fixation rates enough to outweigh fixed nitrogen losses, eventually balancing the oceanic nitrogen cycle.

PART IV: Spanish summary Resumen en español

## Introducción

#### La fijación de N<sub>2</sub> oceánica y sus protagonistas

El océano es el mayor ecosistema de la Tierra. Cubre el 71% de la superficie del planeta y proporciona aproximadamente la mitad de su producción primaria (Barange et al., 2010). A pesar de la gran productividad biológica de los océanos en términos globales, existen amplias regiones donde ésta es muy baja: son los "desiertos oceánicos", localizados en los giros subtropicales de los océanos (Fig. 1). En estas zonas, la columna de agua se mantiene estratificada durante todo el año debido al intenso calentamiento solar. Esta estratificación impide el afloramiento de aguas profundas ricas en nutrientes y por tanto restringe la producción primaria en la parte superior de la columna de agua, donde hay la luz necesaria para que pueda darse la fotosíntesis.



Fig. 1. Distribución global de Clorofila *a* (Chl *a*). Las zonas pintadas de azul oscuro son las menos productivas biológicamente. Imagen extraída de la página web de la NOAA (National oceanic and atmospheric administration (www.noaanews.noaa.gov/stories2008/20080305\_oceandesert.html).

En el océano abierto, la producción biológica se ve fuertemente limitada por la disponibilidad de nitrógeno (Falkowski, 1997). En estas zonas, las principales fuentes de nitrógeno son el afloramiento y la difusión de aguas profundas ricas en nitrato (NO<sub>3</sub>·), la deposición atmosférica de compuestos nitrogenados y la fijación biológica de nitrógeno atmosférico (N<sub>2</sub>) (Gruber, 2008). A pesar de que el N<sub>2</sub> es el principal componente de nuestra atmósfera (78%), este gas está prácticamente indisponible para los productores primarios. Ello se debe a que para romper el triple enlace que une ambos átomos de la molécula N<sub>2</sub>, se requiere una gran cantidad de energía. Solo un grupo restringido de organismos son capaces de llevar a cabo la fijación biológica de N<sub>2</sub>: los *diazótrofos* (del griego *dis* -bis-, *azōos* -inerte- y *trophikos* - nutrición-). Estos organismos albergan el complejo de la enzima nitrogenasa, que se compone de dos proteínas: la dinitrogenasa (una proteína de hierro y molibdeno codificada por el gen *nifDK*), y la dinitrogenasa-reductasa (una proteína de hierro codificada por el gen *nifH*) (Postage, 1982). Existen también algunas nitrogenasas alternativas en las que el molibdeno se sustituye por vanadio o hierro. El complejo de la enzima nitrogenasa permite a los diazótrofos reducir N<sub>2</sub> a amoniaco (NH<sub>3</sub>) según la siguiente estequiometría:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$
 (1)

Como se comentó anteriormente, la fijación de N<sub>2</sub> requiere una gran cantidad de energía (16 ATP). Este proceso enriquece la cadena trófica a través de la liberación de amonio (NH<sub>4</sub><sup>+</sup>) y de aminoácidos que suponen una fuente de nitrógeno para el fitoplancton autotrófico que no puede fijar N<sub>2</sub> (Karl et al., 2002).

Estudiar el ciclo del nitrógeno es de gran importancia. Los microorganismos controlan este ciclo a través de una serie de procesos de ganancias y pérdidas, sustentando la cadena trófica marina (Fig. 2). El nitrógeno inorgánico se fija (o se "gana") a través de la fijación de N<sub>2</sub> y la asimilación de NO<sub>3</sub>-, nitrito (NO<sub>2</sub>-) y NH<sub>4</sub>+, y se remineraliza (o se "pierde") a través de la amonificación, desnitrificación y oxidación anaeróbica del amonio (anammox).

En los últimos años el estudio de la fijación de  $N_2$  ha cobrado importancia debido a su gran contribución a la producción primaria en zonas oceánicas (Capone et al., 2005), pero también debido al desequilibrio en el ciclo del nitrógeno oceánico que encontramos en la actualidad (Tabla 1). Se considera que la fijación de  $N_2$  *versus* la desnitrificación y la anammox, son los procesos que mantienen el reservorio de nitrógeno oceánico (Codispoti, 2007). Hasta la fecha, las estimas de pérdida de nitrógeno a través de la desnitrificación y la anammox en zonas subóxicas, excede ampliamente las estimas de ganancia de nitrógeno (la fijación de  $N_2$ ) en aguas superficiales (Tabla 1). La metodología utilizada para medir fijación de  $N_2$  se ha sometido recientemente a una revisión (Mohr et al., 2010), y en la actualidad se estima que su aplicación pueda aumentar las tasas globales suficientemente como para equilibrar las pérdidas mencionadas anteriormente (Großkopf et al., 2012)..



Fig. 2. El ciclo marino del nitrógeno. Modificado de http://bit.ly/wC3xZo

Recientemente se ha descubierto que los organismos diazótrofos son más diversos (Fig. 3) y están más ampliamente distribuidos de lo que se constataba (Zehr et al., 2001; 2008; Moisander et al., 2010; Riemann et al., 2010; Farnelid et al., 2011). Las **cianobacterias filamentosas heterocísticas** como *Anabaena, Aphanizomenon y Nodularia* abundan frecuentemente en estuarios y en mares semicerrados como el Mar Báltico, siendo menos frecuentes zonas de mar abierto (Zehr, 2011). Las **cianobacterias filamentosas no heterocísticas** como *Katagnymene y Trichodesmium* se encuentran en zonas oceánicas oligotróficas (e.g. Langlois et al., 2005), siendo sin duda más abundante *Trichodesmium*. Esta cianobacteria se ubica en las zonas tropicales y subtropicales de los océanos, donde suele presentarse en forma de floraciones masivas. Debido a su alta abundancia, durante muchos años se creyó que era el principal fijador de N<sub>2</sub> del océano. Su actividad diazotrófica se estudió por primera vez al comienzo de los años 60 (Dugdale et al. 1961) y desde entonces esta organismo se ha estudiado ampliamente.
Gracias a estos estudios, actualmente tenemos un conocimiento bastante profundo de sus controles nutricionales (e.g. Berman-Frank et al., 2001a; Dyhrman et al., 2002), térmicos (e.g. Breitbarth et al., 2007) y físicos (Davis and McGillicuddy, 2006), así como de su distribución global (Luo et al., 2012), y de su comportamiento potencial ante el cambio climático (e.g. Hutchins et al., 2007). Una revisión reciente cubre todos estos aspectos (Bergman et al., 2012).

Proceso	Codispoti et al. (2001)	Galloway et al. (2004)	Gruber (2004)
Ganancias			
Fijación de N2 oceánica	117	106	120
Fijación de N2 bentónica	15	15	15
Aporte fluvial	76	48	80
Deposición atmosférica	86	33	50
Total	294	202	265
Pérdidas			
Exportación de nitrógeno orgánico	1		1
Desnitrificación bentónica	300	206	180
Desnitrificación pelágica	150	116	65
Secuestro en sedimentos	25	16	25
Liberación de N2O a la atmósfera	6	4	4
Total	482	342	275
Ganancias-pérdidas	-188	-140	-10

Tabla 1. Estimas de pérdidas y ganancias globales de nitrógeno. Tabla modificada de Gruber (2008). Todas las tasas están en Tg N y-1.

Otro de los grupos más estudiados son los diazótrofos simbiontes, como las cianobacterias *Richelia y Calothrix*, que suelen encontrarse en simbiosis con las diatomeas *Rhizosolenia, Hemiaulus* o *Chaetoceros* (e.g. Foster et al., 2011). Estas simbiosis pueden contribuir sustancialmente a la bomba biológica del carbono. Por ejemplo, se ha observado que las asociaciones diatomeacianobacteria son la causa principal de los picos de exportación de materia particulada observados durante los meses de verano en el océano Pacífico Norte (Karl et al., 2012). Existen también otras asociaciones menos estudiadas, como la de los organismos diazótrofos anaeróbicos con copépodos (Proctor, 1997).

En los últimos años, la aplicación de técnicas de biología molecular ha permitido descubrir una gran variedad de organismos diazótrofos a través de la detección del gen *nifH*. Zehr et al. (1998; 2001) encontraron por primera vez cianobacterias diazotróficas unicelulares (UCYN) en el océano Pacífico Norte. En los años siguientes, los grupos de UCYN A, B y C se detectaron en el

Atlántico Norte (Langlois et al., 2005; 2008) y el Pacífico (Needoba et al., 2007), observándose que en ocasiones sus tasas de fijación pueden igualar o superar las de Trichodesmium (Falcón et al., 2004; Montoya et al., 2004; Benavides et al., en prensa). El descubrimiento del grupo UCYN-A fue especialmente extraordinario. Este pequeño organismo (<1 μm) todavía no se ha conseguido mantener en cultivo, y según las últimas estimas es la cianobacteria diazótrofa más abundante del océano a nivel global (Luo et al., 2012). En comparación con Trichodesmium y otros diazótrofos que precisan de aguas cálidas para crecer (generalmente >20°C), las UCYN-A se han detectado a latitudes y profundidades mayores (Moisander et al., 2010), e incluso en sistemas de afloramiento costero, donde las temperaturas generalmente son <17ºC y las concentraciones de nitrógeno son altas (Sohm et al., 2011b; N.S. Agawin, datos no publicados). Una peculiaridad importante de las UCYN-A es su metabolismo fotoheterótrofo. Estos organismos carecen de los genes necesarios para desarrollar el fotosistema II, que produce oxígeno y permite la fijación de carbono (Zehr et al., 2008). Por tanto, se cree que las UCYN-A viven en simbiosis o se nutren de compuestos orgánicos producidos por otros organismos (Tripp et al., 2010).



Fig. 3. Diversidad filogenética del gen *nifH* de organismos diazótrofos planctónicos. Extraído de Riemann et al. (2010).

En efecto, en un estudio muy reciente, Thompson et al. (2012) han encontrado miembros del grupo UCYN-A en simbiosis con primnesiofitas en la estación ALOHA, confirmando las sospechas anteriores. Estos autores proponen el nombre *Candidatus* Atelocyanobacterium thalassa para las UCYN-A.

Además de las UCYN, existen otros diazótrofos que no pertenecen a las cianobacterias y se encuentran repartidos entre los cuatro grupos del gen nifH (Chien y Zinder, 1996). Éstos pueden ser  $\alpha$ -,  $\beta$ -,  $\gamma$ - y  $\delta$ -proteobacteria y, menos frecuentemente, arqueas y bacterias anaeróbicas (Riemann et al., 2010). Estudios recientes indican que en aguas superficiales el *nifH* no perteneciente a cianobacterias es más abundante que el *nifH* de las UCYN (Farnelid et al., 2011). También se ha visto que la fijación de N2 heterotrófica es predominante en el océano Pacífico Sur (Halm et al., 2011). En el Mar de los Sargazos, los genes de nifH no pertenecientes a cianobacterias predominan a partir de los 200 m de profundidad (Hewson et al., 2007). También se han medido tasas de fijación de N<sub>2</sub> de hasta 0.3 nmol L<sup>-1</sup> d<sup>-1</sup> asociadas a heterótrofos en zonas hipóxicas del sur de California (Hamersley et al., 2011). Todo esto sugiere que las estimas de fijación de N<sub>2</sub> basadas únicamente en aguas superficiales subestiman claramente la importancia de los fijadores de N2 no cianobacteriales (Riemann et al., 2010). Afortunadamente, la cantidad de estudios de abundancia y distribución de nifH no perteneciente a cianobacterias se encuentra en aumento, pero aun estamos lejos de conocer la contribución de estos organismos a la fijación de N<sub>2</sub> global.

### Factores reguladores y limitantes

Existen varios factores ambientales que controlan la fijación de  $N_2$  oceánica. La influencia de estos factores como inhibidor o favorecedor de la fijación de  $N_2$  varía en función del grupo de diazótrofos. En general, el factor limitante más obvio es el **oxígeno (producto de la fotosíntesis)**, que inhibe la actividad del complejo de la enzima nitrogenasa (Postgate, 1982). Los organismos diazótrofos han desarrollado variedad de estrategias para confrontar esta limitación. Por ejemplo, las cianobacterias filamentosas heterocísticas confinan esta actividad a los heterocistes, que tienen una pared celular más gruesa, inhibiendo la difusión de oxígeno a la célula. Sin embargo, la mayoría de los diazótrofos oceánicos carecen de heterocistes (Zehr, 2011), de modo que precisan de otras estrategias para poder fijar  $N_2$  en presencia de oxígeno. Las cianobacterias diazótrofas unicelulares como *Crocosphaera* (pertenecientes al grupo UCYN-B) o *Cyanothece* (grupo UCYN-C) confinan su actividad diazótrofa a la noche, consiguiendo así evitar el oxígeno que producen durante el día con la fotosíntesis. *Trichodesmium*, sin embargo, a

pesar de no tener heterocistes, fija CO2 y N2 simultáneamente durante el día (Capone et al., 1997; Berman-Frank et al., 2001b). Esta cianobacteria es capaz de fijar N<sub>2</sub> en presencia de oxígeno gracias a su estrategia combinada de segregación espacial y temporal. Entre las estrategias de segregación temporal que se han discutido en la literatura, se encuentran las modificaciones temporales de la nitrogenasa, su rápido ciclado (Capone et al., 1997), o la reducción del oxígeno celular a través del exceso de fijación de carbono, lo que explica los altos ratios carbono : nitrógeno (C:N) por los que se caracterizan generalmente los diazótrofos (Mulholland, 2007). Berman-Frank et al. (2001b) descubrieron la segregación temporal en Trichodesmium cuando constataron que tanto la fijación de N<sub>2</sub> como la fotosíntesis se llevaban a cabo durante el día, pero en franjas horarias distintas. La segregación espacial se consigue gracias a las distintas estructuras de los tricomas. Estos filamentos poseen zonas donde las células son claras y menos granuladas. Es en estas células denominadas diazocitos donde se encuentra la nitrogenasa (Bergman et al., 2012). Una vez que el N<sub>2</sub> se fija, se distribuye rápidamente a lo largo de todo el tricoma (Finzi-Hart et al., 2009).

Otro factor limitante que se ha estudiado ampliamente es la **temperatura**. La disolución del oxígeno es inversamente proporcional a la temperatura del agua, y es por ello que se estima que la temperatura restringe la distribución global de cianobacterias diazótrofas, siendo éstas más abundantes en aguas cálidas donde la disolución de oxígeno es menor y la respiración celular mayor, lo que reduce los niveles intracelulares de oxígeno y favorece la fijación de N<sub>2</sub> (Staal et al., 2003; Stal, 2009). Por ejemplo, la temperatura óptima para el crecimiento de *Trichodesmium* se estima en 27°C (Breitbarth et al., 2007), lo que confina su distribución a las zonas tropicales y subtropicales de los océanos. Sin embargo, aunque los diazótrofos son especialmente abundantes en las zonas tropicales y subtropicales, tanto las UCYN como los diazótrofos heterótrofos se pueden encontrar en latitudes y profundidades mayores. En concreto, estos organismos se han encontrado en sistemas de afloramiento costero (Sohm et al., 2011b), e incluso en el Ártico (Blais et al., 2012), lo que indica que su rango latitudinal es muy amplio.

La fijación de N<sub>2</sub> también puede verse limitada por la disponibilidad *in situ* de **nitrógeno inorgánico** (e.g. Krishnamurthy et al., 2007). Algunos experimentos demuestran que la expresión de los genes *nif* y la producción de diazocitos en *Trichodesmium* se ven inhibidas en presencia de NO<sub>3</sub><sup>-</sup> (Mulholland et al., 2001; Holl and Montoya, 2005). En el caso de las UCYN, Dekaezemacker and Bonnet (2011) estudiaron el efecto del NO<sub>3</sub><sup>-</sup> y el NH<sub>4</sub><sup>+</sup> en la actividad diazotrófica de dos cepas de *Crocosphaera*. Estos autores comprobaron que las tasas de fijación de N<sub>2</sub> disminuían al aumentar las

concentraciones de NH<sub>4</sub><sup>+</sup>, y sin embargo éstas no se veían afectadas al añadir  $NO_3$ <sup>-</sup>. Esto podría explicar porque las UCYN se encuentran generalmente a profundidades mayores que *Trichodesmium*, e incluso en sistemas de afloramiento (Sohm et al., 2011b).



Fig. 4. Distribución global de tasas medias de deposición de polvo atmosférico (g m $^2$  y $^1$ ). Extraído de Jickells et al. (2005).

Las UCYN-A dependen de la materia orgánica disuelta (MOD) como fuente de nutrientes (Tripp et al., 2010), y probablemente es por ello que su actividad nitrogenasa no se ve afectada por las concentraciones de nitrógeno inorgánico *in situ*. Este tipo de metabolismo permite a estos organismos vivir en zonas donde las concentraciones de nitrógeno inorgánico son altas, como en el afloramiento costero de Cabo Ghir (~30-31ºN) (N.S.R. Agawin, datos no publicados), donde las concentraciones de NO<sub>3</sub><sup>-</sup> son generalmente <2  $\mu$ M (Benavides et al., 2011).

La disponibilidad de otros nutrientes como el **hierro** y el **fósforo** influyen sobre la fijación de N<sub>2</sub>. Los diazótrofos tienen cuotas celulares de hierro muy altas (e.g. Berman-Frank et al., 2001a), y el hierro es el principal cofactor de la nitrogenasa reductasa, lo que hace que la fijación de N<sub>2</sub> dependa directamente de la disponibilidad de hierro. El hierro llega a los océanos principalmente a través de la deposición de polvo atmosférico, aunque el flujo difusivo vertical desde aguas profundas hacia aguas superficiales puede ser más importante en algunas ocasiones (Rijkenberg et al., 2012). Dada su proximidad al desierto del Sáhara, el Atlántico Este recibe los mayores aportes de polvo del desierto del mundo (Prospero, 1981; Fig. 4). La distribución de la deposición del polvo sahariano sobre el Atlántico Norte (Fig. 4) se corresponde en gran medida con las zonas donde las tasas de fijación de  $N_2$  son mayores (Fig. 5), lo que sugiere que existe una conexión entre la disponibilidad de hierro y la fijación de  $N_2$ . En efecto, se ha comprobado experimentalmente que la actividad diazótrofa se ve favorecida cuando se añade polvo a muestras de agua de mar (Mills et al., 2004), y las tasas de fijación de  $N_2$  se correlacionan con las concentraciones *in situ* de hierro disuelto en el Atlántico Norte (e.g. Mills et al., 2004; Moore et al., 2009; Fernández et al., 2010).



Geometric Mean N, Fixation Rates in 5 to 25 m (µmol N m<sup>-3</sup> d<sup>-1</sup>)



Geometric Mean N, Fixation Rates in 62.5 to 137.5 m (µmol N m-3 d-1)



60° M

Ge

30

0

30°

Geor



crecimiento, los diazótrofos pueden crecer basándose en el N2 como única fuente de nitrógeno. En teoría, esto plantea un escenario distinto para estos organismos (menos competitivo), ya que en principio crecerían mejor en aguas cálidas, estratificadas y pobres en nutrientes, que en aguas frías y nutritivas. A pesar de que el papel del forzamiento físico sobre la fijación de N<sub>2</sub> no se ha estudiado en detalle, los estudios disponibles indican que las estructuras mesoescalares y los frentes de densidad asociados tienen una influencia sobre las tasas de fijación de  $N_2$  y la diversidad de los organismos diazótrofos (Sohm et al., 2011c). Holl et al. (2007) compararon tasas de fijación de N<sub>2</sub> en el centro de remolinos anticiclónicos y ciclónicos de la costa Oeste de Australia, siendo éstas mayores en los ciclónicos (que son giros cálidos en el hemisferio Sur). Fong et al. (2008) midieron fijación de N<sub>2</sub> y abundancia de diazótrofos en un remolino anticiclónico en del Pacífico Norte, cercano a la estación ALOHA (22°45' N, 158°W), encontrando que las tasas aumentaban hacia el perímetro del remolino, así como una gran abundancia de cianobacterias filamentosas y unicelulares a lo largo del mismo. Church et al. (2009) recopilaron datos de anomalía de la superficie del mar (SSHA, en sus siglas en inglés) tomados en la estación ALOHA entre 2004 y 2007, y detectaron que los picos de fijación de N<sub>2</sub> coincidían con períodos de SSHA positiva. Las estructuras mesoescalares y sus frentes de densidad asociados también pueden afectar a la distribución espacial del plancton. Por ejemplo, Davis y McGillicuddy (2006) encontraron altas abundancias de Trichodesmium asociadas con remolinos anticiclónicos en un transecto transatlántico. Asimismo, Benavides et al. (2011) midieron picos de fijación de N<sub>2</sub> y altas abundancias de Trichodesmium asociados al límite exterior de un filamento de afloramiento en el Nordeste Atlántico. Trichodesmium abunda en aguas cálidas y estratificadas, donde suele acumularse en la superficie gracias a sus vacuolas de gas que le confieren flotabilidad positiva (Villareal and Carpenter, 2003), y es por ello que su proliferación se ve inhibida en aguas turbulentas (Carpenter and Price, 1976). Todos estos estudios indican que el forzamiento físico ejerce cierto control sobre la actividad diazótrofa y la distribución de los organismos diazótrofos.

#### Medir fijación de N<sub>2</sub>

Existen dos grupos principales de métodos para medir fijación de  $N_2$ : los "geoquímicos" y los "biológicos" (o directos).

Los **métodos geoquímicos** se basan en el estudio de la distribución vertical y horizontal de nitrógeno particulado y disuelto con características químicas que indiquen la presencia de actividad diazotrófica. Existen dos métodos geoquímicos principales: el parámetro N\* y el parámetro  $\delta^{15}$ N.

El parámetro N\* se basa en las concentraciones relativas de  $NO_3$ - y  $PO_4^{3-}$ en agua de mar. Redfield et al. (1963) establecieron que el carbono, el nitrógeno y el fósforo se asimilan y se remineralizan en una proporción de 106:16:1 (C:N:P). La fijación de  $N_2$  y la desnitrificación son procesos que no implican pérdidas o ganancias paralelas de fósforo, y es por ello que la fijación de  $N_2$  ocasiona un incremento en el ratio N:P, mientras que la desnitrificación provoca su disminución, i.e.: la producción de nitrógeno en exceso de la estequiometría de Redfield (N:P>16) es indicativa de la actividad diazotrófica, mientras que un consumo de nitrógeno en déficit de la estequiometría de Redfield (N:P<16) es indicativa de desnitrificación. Estos procesos se pueden estimar usando el parámetro N\*, que mide la concentración de  $NO_3$ - en exceso o defecto de lo esperado a través de la remineralización de  $PO_4^{3-}$  en proporciones de la estequiometría de Redfield, siguiendo la siguiente fórmula:

$$N^* = [NO_3^{-}] - 16[PO_4^{3-}]$$
 (2)

donde [NO<sub>3</sub><sup>-</sup>] y [PO<sub>4</sub><sup>3-</sup>] son las concentraciones de NO<sub>3</sub><sup>-</sup> y PO<sub>4</sub><sup>3-</sup>, respectivamente. El parámetro N\* lo introdujeron Michaels et al. (1996). La ecuación se mejoró añadiendo constantes que llevan el ratio global N:P a 16 y la intersección a cero (Michaels et al., 1996; Gruber and Sarmiento, 1997). Los mapas de N\* disponibles en la actualidad se han basado en bases de datos de proyectos como WOCE y GEOSECS.

El segundo método geoquímico más utilizado es el  $\delta^{15}N$ . Este parámetro mide la abundancia relativa de los isótopos  $^{14}N$  y  $^{15}N$  en una muestra determinada con respecto al N<sub>2</sub> atmosférico estándar (Ecuación 3):

$$\delta^{15}N = \left[ ({}^{15}N/{}^{14}N)_{muestra}/({}^{15}N/{}^{14}N)_{estándar} - 1 \right] \times 1000$$
(3)

El N<sub>2</sub> atmosférico tiene un  $\delta^{15}N \sim 0.6\%$ , mientras que el del NO<sub>3</sub>- es de  $\sim 5\%_0$  (Karl et al., 2002). El  $\delta^{15}N$  de un determinado reservorio de nitrógeno fijado viene dado por la composición isotópica de su fuente y el fraccionamiento isotópico experimentado durante su fijación. Por tanto, el nitrógeno orgánico producido a través de la fijación de N<sub>2</sub> atmosférico tiene un  $\delta^{15}N$  bajo, mientras que el nitrógeno orgánico producido gracias a la asimilación de NO<sub>3</sub>- tendrá valores de  $\delta^{15}N$  más elevados.

En general, los métodos geoquímicos presentan el inconveniente de

poder confundir la fijación de N<sub>2</sub> con otros procesos. Por ejemplo, el nitrógeno procedente de la quema de combustibles fósiles y de los fertilizantes usados en agricultura que se emiten a la atmósfera y se deposita en los océanos tiene un ratio N:P alto, al igual que el material procedente de la fijación de N<sub>2</sub> (Zamora et al., 2010). Esto también sucede en el caso del  $\delta^{15}$ N, ya que este nitrógeno atmosférico antropogénico tiene  $\delta^{15}$ N bajos (Hastings et al., 2003). Otros enmascaramiento de la actividad diazótrofa que conllevan los métodos geoquímicos es el fraccionamiento isotópico que tiene lugar durante el ciclado del nitrógeno (Altabet, 1988). A pesar de todos estos inconvenientes, este grupo de métodos tienen la ventaja de poder usarse en amplias áreas oceánicas amplias y a resoluciones espaciales mayores que lo que permiten los más laboriosos métodos biológicos.

Los **métodos biológicos** miden la cantidad de N<sub>2</sub> fijado *in situ* por los organismos diazótrofos durante un período determinado de incubación. En campañas oceanográficas, las incubaciones generalmente se hacen en cubierta, usando incubadores conectados al continuo del barco y con luz atenuada. Alternativamente, las incubaciones se pueden realizar *in situ* en boyas a la deriva o fondeos. Existen dos métodos biológicos principales: el ensayo de reducción de acetileno (ARA, en sus siglas en inglés) y la asimilación del isótopo estable <sup>15</sup>N<sub>2</sub>.

El ARA mide fijación de  $N_2$  de forma indirecta, dado que la enzima nitrogenasa es capaz de reducir acetileno ( $C_2H_2$ ), una molécula con un triple enlace, estructuralmente comparable al  $N_2$ . La reducción de acetileno a etileno ( $C_2H_4$ ) se puede comparar directamente con la reducción de  $N_2$  a  $NH_4^+$ . Para convertir el etileno producido a  $N_2$  fijado hay que aplicar un factor de conversión. Los factores teóricos que se emplean en la literatura son 3:1 o 4:1 ( $C_2H_4$ : $N_2$ ). Sin embargo, los factores de conversión empíricos suelen ser bastante más altos (Mulholland et al., 2004, 2006; Benavides et al., 2011). El uso de un factor u otro depende de considerar el reciclado de hidrógeno o no. El hidrógeno es un producto de desecho de la fijación de  $N_2$  (véase Ecuación 1). Para reducir acetileno a etileno se usan dos electrones, mientras que para reducir  $N_2$  a 2 $NH_3$  se usan ocho, lo que se traduce en un factor de conversión de 4:1 (Ecuación 5). El hidrógeno producido se puede reciclar a través de una enzima asimiladora de hidrógeno, de modo que se usan dos electrones menos y por tanto el factor de conversión sería 3:1 (Ecuación 6):

$$C_2H_2 + 2e^- + 2H^+ \rightarrow C_2H_4 \tag{4}$$

 $N_2 + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2$  (5)

$$N_2 + 6e^- + 6H^+ \rightarrow 2NH_3 \tag{6}$$

El hidrógeno se recicla únicamente cuando la disponibilidad de equivalentes reductores es limitada. Esta baja disponibilidad implica una reducción en la eficiencia de la célula al eliminar el oxígeno intracelular, lo que implicaría que las condiciones necesarias para la actividad diazotrófica no se cumplirían. Por tanto, esta no es la situación habitual de las cianobacterias, que consiguen fijar N<sub>2</sub> en presencia de oxígeno. Por tanto, se considera que el factor de conversión 4:1 es más adecuado (Jensen y Cox, 1983; Stal, 1988).

El método ARA se ha utilizado de forma exitosa en una gran variedad de ambientes marinos y en cultivos de organismos diazótrofos. Sin embargo, el método presenta una serie de problemas metodológicos que merece la pena considerar:

El uso de  $N_2$  por la enzima nitrogenasa se inhibe en presencia de concentraciones saturantes de acetileno. Por tanto, cabe esperar que el metabolismo del nitrógeno de la célula se vea alterado cuando se aplica el ARA (Capone, 1993). Este problema se puede atajar usando tiempos de incubación cortos (Stal, 1988). Otro problema habitual es la baja disolución de acetileno y etileno en muestras acuosas. Esto se soluciona generalmente dejando que la proporción aire/muestra líquida en el tubo de ensayo estanco sea alta. Un problema adicional es que la difusión de acetileno hacia la célula pueda verse reducida en muestras líquidas (como en cultivos de microalgas o en muestras planctónicas), debido a los gradientes de oxígeno y  $CO_2$ . Es por ello que en experimentos de ARA con muestras planctónicas se suele filtrar la muestra sobre un filtro de fibra de vidrio, que se mantiene humidificado durante el período de incubación, mientras las células están expuestas al acetileno. Esto proporciona un contacto directo entre los organismos y la fase gaseosa (Staal et al., 2001).

El otro método biológico principal es el trazador isotópico  ${}^{15}N_2$ . Este método se introdujo ya en los años 40 (Burris y Miller, 1941), aunque no fue hasta años más tarde que los espectrómetros de masas de relaciones isotópicas (IRMS, en sus siglas en inglés) fueron suficientemente sensibles como para medir las tasas de fijación tan bajas que se dan en aguas oceánicas, y el  ${}^{15}N_2$  disponible comercialmente fue lo suficientemente puro para este tipo de análisis. El protocolo establecido por Montoya et al. (1996) se ha usado ampliamente durante la última década, proporcionando una gran cantidad de datos de fijación de N<sub>2</sub>, siendo éstos especialmente numerosos en el Atlántico y el Pacífico Norte (Luo et al., 2012). Brevemente, el método consiste en inyectar un volumen determinado de  ${}^{15}N_2$  a una muestra de agua que se incuba durante un tiempo determinado (en cubierta o *in situ*), y finalmente se filtra sobre un filtro de fibra de vidrio. Más tarde, los filtros se analizan por IRMS y se calcula

la cantidad de  $^{15}\rm{N}_2$  que se ha transferido desde la fase acuosa hacia el material particulado (i.e. hacia las células).

Recientemente, se ha señalado que este método subestima las tasas de fijación de N<sub>2</sub> de forma variable. Una de las principales asunciones del método del <sup>15</sup>N<sub>2</sub> es que el enriquecimiento en <sup>15</sup>N del medio acuoso permanece constante durante el período de incubación (Fry, 2006). Sin embargo, esta condición básica no se cumple cuando el <sup>15</sup>N<sub>2</sub> se añade en forma de gas ("método de la burbuja"). Mohr et al. (2010) comprobaron que el enriquecimiento isotópico del medio acuoso aumenta a medida que la burbuja de <sup>15</sup>N<sub>2</sub> se disuelve (Fig. 6). Cuando se aplica el método de la burbuja, para los cálculos se considera que el <sup>15</sup>N<sub>2</sub> añadido se disuelve completamente, y para ello se usan las ecuaciones de disolución de gases de Weiss (1970). Sin embargo, si la burbuja de <sup>15</sup>N<sub>2</sub> no se disuelve completamente, el enriquecimiento isotópico real es menor de lo esperado teóricamente, lo que produce un error en el cálculo de las tasas de fijación de N<sub>2</sub> según la siguiente ecuación:

Fijación N<sub>2</sub> = [(at%XS PON)/(at%XS fuente x tiempo incubación)] x [PON] (7)

siendo at%XS el porcentaje de <sup>15</sup>N en exceso de la abundancia natural (siendo éste ~0.36558%) en el nitrógeno orgánico particulado (PON, en sus siglas en inglés) o en la fuente de N<sub>2</sub>, y [PON] la concentración de nitrógeno orgánico particulado.

Mohr et al. (2010) utilizaron cultivos de la cianobacteria diazotrófica unicelular *Crocosphaera* para comparar las tasas de fijación que se obtenían con ambos métodos ( $^{15}N_2$  en burbuja y en forma disuelta). Estos autores vieron que cuando utilizaban el método de la burbuja, las tasas de fijación de N<sub>2</sub> eran un 60% inferiores a las obtenidas usando el método disuelto. Este gran descubrimiento puso de manifiesto la urgencia de aplicar este nuevo método en medidas de campo y de tratar de recalcular las tasas que se habían publicado hasta ahora utilizando el método de la burbuja. Hasta la fecha existen apenas unas pocas comparaciones de los dos métodos. Wilson et al. (2012) compararon ambos métodos en la estación ALOHA y observaron que las tasas medidas con <sup>15</sup>N<sub>2</sub> disuelto eran de 2 a 6 veces mayores que las tasas medidas con burbuja de <sup>15</sup>N<sub>2</sub>. Großkopf et al. (2012).



Fig. 6. Círculos blancos: porcentaje de  ${}^{15}N_2$  disuelto real en función del  ${}^{15}N_2$  disuelto teórico (calculado con las ecuaciones de Weiss, 1970) cuando se añade como burbuja (círculos blancos), o en forma disuelta (círculos grises), a lo largo de 24 h de incubación. Los círculos grises muestran que el enriquecimiento isotópico es constante cuando el  ${}^{15}N_2$  se añade de forma disuelta. Figura adaptada de Mohr et al. (2010).

Las diferencias entre los resultados obtenidos con ambos métodos pueden deberse a una serie de factores: (1) la temperatura del agua –dado que cuanto mayor sea la temperatura, menor es la disolución del gas en el líquido-, (2) la agitación de las botellas de incubación, (3) el volumen de las botellas de incubación, (4) el volumen de  ${}^{15}N_2$  añadido a las muestras, (5) la duración de la incubación, (6) la hora a la que se inicia la incubación con respecto a la hora en la que comienza la actividad nitrogenasa -que es distinta según el organismo diazótrofo-, y (7) el DOM que recubre la burbuja de  ${}^{15}N_2$  (Mohr et al., 2010). Además de estos factores, Großkopf et al. (2012), encontraron que la subestima de las tasas de un método respecto al otro es menor cuando la comunidad diazotrófica está dominada por Trichodesmium (subestima del 62%), y mayor cuando la comunidad está dominada por otros diazótrofos (simbiontes, cianobacterias unicelulares, diazótrofos heterótrofos) (hasta el 570% de subestima). Todos estos factores varían en gran medida entre los trabajos de fijación de N<sub>2</sub> publicados anteriormente, lo que implica que el recálculo de las tasas publicadas sea difícil, acaso imposible (Großkopf et al., 2012).

Introducción

Otro factor que puede subestimar las tasas de fijación de N<sub>2</sub> en gran medida y que, sin embargo ha recibido mucha menos atención, es la exudación de nitrógeno fijado recientemente. Glibert y Bronk (1994) midieron la exudación de nitrógeno orgánico disuelto (DON, en sus siglas en inglés) en poblaciones naturales de Trichodesmium. Estas autoras encontraron que el 50% del N<sub>2</sub> fijado se exudaba en forma de DON. Esta "pérdida" de N<sub>2</sub> fijado es considerable v sigue sin tenerse en cuenta debido a que, generalmente, solo se recupera el material particulado para su análisis en el IRMS, ignorando cuánto N<sub>2</sub> se ha fijado y ha ido a parar a la fase disuelta, en vez de a la particulada. Esto supone una subestima de las tasas de fijación de N<sub>2</sub> reales (Bronk et al., 1994; Glibert y Bronk, 1994). La estima de este flujo seguramente sea en cierto modo poco popular entre la comunidad científica debido a las dificultades que presenta. Gallon et al. (2002) y Mulholland et al. (2004) propusieron un método simple: medir fijación de N<sub>2</sub> mediante el método ARA y el del  $^{15}N_2$  al mismo tiempo. Como el ARA se considera una estima de la fijación "bruta" (N<sub>2</sub> fijado total), y el <sup>15</sup>N<sub>2</sub> una estima de la fijación "neta" (N<sub>2</sub> fijado e incorporado como biomasa), la diferencia entre ambos debe de darnos una estima del N2 fijado y posteriormente exudado. Una alternativa a este método es medir el enriquecimiento isotópico del DON extracelular. Slawyk y Raimbault (1995) propusieron un método consistente en extraer el <sup>15</sup>N del nitrógeno inorgánico y orgánico de la muestra en varios pasos bajo altas condiciones de pH y temperatura. Estas condiciones pueden ser adversas para la estabilidad de la muestra, conllevando la rotura de las moléculas de DON en moléculas más pequeñas y por tanto subestimar las tasas de exudación de DON (McCarthy and Bronk, 2008). Bronk y Glibert (1991) adaptaron otro método más exitoso usando columnas de intercambio iónico. Sin embargo, la marca comercial que fabricaba dichas columnas ha disminuido su calidad, de modo que este método ha dejado de utilizarse. Los intentos de manufacturar las columnas en el laboratorio tampoco han sido exitosos (D. Bronk, comunicación personal), y por tanto aun aguardamos un método para medir exudación de DON fiable. Sin embargo, los datos indican que este flujo podría ser importante y que no debería de obviarse a la hora de medir fijación de N<sub>2</sub>.

Como hemos visto anteriormente, los métodos geoquímicos y biológicos son muy distintos. Cabe esperar por tanto que las tasas obtenidas por ambos sean también muy distintas. En el Atlántico Norte, las tasas de fijación de  $N_2$ obtenidas por métodos geoquímicos oscilan entre 0.84 y ~90 Tg N y<sup>-1</sup> (véase la Tabla 1 en Mahaffey et al., 2005), mientras que la estima más reciente de fijación de  $N_2$  a nivel de todo el Atlántico Norte se ha establecido en 12.7 Tg N y<sup>-1</sup> (véase Tabla S3 en la información suplementaria de Großkopf et al., 2012). En un futuro próximo las tasas estimadas por ambos métodos podrían asemejarse, a medida que el método del <sup>15</sup>N<sub>2</sub> disuelto se vaya aplicando y la tasa media vaya aumentando (Mohr et al., 2010; Großkopf et al., 2012).

#### La fijación de N<sub>2</sub> y el cambio climático

Para finales de este siglo, se espera que las presiones parciales de  $CO_2$  (*p*CO2) actuales se multipliquen por dos (IPCC, 2007). Las consecuencias principales de este aumento para los organismos planctónicos son: (1) la acidificación del agua de mar, (2) el aumento de la temperatura del agua de mar, (3) la estratificación de la columna de agua, (4) la expansión de las zonas mínimas de oxígeno (OMZ, en sus siglas en inglés), (4) la desertificación y el consecuente aumento de deposición de polvo sobre los océanos (Boyd and Doney, 2002; Jickells et al., 2005; Stramma et al., 2008).

Mientras que la acidificación (el aumento de  $CO_2$  disuelto en agua de mar) puede provocar un aumento en la actividad fotosintética en determinadas especies, o ser perjudicial para otras (como los cocolitofóridos con sus exoesqueletos calcáreos; Doney et al., 2009), el calentamiento y la estratificación del océano sin embargo podrían propiciar la proliferación de diazótrofos como *Trichodesmium*. Algunos experimentos demuestran que tanto la tanto longitud de los tricomas como la fijación de N<sub>2</sub> en *Trichodesmium* aumenta ante niveles crecientes de  $CO_2$  (e.g. Levitan et al., 2007).



Fig. 7. El ciclo de retroalimentación de la fijación de  $N_2$  basado en el clima. Extraído de Michaels et al. (2001).

Además, la fijación de  $N_2$  por heterótrofos en zonas hipóxicas (Hamersley et al., 2011), sugiere que la expansión de las OMZ (Stramma et al., 2008) pueda serles favorables a estos organismos, aumentando las tasas de fijación de  $N_2$  a nivel global.

*Trichodesmium* y otros diazótrofos también podrían verse beneficiados por el aumento en la disponibilidad de hierro a través de la deposición de polvo del desierto. Este concepto se explica en el trabajo de Michaels et al. (2001), donde se propone un ciclo de retroalimentación de la fijación de N<sub>2</sub> basado en el clima (Fig. 7). En este esquema, la desertificación propicia un aumento global de las tasas de fijación de N<sub>2</sub> gracias al aporte de hierro. Ello provoca una disminución de los niveles atmosféricos de CO<sub>2</sub>, lo que a su vez provoca una disminución de la temperatura global. El enfriamiento global, en cambio, ocasionaría una disminución del aporte de polvo a los océanos, lo que disminuiría la fijación de N<sub>2</sub> y por tanto provocaría un aumento del CO<sub>2</sub> atmosférico, volviendo a aumentar la temperatura global. En conclusión, las respuestas de los diazótrofos frente a los niveles atmosféricos de CO<sub>2</sub> pueden jugar un papel importante en el ciclo del carbono y del nitrógeno, y consecuentemente en la productividad de los océanos en el futuro.

### Objetivos y planteamiento de la investigación

La mayoría de los estudios de fijación de  $N_2$  oceánica de que disponemos en la actualidad se han realizado en el Atlántico Norte, siendo éstos especialmente numerosos en la cuenca Oeste.

Dado que los estudios en la cuenca Este son mucho más escasos, el propósito principal de esta tesis es ahondar en el estudio de la fijación de  $N_2$  en el Atlántico Nordeste subtropical, con objeto de conocer su magnitud y los factores que la controlan en estas aguas, así como estudiar la diversidad de los organismos diazótrofos que habitan en esta zona. Por tanto, esta tesis pretende dar respuesta a las siguientes preguntas:

1. ¿Cuál es la magnitud y la distribución espacial de la fijación de  $N_2$  y de la exudación de nitrógeno disuelto en el Atlántico Nordeste subtropical? ¿Cuál es la importancia relativa de las distintas fracciones de tamaño? **Capítulo 1.** 

Para responder a estas preguntas medimos la fijación de N<sub>2</sub> bruta y neta en organismos >10  $\mu$ m y <10  $\mu$ m en dos zonas de afloramiento y una de mar abierto sobre la Corriente de Canarias (desde ~42°N hasta ~28°N). El fraccionamiento de las muestras nos permitió estimar la importancia relativa de los diazótrofos filamentosos y simbiontes (>10  $\mu$ m), frente a los unicelulares (<10  $\mu$ m) La exudación de nitrógeno disuelto se estimó como la diferencia entre la fijación de N<sub>2</sub> bruta y la neta.

### 2. ¿Cuánto nitrógeno orgánico disuelto (DON) exudan los diazótrofos unicelulares?: un estudio experimental con cultivos. **Capítulo 2.**

Para responder a esta pregunta medimos la exudación de DON en cultivos axénicos de la cianobacteria diazótrofa unicelular *Cyanothece* sp. Miami BG 043511. Para ello medimos la transferencia de al DON extracelular mediante experimentos con isótopos estables.

3. ¿Las poblaciones de diazótrofos unicelulares oceánicos también exudan DON? ¿Cómo se compara con la exudación de DON en otros diazótrofos? ¿Existen tendencias geográficas? ¿Qué las controla? **Capítulo 3.**  Para responder a esta pregunta medimos la exudación de DON en organismos >10  $\mu$ m y <10  $\mu$ m en muestras naturales recogidas a lo largo del paralelo 24.5ºN en el Atlántico Norte subtropical. Para averiguar qué factores pueden afectar a la actividad diazotrófica, comparamos las tasas fraccionadas de fijación de N<sub>2</sub> y exudación de DON con factores como la temperatura, la salinidad, la concentración de nutrientes y el polvo atmosférico.

## 4. ¿Cómo afecta la deposición de polvo sahariano a las tasas de fijación de $N_2$ y a la diversidad de organismos diazótrofos en el Atlántico Nordeste subtropical? **Capítulo 4.**

Para responder a esta pregunta medimos tasas de fijación de  $N_2$  y la abundancia de *Trichodesmium* y diazótrofos unicelulares, conjuntamente con tasas de deposición y de concentración de hierro del polvo atmosférico en las Islas Canarias.

### 5. ¿Cuál es la diversidad molecular de los diazótrofos en el Nordeste Atlántico subtropical? **Capítulo 5.**

Para responder a esta pregunta analizamos la diversidad del gen *nifH* (que codifica la enzima nitrogenasa, responsable de la fijación de  $N_2$ ), en un muestreo sobre la Corriente de Canarias.

## 6. ¿Cuál es la contribución de la fijación de $N_2$ a la producción nueva y a la anomalía de nitrógeno en el Atlántico Nordeste subtropical? **Capítulo 6.**

Para responder a esta pregunta medimos la asimilación de  $NO_3$ - y  $NH_4$ + sobre la Corriente de Canarias y lo comparamos con tasas de fijación de  $N_2$  obtenidas en la misma zona. También comparamos la fijación de  $N_2$  con la anomalía de nitrógeno observada en estudios anteriores realizados en esta zona.

### Metodología

Para lograr los objetivos planteados en el apartado anterior utilizamos una amplia variedad de técnicas biogeoquímicas y de biología molecular. A continuación describimos en términos generales las técnicas utilizadas:

### Fijación y regeneración de $N_2$ , $NO_3^-$ y $NH_4^+$ , y exudación de DON asociada

las tasas de **fijación de N**<sub>2</sub> **bruta** se midieron mediante el método ARA. El contenido en etileno y acetileno de las muestras se midió con cromatógrafos de gases (HP 5890 o CP9001 Chrompack), equipados con un detector ionizador de flama (FID, en sus siglas en inglés). El acetileno reducido se convirtió a tasas de fijación de N<sub>2</sub> aplicando un factor de conversión de 4:1 y las ecuaciones de Stal (1988).

La **fijación de N<sub>2</sub> neta** se midió inyectando concentraciones traza de  ${}^{15}N_2$  en forma gaseosa a las muestras, tal y como se indica en Montoya et al. (1996). En el Capítulo 3, el  ${}^{15}N_2$  se añadió de forma disuelta, como se indica en Mohr et al. (2010).

La asimilación y regeneración de NO<sub>3</sub><sup>-</sup> y NH<sub>4</sub><sup>+</sup> y la exudación de DON asociada se midieron añadiendo sustratos marcados con <sup>15</sup>N ( $^{15}NO_3$ <sup>-</sup> y  $^{15}NH_4$ <sup>+</sup>) a muestras de agua de mar. La asimilación de NO<sub>3</sub><sup>-</sup> y NH<sub>4</sub><sup>+</sup> se calculó según las ecuaciones de Dugdale and Wilkerson (1986). La regeneración de NO<sub>3</sub><sup>-</sup> y NH<sub>4</sub><sup>+</sup> y la exudación de DON resultante de la asimilación de estos sustratos se midió usando el protocolo de Slawyk y Raimbault (1995). La tasas de regeneración de NO<sub>3</sub><sup>-</sup> y NH<sub>4</sub><sup>+</sup> se calcularon aplicando las ecuaciones de Fernández y Raimbault (2007). La regeneración de NH<sub>4</sub><sup>+</sup> se calculó según Glibert et al. (1982). La exudación de DON asociada a la asimilación de NO<sub>3</sub><sup>-</sup> y NH<sub>4</sub><sup>+</sup> se calculó con las ecuaciones de Slawyk et al. (1998).

El **enriquecimiento isotópico (**<sup>15</sup>N **atom%)** de las muestras se analizó con un analizador elemental Thermo Flash EA 1112 conectado a un IRMS Thermo Delta Advantage, con un analizador elemental EA1108 Carlo Erba conectado a un IRMS MAT253 Thermo Finnigan IRMS, o con un IRMS Europa GEO 20/20 IRMS equipado con un analizador automatizado de carbono y nitrógeno para sólidos y líquidos (ANCA-SL).

En el Capítulo 3, el <sup>15</sup>N atom% del nitrógeno disuelto total (TDN, en sus

siglas en inglés) se analizó mediante la oxidación del TDN a  $NO_3^{-}$  a través del método de oxidación por persulfato de Valderrama (Valderrama, 1981). El  $NO_3^{-}$  obtenido se convirtió entonces a óxido nitroso ( $N_2O$ ) mediante la actividad de bacterias carentes de actividad  $N_2O$ -reductasa (Sigman et al., 2001). Las razones isotópicas del  $N_2O$  se midieron con un concentrador de gases traza Thermo Finnigan Gas Bench + PreCon conectado a un IRMS Thermo Scientific Delta V en el Centro de Isótopos Estables de la Universidad de California Davis (Davis, California, EE UU). El mismo procedimiento sin el paso de la oxidación por persulfato nos sirvió para analizar el <sup>15</sup>N atom % del  $NO_3^{-}$ .

En el Capítulo 2, la asimilación de carbono y la exudación de carbono orgánico disuelto (DOC, en sus siglas en inglés) se midió mediante la adición de sustratos radiactivos (NaH<sup>14</sup>CO<sub>3</sub>). Las radioactividad se contó usando el cocktail de contaje Insta-Gel (Perkin Elmer), y el contador de centelleo Packard Tricarb 2300. En el Capítulo 5, se midió asimilación de carbono mediante adición de sustratos marcados con el isótopo estable <sup>13</sup>C (Hama et al., 1983).

#### Nitrógeno y carbono, orgánico e inorgánico, disuelto y particulado

las concentraciones de NO<sub>3</sub><sup>-</sup>, nitrito (NO<sub>2</sub><sup>-</sup>), (PO<sub>4</sub><sup>3-</sup>) y silicato (SiO<sub>2</sub>) se midieron usando autoanalizadores (AA3 Bran Luebbe, Prescop Alpkem, SEAL Technicon II y Lachat QuikChem 8500).

Las concentraciones de  $\mathbf{NH}_4^+$  se midieron por espectrofluorimetría aplicando el método de Holmes et al. (1999), por espectrofotometría con el método del fenol-hipoclorito de Hansen y Koroleff, (1983), con un espectrofotómetro Shimadzu UV-1601, o mediante un autoanalizador SEAL Technicon II.

Las concentraciones de **TDN** se analizaron con un TOC Shimadzu V-CSH equipado con un módulo TNM, o en midiendo las concentraciones de nitrógeno inorgánico tras la oxidación de persulfato (Valderrama, 1981).

Las concentraciones de **DON** se calcularon restándole las concentraciones de  $NO_3^-$ ,  $NO_2^-$  y  $NH_4^+$  a las de TDN.

El **DOC** se midió restando a las concentraciones de TOC (carbono orgánico total, en sus siglas en inglés) las de POC, o en muestras filtradas usando un TOC Shimadzu V-CSH TOC o un TOC Skalar Formacs de flujo continuo.

El **carbono y nitrógeno orgánico particulado (POC y PON**, respectivamente, en sus siglas en inglés) se analizaron con un analizador

elemental CHN Perkin Elmer 2400 CHN siguiendo los métodos de los protocolos de JGOFS (UNESCO, 1994), o conjuntamente con el <sup>15</sup>N o el <sup>13</sup>C atom % utilizando los IRMS descritos anteriormente (acoplados a equipos de análisis elemental).

### *Concentraciones de Clorofila* a, *y abundancias de* Trichodesmium *y UCYN*

Las concentraciones de **Clorofila** *a* se midieron filtrando muestras de agua de mar en filtros de fibra de vidrio y extrayendo la clorofila en acetona (90%) durante 24 h. En el Capítulo 2, las muestras de clorofila se analizaron mediante un espectrofotómetro Cary Eclipse y las concentraciones se calcularon mediante las ecuaciones de Ritchie (2006). En el resto de capítulos, las muestras de clorofila se midieron en un fluorómetro Turner AU-10 siguiendo las recomendaciones de Holm-Hansen (1965).

Las muestras de **Trichodesmium** se recogieron arrastrando una red de 50  $\mu$ m de luz de malla desde el máximo profundo de clorofila (DCM, en sus siglás en inglés) hasta la superficie. Las muestras se preservaron con un 10% de formaldehído tamponado con bórax, obteniendo una concentración final de formaldehído en la muestra del 4%. Los tricomas y colonias se contaron en un microscopio invertido Zeiss.

Para determinar la **abundancia de UCYN** se usaron técnicas de biología molecular: detección del gen *nifH* mediante hibridación *in situ* fluorescente con señal amplificada por tiramida (TSA-FISH, en sus siglas en inglés) usando la sonda Nitro821 (Biegala y Raimbault, 2008), y por PCR-anidada del gen *nifH* (Langlois et al., 2005).

### **Resultados**

*Capítulo 1:* Fijación de  $N_2$  por Trichodesmium y pequeños diazótrofos en el Atlántico Nordeste subtropical

Benavides M, Agawin NSR, Arístegui J, Ferriol P, Stal LJ (2011). *Aquatic Microbial Ecology* 65:43-53

En este estudio medimos fijación de N<sub>2</sub> en muestras fraccionadas (>50  $\mu$ m, >10  $\mu$ m y <10  $\mu$ m) en una zona de mar abierto y en dos zonas de afloramiento costero de la Corriente de Canarias: Cabo Silleiro (Noroeste de la península Ibérica) y Cabo Ghir (Noroeste de África). Los objetivos de este estudio fueron: (1) obtener las primeras tasas de fijación de N<sub>2</sub> en zonas de afloramiento del Nordeste Atlántico, y (2) comparar la contribución relativa de *Trichodesmium* con los diazótrofos más pequeños (<10  $\mu$ m). Para ello combinamos los dos métodos más comunes para medir fijación de N<sub>2</sub> bruta (el método ARA; Stal, 1988) y neta (el método del <sup>15</sup>N<sub>2</sub>; Montoya et al., 1996). La diferencia entre las tasas obtenidas por ambos métodos sirve para estimar la exudación de DON (Gallon et al., 2002; Mulholland et al., 2004).

La abundancia de *Trichodesmium* fue baja en toda la zona de estudio (<0.5 tricomas L<sup>-1</sup>), apareciendo mayormente en forma de tricomas libres. Encontramos algunos picos de abundancia coincidiendo con zonas de frente, como en el de las Azores (situado durante esta campaña aproximadamente a 37º 30' N), y en el extremo exterior del filamento de Cabo Ghir.

Las tasas de fijación de N<sub>2</sub> bruta asociadas a *Trichodesmium* (método ARA aplicado a las muestras recogidas con una red de 50  $\mu$ m) fueron bajas en toda la zona de estudio (entre 0.01 y 0.1  $\mu$ mol N m<sup>-2</sup> d<sup>-1</sup>). Las tasas fueron más altas al norte del frente de las Azores, disminuyendo después hasta las proximidades de la costa africana.

El efecto del frente de las Azores también pudo notarse en las tasas de fijación de N<sub>2</sub> de las muestras >10 µm y <10 µm. Las tasas medias de fijación de N<sub>2</sub> bruta total (>10 µm + <10 µm) antes y después de este frente fueron de 0.07  $\pm$  0.02 nmol N L<sup>-1</sup> d<sup>-1</sup> y 0.11  $\pm$  0.05 nmol N L<sup>-1</sup> d<sup>-1</sup>, siendo éstas estadísticamente distintas (*t* de Student, p = 0.04, n = 8). Estas tasas también se correlacionaron significativamente con la temperatura superficial del mar (SST, en sus siglas en inglés) (r<sup>2</sup> = 0.56, p < 0.01).

La fijación de  $N_2$  bruta en la fracción >10  $\mu m$  fue similar en todas las

zonas muestreadas. Sin embargo, la de la fracción <10  $\mu$ m fue mayor en las zonas de afloramiento (Cabo Silleiro y Cabo Ghir) que en la zona de mar abierto. De forma similar, la fracción <10  $\mu$ m presentó tasas de fijación de N<sub>2</sub> neta mayores en las zonas de afloramiento que en la zona de mar abierto.

A continuación, se muestra la contribución de cada una de las fracciones a la fijación de  $N_2$  total (bruta + neta) en cada una de las zonas muestreadas. La zona de mar abierto se halla dividida en dos por el frente de las Azores (Tabla 1).

	%Exudación de DON (respecto a la fijación de N₂ total)			%Contribución a la fijación de N2 bruta total (>10+<10)			%Contribución a la fijación de N₂ neta total (>10+<10)	
	Total	>10 µm	<10 µm	>10 µm	<10 µm	>50 µm	>10 µm	<10 µm
Al Norte del frente	41.1	99.7	24.2	40.25	59.8	0.2	5.7	94.3
Al Sur del frente	76.4	28.5	58.12	42.28	57.8	0.4	35.7	64.3
Cabo Silleiro	44.1	42.7	64.07	29.65	70.4	0.5	26.3	73.7
Cabo Ghir	54.1	14.9	89.73	7.62	92.4	0.2	12.8	87.2

Tabla 1. Porcentaje medio de exudación de DON respecto a la fijación de N<sub>2</sub> total, porcentaje de contribución a la fijación bruta y a la fijación neta total, por cada fracción.

Como vemos, la fracción <10 µm (en la que se encuentran los fijadores de N<sub>2</sub> unicelulares) es la que más aporta a la fijación de N<sub>2</sub> total. Esto destaca el papel de las UCYN en esta zona del Atlántico, y amplía de forma considerable las zonas de actividad diazotrófica, que generalmente se han restringido a las bandas tropicales de los océanos.

### *Capítulo 2: Exudación de nitrógeno y carbono orgánico disuelto en una cianobacteria marina diazótrofa unicelular*

Benavides M, Agawin NSR, Arístegui J, Peene J, Stal LJ. *Aquatic Microbial Ecology*. En revisión.

Las tasas de fijación de N<sub>2</sub> pueden verse subestimadas en gran medida si no se incluye el N<sub>2</sub> recientemente fijado y exudado en forma de DON. La exudación de DON en poblaciones naturales de *Trichodesmium* es del 50% (Glibert y Bronk, 1994), pero hasta la fecha se desconoce si los fijadores de N<sub>2</sub> unicelulares también exudan DON (Mulholland, 2007). En este trabajo empleamos cultivos de una cianobacteria unicelular *Cyanothece* sp. Miami BG 043511, (de ahora en adelante *Cyanothece*) como modelo para medir la exudación de DON en diazótrofos unicelulares.

En un experimento de 10 días de duración (experimento #1), usando el isótopo estable  ${}^{15}N_2$  medimos la fijación de N<sub>2</sub> neta, la exudación de DON (DONr, en sus siglas en inglés) y la producción de DON intracelular (inDONp, en sus siglas en inglés). Para estimar la fijación de N<sub>2</sub> neta se considera el N<sub>2</sub> que pasa del medio de cultivo al material celular (a la biomasa). Para el DONr se considera cuánto del N<sub>2</sub> del medio de cultivo se fija y se encuentra en forma de DON marcado con  ${}^{15}N$  de nuevo en el medio de cultivo, tras un período de incubación. Para la inDONp se considera el N<sub>2</sub> que pasa del medio al DON al interior de la célula, tras un período de incubación.

En un segundo experimento de 8 días de duración (experimento #2), medimos los mismos flujos pero con  $^{14}$ C, para estudiar el metabolismo del carbono en *Cyanothece*.

Los resultados del experimento #1 mostraron que la fijación neta disminuye a lo largo del experimento, mientras que la DONr y la inDONp se mantienen más o menos constantes en los días centrales del experimento, siendo menores en los primeros y últimos días del mismo. La DONr contribuyó únicamente ~1% a la fijación de N<sub>2</sub> total (siendo la fijación de N<sub>2</sub> total = fijación neta + DONr + inDONp), véase la Tabla 1.

En el experimento #2, la fijación de carbono neta también aumentó a lo largo del mismo. La exudación de DOC (DOCr) siguió la misma tendencia, a pesar del pico observado en el día 1. La producción de DOC intracelular (inDOCp) aumentó entre los días 1 y 4, manteniendo unos niveles más o menos constantes durante el resto del experimento. En promedio, el DOCr representó un  $\sim 2\%$  del carbono fijado total. Véase la Tabla 2.

Día	Fijación N₂ neta (A)	DONr (B)	inDONp (C)	Fijación № total (A+B+C)	% DONr
1	3.66 (0.27)	0.00 (0.00)	0.06 (0.04)	3.72	0.08
2	7.36 (3.06)	0.07 (0.10)	0.15 (0.10)	7.59	0.98
3	4.85 (0.52)	0.04 (0.02)	0.10 (0.02)	4.99	0.72
4	4.28 (0.11)	0.03 (0.03)	0.20 (0.03)	4.51	0.69
5	3.44 (1.18)	0.03 (0.01)	0.18 (0.01)	3.65	0.76
6	3.47 (0.38)	0.03 (0.01)	0.06 (0.01)	3.56	0.95
7	2.76 (1.01)	0.04 (0.01)	0.13 (0.01)	2.93	1.35
8	3.08 (0.19)	0.06 (0.02)	0.01 (0.02)	3.15	1.78
9	2.90 (0.08)	0.03 (0.01)	0.00 (0.01)	2.94	1.09
10	1.83 (0.21)	0.02 (0.01)	0.00 (0.01)	1.85	1.01

Tabla 1: Tasas medias de fijación de  $N_2$  neta (A), DONr (B) e inDONp (C), todas en µmol N L<sup>-1</sup> h<sup>-1</sup>. Las desviaciones estándar se dan entre paréntesis.

**Tabla 2:** Tasas medias de fijación de carbono (C) neta (A), DOCr (B) e inDOCp (C), todas en µmol C  $L^{\cdot 1} h^{\cdot 1}$ . Las desviaciones estándar se dan entre paréntesis.

	OCr (B) inDOCp (C) Fijación de C % DOCr total (A+B+C) % DOCr	r (B)	Fijación de C (A) DO	Día
$1 \qquad 37.47 (4.1) \qquad 2.41 (0.12) \qquad 14.55 (3.6) \qquad 54.43 (5.8) \qquad 4.4$	41 (0.12) 14.55 (3.6) 54.43 (5.8) 4.43	(0.12)	37.47 (4.1) 2.4	1
2 61.38 (7.7) 0.70 (0.4) 37.44 (7.6) 99.52 (12.9) 0.7	.70 (0.4) 37.44 (7.6) 99.52 (12.9) 0.70	(0.4)	61.38 (7.7) 0.2	2
3 89.31 (7) 0.96 (0.3) 54.80 (6.32) 145.07 (11.8) 0.6	.96 (0.3) 54.80 (6.32) 145.07 (11.8) 0.66	(0.3)	89.31 (7) 0.9	3
4 82.62 (10.3) 1.88 (0.2) 52.50 (1.2) 137 (16.9) 1.3	.88 (0.2) 52.50 (1.2) 137 (16.9) 1.37	(0.2)	82.62 (10.3) 1.8	4
5 97.6 (4.5) 3.11 (0.8) 46.10 (2) 146.81 (7.8) 2.1	.11 (0.8) 46.10 (2) 146.81 (7.8) 2.11	(0.8)	97.6 (4.5) 3.2	5
6 84.7 (1.9) 2.54 (0.2) 46.04 (2.8) 133.28 (3.4) 1.9	.54 (0.2) 46.04 (2.8) 133.28 (3.4) 1.91	(0.2)	84.7 (1.9) 2.5	6
7 101 (2.7) 2.70 (0.1) 47.52 (12.2) 151.22 (4.1) 1.7	.70 (0.1) 47.52 (12.2) 151.22 (4.1) 1.79	(0.1)	101 (2.7) 2.7	7
8 98.59 (9.01) 2.43 (0.2) 49.34 (14.5) 150.36 (13.8) 1.6	.43 (0.2) 49.34 (14.5) 150.36 (13.8) 1.62	(0.2)	98.59 (9.01) 2.4	8

Estudios anteriores demuestran que poblaciones naturales de *Trichodesmium* pueden exudar hasta el 50% del N<sub>2</sub> fijado en forma de DON (Glibert y Bronk, 1994), mientras que *Trichodesmium en* cultivo apenas exuda aproximadamente el 8% (Mulholland et al., 2004). En el estudio que se presenta en el capítulo siguiente (Capítulo 3) veremos que la fracción <10  $\mu$ m en el Atlántico Norte exuda alrededor del 20% del N<sub>2</sub> fijado en forma de DON, mientras que las tasas de exudación de DON obtenidas con los cultivos de *Cyanothece* en el presente capítulo son apenas del ~1%. Parece por tanto que

las diferencias entre el medio y los cultivos podrían ser similares en ambos grupos de diazótrofos.

**Capítulo 3:** Variabilidad longitudinal de tasas fraccionadas de fijación de  $N_2$  y exudación de DON en el Atlántico Norte subtropical a lo largo del paralelo 24.5<sup>o</sup>N

Benavides M, Bronk DA, Agawin NSR, Pérez-Hernández MD, Hernández-Guerra A, Arístegui J. Enviado a *Journal of Geophysical Research*.

Para saber si las cianobacterias unicelulares en el medio marino también exudan DON, medimos fijación neta de N<sub>2</sub> y la DONr en organismos >10  $\mu$ m y <10  $\mu$ m en el Atlántico subtropical a lo largo del paralelo 24.5°N. Encontramos que la fracción <10  $\mu$ m exuda ~23% del N<sub>2</sub> fijado, mientras que la fracción >10  $\mu$ m exuda aproximadamente el 14%.

Las tasas de fijación neta oscilaron entre 0.01 y 0.4 nmol N L<sup>-1</sup> h<sup>-1</sup>, y las de DONr entre 0.001 y 0.09 nmol N L<sup>-1</sup> h<sup>-1</sup>. La fijación neta disminuyó de Este a Oeste (Fig. 1) y se correlacionó significativamente con AOD 550 nm (Fig. 2), indicando que la actividad diazotrófica aumenta como respuesta a los aportes de polvo atmosférico. Las tasas de DONr sin embargo no siguieron una tendencia longitudinal clara, ni se correlacionaron significativamente con la AOD 550 nm.



Fig. 1: Tasas de fijación en la fracción >10  $\mu m$  sumadas a las de <10  $\mu m$  a lo largo del paralelo 24.5°N.

#### Resultados



Fig. 2. Valores de AOD 550 nm en la zona muestreada entre el 28 de Enero y el 9 de Marzo de 2011.

En este estudio utilizamos una técnica novedosa para medir fijación de N<sub>2</sub>, consistente en usar <sup>15</sup>N<sub>2</sub> en forma disuelta (Mohr et al., 2010), en vez de en forma gaseosa como se había hecho hasta la fecha (Montoya et al., 1996). Los resultados obtenidos demuestran que el método antiguo (<sup>15</sup>N<sub>2</sub> en burbuja) subestima considerablemente las tasas de fijación de N<sub>2</sub>. En concreto, las tasas obtenidas usando el nuevo método (<sup>15</sup>N<sub>2</sub> disuelto) fueron un ~50% (49 ± 39%) superiores a las obtenidas utilizando el método antiguo.

Los resultados de este estudio sugieren que mediante la combinación del método del  ${}^{15}N_2$  disuelto y las medidas de DONr, las tasas de fijación de  $N_2$  globales podrían aumentar suficientemente como para equiparar las de desnitrificación, que en la actualidad se estima que superan a las de fijación por  $\sim$ 200 Tg N y-1 (Mahaffey et al., 2005; Codispoti, 2007).

# **Capítulo 4:** Aumento de las tasas de fijación de $N_2$ en diazótrofos unicelulares versus Trichodesmium tras un evento de deposición de polvo atmosférico en las Islas Canarias

Benavides M, Arístegui J, Agawin NSR, López Cancio J, Hernández-León S. Aceptado para su publicación en *Limnology & Oceanography*.

En este capítulo estudiamos la variabilidad a corto plazo de la fijación de N<sub>2</sub> y la diversidad de diazótrofos en las Islas Canarias ante un evento de deposición de polvo sahariano. Este polvo es rico en hierro, elemento que limita la fijación de N<sub>2</sub> (Mills et al., 2004).



Fig. 1: Ejemplos de diazótrofos unicelulares asociados a partículas. Las células se hibridaron con la sonda Nitro821, teñidas con fluorescencia verde (FITC) mediante la técnica TSA-FISH (ver la sección "Metodología").

Encontramos que las tasas de fijación en unicelulares aumentan un  $\sim$ 90% tras un evento de deposición de polvo sahariano, mientras que las tasas de *Trichodesmium* disminuyen entre el 34 y el 92%. Curiosamente, tras la deposición de polvo, los diazótrofos unicelulares aparecen asociados a partículas de materia orgánica (Fig. 1).

Dado que la materia orgánica propicia la biodisponibilidad del hierro contenido en el polvo (Barbeau, 2006), planteamos la hipótesis de que ésta sea una estrategia de los diazótrofos unicelulares para aprovechar el hierro más eficientemente. Sin embargo, tras el evento de deposición, la abundancia de *Trichodesmium* disminuye. *Trichodesmium* solo puede aprovechar las partículas de polvo cuando ésta se halla en forma de colonia (Rubin et al., 2011). Sin embargo, en estas aguas Trichodesmium aparece predominantemente como tricomas sueltos y no como colonias (Benavides et al., 2011), y por ello no puede beneficiarse del hierro contenido en las partículas de polvo.

# **Capítulo 5:** Comunidad cianobacteriana fijadora de $N_2$ en el Atlántico Nordeste: predominancia de cianobacterias unicelulares (UCYN-A)

Agawin NSR, Benavides M, Busquets A, Ferriol P, Stal LJ, Arístegui J. Manuscrito en preparación.

En este capítulo estudiamos la diversidad de diazótrofos en zonas de afloramiento y mar abierto de la Corriente de Canarias, mediante técnicas de biología molecular (PCR-anidada y TSA-FISH). Encontramos que los diazótrofos <3  $\mu$ m predominan en la zona, siendo la mayoría pertenecientes al grupo UCYN-A. Este grupo además contribuyó un ~50% a la fijación de N<sub>2</sub> total.

Se encontraron correlaciones positivas de la abundancia y la actividad diazotrófica de las UCYN-A con la temperatura, y correlaciones inversas con los niveles de oxígeno disuelto.

Las UCYN-A abundan en las zonas de afloramiento, lo que sugiere que su actividad no se ve tan afectada por la disponibilidad de nitrógeno inorgánico in situ, a diferencia de otras especies de diazótrofos. Este hecho aumenta la distribución espacial de diazótrofos en el océano, lo que indica que las tasas de fijación de N<sub>2</sub> globales pueden ser mayores de lo que se estima actualmente.

### **Capítulo 6:** Contribución de la fijación de $N_2$ a la producción nueva y el exceso de nitrógeno en el Atlántico Nordeste subtropical

Benavides M, Arístegui J, Agawin NSR, Álvarez-Salgado XA, Álvarez M, Troupin C. Manuscrito en preparación.

En este estudio usamos sustratos marcados con <sup>15</sup>N para medir fijación de N<sub>2</sub>, asimilación de NO<sub>3</sub><sup>-</sup> y de NH<sub>4</sub><sup>+</sup>, su regeneración y DONr asociada, en una zona de afloramiento costero y otra de mar abierto sobre la Corriente de Canarias, durante el verano de 2009. La producción nueva (Pnew = asimilación de NO<sub>3</sub><sup>-</sup> + fijación de N<sub>2</sub> + exudación de DON procedente de la asimilación de NO<sub>3</sub><sup>-</sup> - regeneración de NO<sub>3</sub><sup>-</sup>) fue mayor en la zona de afloramiento que en la de mar abierto (0.126 y 0.014 µmol N L<sup>-1</sup> h<sup>-1</sup>, respectivamente), mientras que la producción regenerada (Preg = asimilación de NH<sub>4</sub><sup>+</sup> + exudación de DON procedente de la asimilación de NH<sub>4</sub><sup>+</sup>) fue similar en ambas zonas: 0.157 y 0.133 µmol N L<sup>-1</sup> h<sup>-1</sup>, respectivamente. El f-ratio resultante (Pnew/Pnew+Preg) fue de 0.08 y 0.48 respectivamente en la zona de mar abierto y en la zona de afloramiento costero.

Tabla 1: Tasas medias de fijación de N<sub>2</sub>, producción nueva (Pnew) y regenerada (Preg), y f-ratio en las zona muestreadas: zona afectada por el afloramiento costero y zona de mar abierto no afectada por el mismo. Las tasas están en in  $\mu$ mol L<sup>-1</sup> h<sup>-1</sup>.

	Fijación N <sub>2</sub>	Pnew	Preg	f-ratio
Mar abierto	1.3x10 <sup>-6</sup>	0.014	0.157	0.084
Afloramiento	4.6x10-6	0.126	0.133	0.487

El exceso de nitrógeno en el Atlántico Norte (respecto a lo esperado por el ratio de Redfield, C:N:P=106:16:1) se atribuye a la fijación de N<sub>2</sub> (Gruber y Sarmiento, 1997; Hansell et al., 2004), proceso que se estima sustenta el 50% de la producción nueva oceánica (Capone et al., 2005). En este trabajo también comparamos la Pnew con la fijación de N<sub>2</sub>. Encontramos que la fijación de N<sub>2</sub> aporta <1% a la producción nueva en la zona (Tabla 1), y que por tanto difícilmente puede explicar el exceso de nitrógeno observado en la misma. Aportando nuevos datos de materia orgánica disuelta (DOM, en sus siglas en inglés) y de intercambio de  $NO_3$ - a través del Estrecho de Gibraltar, recalculamos el exceso de nitrógeno en la zona y obtuvimos una nueva estima de exceso de nitrógeno de aproximadamente la mitad de lo que se había estimado anteriormente ( $22 \pm 19 \times 10^{10}$  mol N y<sup>-1</sup> frente a 40 x 10<sup>10</sup> mol N y<sup>-1</sup>). La tasa volumétrica media de fijación de N<sub>2</sub> fue de apenas 1.3 x 10<sup>-3</sup> nmol N L<sup>-1</sup> d<sup>-1</sup>, indicando que su influencia en la Pnew en esta zona es insignificante.

### Discusión general

### Magnitud y distribución de tasas de fijación de $N_2$ en el Atlántico Nordeste subtropical

Recientemente, Luo et al. (2012) recopilaron todas las tasas de fijación de  $N_2$  y abundancia de organismos diazótrofos disponibles en la literatura hasta la fecha, componiendo una base de datos que se puede consultar a través de internet (<u>http://doi.pangaea.de/10.1594/PANGAEA.774851</u>). Comparando las tasas de fijación de  $N_2$  por unidad de área entre las distintas cuencas oceánicas del mundo (Tabla 1), observamos que las más bajas se dan en el Atlántico Sur, mientras que las del Atlántico Norte son aproximadamente la mitad de lo que se ha medido en otras cuencas.

Tabla 1: Medias ponderadas globales de tasas anuales de fijación de  $N_2$ . Tabla modificada de la Tabla S3 en el material suplementario de Großkopf et al. (2012).

Cuenca	Límite Norte/Sur (ºN)	Tasa (µmol N m² d¹)	Tasa (mol N m <sup>-2</sup> y <sup>-1</sup> )	Área (10º km²)	Tasa por cuenca (Tg N y <sup>-1</sup> )
Atlántico N	50/-5	64	0.023	39.2	12.7
Atlántico S	-5/-30	19	0.07	15.9	1.5
Pacífico N	30/0	115	0.042	56.3	33.0
Pacífico S	0/-35	103	0.038	56.1	29.5
Índico N	25/0	115	0.042	15.8	9.3
Índico S	0/-35	103	0.038	31.8	16.7

A pesar de que la gran mayoría de las tasas de fijación de  $N_2$  de que se disponen a nivel global corresponden al Atlántico Norte (Luo et al., 2012), la mayor parte de estos estudios se han centrado en el Atlántico Noroeste tropical, donde las altas tasas de fijación de  $N_2$  se atribuyen principalmente a la cianobacteria colonial *Trichodesmium* (e.g. Carpenter and Romans 1991; Capone et al., 1997, 2005). En el Atlántico Nordeste, sin embargo, la cantidad de datos disponibles es mucho menor. Las tasas de fijación de  $N_2$  en esta zona provienen de un número limitado de estudios, concretamente los de Moore et al. (2009), Fernández et al. (2010), Turk et al. (2011) and Großkopf et al. (2012). Los datos incluidos en esta tesis aumentan considerablemente la base de datos de tasas de fijación de  $N_2$  en esta región (ver el **Anexo II**).

La Tabla 2 muestra tasas volumétricas medias de fijación de  $N_2$  en las bandas latitudinales tropical, subtropical y templada del Atlántico Norte,

basadas en los datos recopilados por Luo et al. (2012), añadiendo los datos aportados por esta tesis.

Tabla 2: Tasas medias de fijación de  $N_2$  en agua de mar no prefiltrada en el Atlántico Norte. La desviación estándar se da entre paréntesis.

Zonas del Atlántico Norte	Fijación de N2 media (nmol L·1 d·1 )
Tropical (0 - 23.5ºN)	3.59 (17.87)
Subtropical (23.5 - 40ºN)	1.60 (4.56)
*Templada (>40ºN)	2.28 (4.34)
Todo NE Atlántico	3.10 (15.16)
NE Atlántico subtropical	1.16 (1.93)
%Contribución del NE subtropical Atlántico a	72.45%
	0.1X

\*la tasa disponible a mayor latitud corresponde a ~56ºN.

Como se ha mencionado anteriormente, la tasas de fijación de  $N_2$  más altas del Atlántico Norte se encuentran en la banda tropical, siendo éstas más del doble de las medidas en la banda subtropical. Sorprendentemente, la tasa media de fijación de  $N_2$  en la banda templada es bastante alta (Tabla 2). Sin embargo, debe tenerse en cuenta que esta media está basada en un número muy limitado de datos (ver Figura 3b en Luo et al., 2012).

En general, estimamos que el Atlántico Nordeste subtropical aporta >70% del N<sub>2</sub> fijado en toda la banda subtropical del Atlántico Norte. Los aportes de polvo rico en hierro provenientes del Sáhara que recibe este área, lo explican parcialmente (Prospero, 1981). Las tasas de fijación de N2 en el Atlántico Nordeste se correlacionan con la concentración de hierro en disolución (Moore et al., 2009), y con la AOD 550 nm (Fernández et al., 2010; Capítulo 3). A pesar de ello, las bajas temperaturas y altas concentraciones de nitrógeno inorgánico disuelto (DIN, en sus siglas en inglés) asociadas al afloramiento costero del Noroeste de África pueden limitar la actividad diazotrófica (Gruber y Sarmiento, 1997). Estas condiciones parecen seleccionar a las UCYN sobre *Trichodesmium* en esta zona. En el **Capítulo 1** estimamos que la fracción  $<10 \ \mu m$  contribuye 60-90% de la fijación de N<sub>2</sub> bruta total. En el **Capítulo 3** observamos que, al Este de 45ºO sobre el paralelo 24.5ºN, esta fracción contribuye ~39% (±20%) a la fijación de N<sub>2</sub> total. En el Capítulo 4, observamos que las tasas asociadas a diazótrofos unicelulares son de 3 a >100 veces mayores que las asociadas a Trichodesmium. La predominancia de la fijación de N2 asociada a la fracción <10 µm observada en los estudios incluidos en esta tesis concuerda con otros estudios realizados en el Atlántico Norte a latitudes menores (Voss et al., 2004; Montoya et al., 2007; Goebel et al., 2010).

No obstante, podríamos estar subestimando la verdadera magnitud de las tasas de fijación de N<sub>2</sub> correspondientes a la fracción <10  $\mu$ m, dado que la mayoría de los datos disponibles en la actualidad corresponden a la capa superficial del océano (generalmente <200 m), donde proliferan preferentemente *Trichodesmium* y diazótrofos en simbiosis con diatomeas, mientras que las UCYN y especialmente los diazótrofos que no son cianobacterias (bacterias y arqueas) habitan a mayores profundidades (Hewson et al., 2007; Riemann et al., 2010; Hamersley et al., 2011). Estudios recientes sugieren que el *nifH* perteneciente a diazótrofos no-cianobacteriales es más abundante que el de las UCYN y *Trichodesmium* juntos a nivel global (Farnelid et al., 2011). Esto indica que la distribución global de la actividad diazotrófica debe ser reevaluada incluyendo estudios a mayores profundidades.

### Diversidad de organismos diazótrofos

En esta tesis hemos evaluado la diversidad y abundancia de organismos diazótrofos a través de contajes de *Trichodesmium* por microscopía y técnicas de biología molecular que nos han permitido identificar el gen *nifH* (TSA-FISH y PCR-anidada).

En el **Capítulo 1** estimamos la abundancia de *Trichodesmium* durante el verano de 2009 entre ~42ºN y ~28ºN, a lo largo del meridiano 20ºO, encontrando abundancias de hasta 0.43 tricomas L-1, mientras que en los afloramientos de Cabo Silleiro y Cabo Ghir la abundancia de Trichodesmium fue generalmente <0.1 tricomas L<sup>-1</sup>. Trichodesmium es un organismo que se ve seriamente afectado por la disponibilidad de nitrógeno inorgánico in situ (Mulholland et al., 2001; Holl y Montoya, 2005), y por tanto, es predecible que las altas concentraciones de DIN, frecuentes en estas zonas de afloramiento (Arístegui et al., 2006) impidan la proliferación de Trichodesmium. Durante nuestros muestreos en la zona en verano de 2009, las concentraciones de DIN variaron entre  $\sim 0.7$  y >2  $\mu$ M. En el **Capítulo 4** estimamos la abundancia de *Trichodesmium* en una estación  $\sim$ 10 millas náuticas al Norte de la isla de Gran Canaria, desde Febrero hasta Mayo de 2010. En este caso, observamos abundancias mayores que en el **Capítulo 1** (0.2 - 1.2 tricomas L<sup>-1</sup>), y concentraciones de DIN menores ( $\sim$ 0.5  $\mu$ M). La mayoría de los individuos de Trichodesmium observados en estos estudios aparecieron como tricomas sueltos, en vez de colonias, que es cómo se suelen observar en el Atlántico Noroeste tropical (Capone et al., 1997). Los pocos estudios en los que se incluyen contajes de *Trichodesmium* en el Atlántico Nordeste subtropical, también indican que predomina la presencia de *Trichodesmium* como tricomas sueltos (Fernández et al., 2010; González-Taboada et al., 2010).

*Trichodesmium* crece en aguas cálidas (>20°C) y estratificadas, donde habitualmente forma proliferaciones masivas ("blooms"), que se acumulan en superficie gracias a sus vesículas de gas (Villareal y Carpenter, 2003). Las bajas temperaturas y la turbulencia, inherentes a las aguas subtropicales del Nordeste Atlántico como la región de la Corriente de Canarias, suponen seguramente condiciones poco adecuadas para la proliferación de *Trichodesmium* (Carpenter y Price, 1976). No obstante, los blooms pueden darse en zonas determinadas como, por ejemplo, al Sur de las Islas Canarias. Estas zonas se encuentran a sotavento de los vientos alisios y por tanto son cálidas y estratificadas (Basterretxea et al., 2002). Aquí también los aportes de hierro son frecuentes gracias a la proximidad del desierto del Sáhara, de manera que se reúnen todas las condiciones necesarias para un bloom de *Trichodesmium*. Los blooms al Sur de las islas se han observado con frecuencia en los últimos años (e.g. Ramos et al., 2005), llamando la atención de la prensa local (Fig. 1).



Fig. 1. Concentraciones de *Trichodesmium* en la costa Sur de las Islas Canarias. Fotos publicadas en la prensa local, extraídas de <u>http://www.isladelanzarote.org/?p=19290</u> y <u>www.universocanario.com/siete-islas/microalgas-/menos-toxicas/trichodesmium/poco-agresivas/5061</u> el 29 de Octubre de 2012.

En la recopilación de datos de Luo et al. (2012) también se incluyen todos los datos de abundancia de UCYN disponibles en la literatura. Como se muestra en la Tabla 3, la mayor parte de las UCYN del Atlántico Norte pertenecen al grupo A, siendo éstas especialmente abundantes en la banda tropical. Sin embargo, en la banda subtropical son más abundantes las UCYN-B,
mientras que las UCYN-C se han detectado únicamente en la banda tropical. Miembros de las UCYN-B y UCYN-C están disponibles en cultivo (Webb et al., 2009; Taniuchi et al., 2012), pero las UCYN-A no se han conseguido aislar y cultivar aun (Zehr et al., 2008).

Tabla 3: Valores medios de número de copias del gen *nifH* en el Atlántico Norte. Los valores se dan en x10<sup>6</sup> *nifH* copias m<sup>-3</sup>. Datos extraídos de la base de datos online presentada en Luo et al. (2012).

	UCYN-A	UCYN-B	UCYN-C
Tropical (0 - 23.5ºN)	53747	9.87	6.83
Subtropical (23.5 - 40ºN)	963	19230	0.00
*Templada (>40ºN)	271	0.00	0.00

\*el valor de copias de *nifH* más al Norte disponible se ha medido a  $\sim$ 42ºN.

Las UCYN-A son cianobacterias de pequeño tamaño (<1  $\mu$ m) y metabolismo fotoheterotrófico. Carecen del fotosistema II y no son capaces de fijar carbono (Zehr et al., 2008). Al contrario que las UCYN-B y -C, este tipo de metabolismo les permite a las UCYN-A fijar N<sub>2</sub> durante el día (Church et al., 2005), mientras que su incapacidad para fijar carbono les hace depender de otros organismos para obtener compuestos esenciales (Tripp et al., 2010). A pesar de que aun no disponemos de información más concreta sobre su metabolismo y ecología, en la actualidad sabemos que las UCYN-A son las cianobacterias diazótrofas más abundantes del océano a nivel global (Luo et al., 2012).

En los **Capítulos 4 y 5** medimos la abundancia de UCYN a través de la técnica de TSA-FISH usando la sonda Nitro821. Esta técnica no proporciona información filogenética de las células diazotróficas, pero sin embargo proporciona otro tipo de información interesante, como es la forma de los organismos o sus asociaciones simbióticas. Aproximadamente el 20% de las células contadas en el **Capítulo 4** tenían un tamaño <1  $\mu$ m, y por tanto es muy probable que pertenecieran al grupo UCYN-A. En el **Capítulo 5** también estudiamos la diversidad del gen *nifH* mediante PCR-anidada en una zona de mar abierto y en dos zonas de afloramiento costero sobre la Corriente de Canarias. En 15 muestras representativas recogidas durante estas campañas (**Capítulo 5**), los análisis filogenéticos mostraron que el 95% de las secuencias eran similares al grupo UCYN-A en un 98-100% (ver Figura 2).

Un resultado interesante de los análisis de TSA-FISH realizados en los **Capítulos 4 y 5** es la relativa gran cantidad de UCYN que aparecieron

asociadas a partículas (Tabla 4). Este "modo de vida pegado a partículas" proporciona una serie de ventajas a los microorganismos: las bacterias colonizan partículas orgánicas que se hunden en la columna de agua (Azam y Long, 2001), propiciando altas tasas de respiración como consecuencia de la oxidación de la materia orgánica, propiciando las condiciones subóxicas necesarias para la fijación de N<sub>2</sub> (Riemann et al., 2010). Además, las moléculas orgánicas de la partícula pueden ayudar a ligar elementos traza como el hierro (**Capítulo 4**), que son limitantes de la fijación de N<sub>2</sub> (e.g. Kustka et al., 2002).

Tabla 4: Porcentaje de UCYN encontradas, asociadas a partículas, libres o en simbiosis con otras células mediante la técnica TSA-FISH, usando la sonda Nitro821, en distintas campañas oceanográficas realizadas durante esta tesis.

Campaña	Latitud/Longitud	Fecha	% asociado a partículas	% libres	% en simbiosis
CAIBOX	~42-28ºN/~9-20ºO	Jul-Ago 09	14.50	81.65	3.86
Ghir	~30-32ºN/~9-12ºO	Ago-Sep 09	7.63	90.10	2.27
Silleiro	~41-42ºN/~9ºO	Jul 09	13.92	65.71	20.38
Lucifer	28º21'N/15º23'0	Feb-May 10	24.71	73.97	1.36

En general, las asociaciones diazótrofo-partícula se han descrito para los diazótrofos no-cianobacteriales del Grupo III (véase la revisión de Riemann et al., 2010), aunque es muy probable que éstas también tengan lugar con algunas UCYN. Tripp et al. (2010) sugirieron que el metabolismo y el genoma reducido del grupo UCYN-A (1.44 Mb) indican que es probable que viva en simbiosis (Pérez-Brocal eta l., 2006), o que dependa de la materia orgánica producida por otros organismos. En el trabajo de Tripp et al. (2010), prácticamente la totalidad del nifH perteneciente al grupo UCYN-A se recogió de la fracción <1  $\mu$ m, lo que contradice la suposición de que estos organismos viven en simbiosis con organismos de mayor tamaño. En el sistema de afloramiento de Benguela, Sohm et al. (2011b) encontraron altas abundancias de UCYN-A en estaciones donde las tasas de fijación de N<sub>2</sub> <10  $\mu$ m representaban apenas el ~30% de la fijación total. En este estudio, el otro único diazótrofo detectable fue Crocosphaera, que apareció en abundancias mucho menores. La única explicación para esta incongruencia es que las UCYN-A también formaran parte de la fijación de N<sub>2</sub> >10  $\mu$ m (i.e. células de UCYN-A en simbiosis con células de mayor tamaño o asociadas a partículas no pasarían a través de un filtro de 10 µm de poro). En el Capítulo 4, la mayoría de las células asociadas a partículas tenían un tamaño entre 0.6 y ~1 μm (véase la Fig. 5 del **Capítulo 4**). De acuerdo con este rango, es probable que estas células pertenecieran al grupo UCYN-A. Además, también se han observado



Fig. 2. Árbol filogenético de secuencias del gen *nifH*. Las secuencias obtenidas en este estudio se referencian según (i) la campaña oceanográfica de CAIBEX, (ii) las estaciones en las que se tomaron las muestras, (iii) la profundidad en la columna de agua (A=superficie, B=límite de la capa de mezcla, C=máximo profundo de clorofila), y (iv) el número de clon. Los valores de bootstrap >50% se indican en los nodos. Barra de escala=0.05 sustituciones por nucleótidos.

diazótrofos picoplanctónicos en asociación con partículas en el Pacífico Suroeste (Biegala y Raimbault, 2008) y Ecuatorial (Bonnet et al., 2009), así como en el Mar Mediterráneo (Le Moal y Biegala, 2009; Le Moal et al., 2011). Finalmente, en un estudio muy reciente, Thompson et al. (2012) han encontrado miembros del grupo UCYN-A en simbiosis con primnesiofitas. Según estos autores, estas asociaciones no se habían observado anteriormente debido al fraccionamiento de las muestras y a su manipulación, que puede desprender a las UCYN-A de la superficie de sus hospedadores. Estos problemas de manipulación de muestras dificultan el estudio de este tipo de asociaciones de simbiosis, así como las asociaciones organismo-partícula (Simon et al., 2002).

Todas las evidencias recogidas en estos estudios sugieren que, al igual que ocurre con algunos diazótrofos heterótrofos, las UCYN-A pueden presentar un modo de vida asociado a partículas bajo determinadas circunstancias. En general, parece que estas asociaciones predominan en ambientes con altas concentraciones de DIN y materia orgánica particulada, como en los afloramientos costeros (Sohm et al., 2011b; **Capítulo 5**), y en otras zonas costeras eutróficas (Biegala y Raimbault, 2008). Las asociaciones cianobacteria-partícula también se han observado en otros grupos de UCYN. Por ejemplo, Sohm et al. (2011a) observaron que *Crocosphaera* produce polisacáridos extracelulares (EPS, en sus siglas en inglés) que se acumulan alrededor de estas UCYN, formando partículas.

En todos los estudios incluidos en esta tesis, otros grupos de diazótrofos como las cianobacterias filamentosas en simbiosis con diatomeas o copépodos fueron raramente observados. Generalmente, los diazótrofos en simbiosis representaron <5% de las células contadas en los análisis de TSA-FISH, mientras que bajo el microscopio óptico nunca se observaron simbiosis diatomea-cianobacteria.

#### Exudación de N<sub>2</sub> fijado recientemente

La mayoría de las tasas de fijción de N<sub>2</sub> disponibles en la literatura son tasas de fijación neta (incorporación de N<sub>2</sub> a biomasa). Para medir fijación de N<sub>2</sub>, generalmente se filtran las muestras de agua de mar a través de filtros de fibra de vidrio de 0.7  $\mu$ m de abertura de poro. El agua que ha atravesado el filtro raramente se recoge, de modo que el N<sub>2</sub> que se fija y se exuda extracelularmente no se contabiliza a la hora de calcular las tasas. Si este flujo es sustancial, las verdaderas tasas de fijación de N<sub>2</sub> pueden verse subestimadas en gran medida (Bronk et al., 1994). Discusión

Dado que en la actualidad existe un desequilibrio entre las tasas de "ganancia" y "pérdida" de nitrógeno (la desnitrificación y la anammox superan a la fijación de N<sub>2</sub> en  $\sim$ 200 Tg N y<sup>-1</sup>; Mahaffey et al., 2005; Codispoti, 2007), precisamos de medidas fiables de fijación de N<sub>2</sub> que nos avuden a equilibrar el ciclo oceánico del nitrógeno. Recientemente, Mohr et al. (2010) descubrieron que las tasas de fijación de N2 basadas en el método de la burbuja de <sup>15</sup>N2 publicados hasta la fecha, estaban subestimadas en gran medida (véase también Großkopf et al., 2012; Wilson et al., 2012; Capítulo 3). Es probable que la aplicación del método mejorado (<sup>15</sup>N<sub>2</sub> disuelto) pueda aumentar las tasas de fijación de N<sub>2</sub> suficientemente como para equilibrar las pérdidas de nitrógeno fijado por desnitrificación y anammox, pero hasta que no apliquemos el nuevo método a lo largo y ancho de nuestros océanos no podremos confirmarlo. Este desequilibrio podría verse aun más reducido si se midiera la exudación de N<sub>2</sub> fijado asiduamente, utilizando el método del <sup>15</sup>N<sub>2</sub> disuelto, evitando por tanto todos los factores que pueden subestimar las tasas reales.

Glibert y Bronk (1994) estimaron que poblaciones naturales de *Trichodesmium* pueden exudar ~50% del N<sub>2</sub> que fijan en forma de DON, mientras que cultivos de *Trichodesmium* IMS101 únicamente exudan ~8% (Mulholland et al., 2004). De manera similar, en el **Capítulo 2** observamos que cultivos de la cianobacteria diazótrofa unicelular *Cyanothece* sp. Miami BG 043511 exuda únicamente ~1% del N<sub>2</sub> que fija, mientras que en el **Capítulo 3**, los diazótrofos de la fracción <10  $\mu$ m en aguas del Atlántico Nordeste subtropical exudaron ~23%. Estas diferencias en la dinámica de exudación de DON probablemente estén asociadas a las condiciones inherentes de aguas oceánicas y difícilmente imitables en sistemas de cultivo, tales como la variabilidad en la intensidad de la luz, la turbulencia, el estrés nutritivo y térmico, la bacterivoría, la lisis vírica y la predación.

La exudación de DON se puede medir indirectamente como la diferencia entre la fijación de N<sub>2</sub> bruta (método ARA) y neta (método del <sup>15</sup>N<sub>2</sub>). El aumento observado en las tasas de fijación de N<sub>2</sub> cuando se aplica el método del <sup>15</sup>N<sub>2</sub> disuelto en vez del <sup>15</sup>N<sub>2</sub> en burbuja (Großkopf et al., 2012; Wilson et al., 2012), sugiere que las diferencias observadas entre las tasas estimadas mediante ARA o burbuja de <sup>15</sup>N<sub>2</sub> en estudios anteriores (e.g. Gallon et al., 2002; Mulholland et al., 2004) podrían ser, en realidad, insignificantes (Mohr et al., 2010). Sin embargo, cuando medimos el enriquecimiento en <sup>15</sup>N del DON extracelular tras un período de incubación, inequívocamente estamos midiendo tasas de exudación de DON, o en el peor de los casos, N<sub>2</sub> fijado como DON que ha salido de la célula tras la ruptura de la pared celular por cualquier otra razón (Glibert y Bronk, 1994; Mulholland et al., 2004; **Capítulo 3**).

Medir el DON marcado con <sup>15</sup>N requiere un gran esfuerzo científico, además de que conlleva una serie de problemas metodológicos, previamente discutidos en la Introducción General de esta tesis. Es preciso diseñar un método más sencillo para poder aplicarlo de manera rutinaria en campañas oceanográficas y experimentos de laboratorio.

# Contribución de la fijación de $N_2$ a la producción nueva en el Atlántico Nordeste subtropical

Los giros subtropicales de los océanos son pobres en nutrientes debido a la fuerte estratificación de la columna de agua en estas zonas, la cual persiste a lo largo de todo el año, impidiendo la entrada de aguas profundas ricas en nutrientes a la capa superficial (Falkowski, 1997). En estos sistemas oligotróficos, se cree que el N<sub>2</sub> fijado por los organismos diazótrofos sustenta en gran medida la producción primaria (Karl et al., 2002; Capone et al., 2005). Esencialmente, esta idea surgió de las altas tasas de fijación de N<sub>2</sub> a nivel de cuenca derivadas de estudios de carácter geoquímico (Tabla 5). Sin embargo, las tasas derivadas de métodos geoquímicos son típicamente un orden de magnitud mayores que las derivadas de métodos biológicos (Tabla 1). Esto puede significar tanto que los métodos geoquímicos las subestiman.

Referencia	Método	Tasa (μmol N m <sup>-2</sup> d <sup>-1</sup> )	Área (10º km²)	Tasa por cuenca (39.2 x 10 <sup>6</sup> km²)
Michaels et al. (1996)	N*	500-2500	7-19	100.2-500.8
Gruber y Sarmiento (1997)	N*	197	27.8	39.5
Hansell et al. (2004)	N* modificado	70-208	6.8	14-41.7
Bates y Hansell (2004)	N* modificado	45-259	16	9-52
Capone et al. (2005)	$\delta^{15}N$	850	17.8	170.3

Tabla 5: Estimas de fijación de  $N_2$  obtenidas con métodos geoquímicos en distintas áreas del Atlántico Norte. Tabla modificada de Mahaffey et al. (2005).

En el **Capítulo 6** medimos tasas de producción nueva en una zona de océano abierto y otra de afloramiento costero de la Corriente de Canarias, y las comparamos con las tasas de fijación de  $N_2$  obtenidas en las mismas estaciones (en el **Capítulo 1**). La contribución de la fijación de  $N_2$  a la producción nueva fue <1%, lo que nos indica que la contribución de la actividad diazotrófica al reservorio de nitrógeno en el Atlántico Nordeste subtropical es ínfimo (al

menos durante el verano, que es cuando se realizaron estas campañas). De forma similar, Mouriño-Carballido et al. (2011) midieron las entradas de  $NO_3$ <sup>-</sup> a la capa eufótica conjuntamente con tasas de fijación de  $N_2$  en un transecto Norte-Sur a lo largo del Atlántico central. Estos autores estimaron que la fijación de  $N_2$  representa un ~2% del aporte total de nitrógeno nuevo a la zona fótica.

Las bajas tasas de fijación de N2 medidas en el Atlántico Nordeste subtropical (véase el Anexo II) no se corresponden con los valores de los parámetros N\* y δ<sup>15</sup>N medidos en esta región (e.g. Mahaffey et al., 2003; Reynolds et al., 2007; Bourbonnais et al., 2009). Todos estos autores concluyeron que la fijación de N<sub>2</sub> contribuía sustancialmente a la producción nueva en el Atlántico Nordeste. Sin embargo, debemos tener en cuenta que los parámetros que utilizaron (N\* y  $\delta^{15}$ N) pueden verse enmascarados fácilmente por otros procesos, como la deposición de nitrógeno atmosférico y el fraccionamiento isotópico (véase la Introducción General de esta tesis). En efecto, estudios recientes indican que las tasas de deposición de nitrógeno atmosférico se están acercando a las estimas de fijación de N2 actuales (Duce et al., 2008). De acuerdo con las tasas de deposición de la Tabla 6, la deposición de nitrógeno atmosférico podría representar el ~2-100% de las tasas de fijación de N<sub>2</sub> a nivel de cuenca estimadas por métodos geoquímicos (Tabla 5). Sin embargo, el exceso de nitrógeno fijado, observado en esta zona por Álvarez y Álvarez-Salgado (2007) y revisado en el **Capítulo 6**, no se explica por estos flujos.

Referencia	Tasa de deposición (Tg N y-1)			
Duce et al., 1991	8.7			
Prospero et al., 1996	5.7			
Dentener et al., 2006	6.9			
Luo et al., 2007	6.5			
Baker et al., 2010	6.4-10			

Tabla 6: Tasas de deposición de nitrógeno atmosférico a nivel de Cuenca (Atlántico Norte).

En resumen, las diferencias entre métodos geoquímicos y biológicos provienen de: (1) las distintas escalas espaciales y temporales involucradas, i.e. los métodos geoquímicos estudian cuencas enteras a escala de décadas, mientras que los biológicos a penas usan unos cuantos litros e incubaciones de hasta 24 h, (2) sobreestimas ocasionadas al aplicar métodos geoquímicos ya que éstos se ven enmascarados por otros procesos como la deposición de nitrógeno atmosférico, o (3) subestimas ocasionadas al aplicar métodos biológicos (Mohr et al., 2010).

En el futuro, los métodos geoquímicos deberían ajustarse para poder discernir qué porcentaje de su señal se debe realmente a la actividad diazotrófica, y cuál a otros procesos. La tasa resultante podría ser lo suficientemente baja como para ser compensada por las tasas de fijación de  $N_2$  obtenidas usando el método de  $^{15}N_2$  disuelto (Mohr et al., 2010), de forma que se equilibraría el ciclo oceánico del nitrógeno.

## Conclusiones

Las principales conclusiones de esta tesis son:

- Las tasas de fijación de N<sub>2</sub> en el Atlántico Nordeste subtropical son bajas en comparación con otras áreas de esta cuenca. En la banda latitudinal tropical, las tasas de fijación de N<sub>2</sub> más altas se encuentran en la mitad Oeste. Sin embargo, en la banda latitudinal subtropical, la mitad Este proporciona >70% del N<sub>2</sub> fijado total. En los estudios incluidos en esta tesis, la fracción <10 µm contribuyó 60-90% de la fijación de N<sub>2</sub> bruta total, indicando que los diazótrofos unicelulares predominan en el Atlántico Nordeste subtropical. Las diferencias entre las tasas de fijación de N<sub>2</sub> brutas y netas indicaron que los diazótrofos en la fracción >10 µm exudaron entre el 15 y el ~90% del N<sub>2</sub> recientemente fijado, mientras que la fracción <10 µm exudó entre el 25 y el 90%. La exudación de N<sub>2</sub> fijado por la fracción >10 µm fue mayor en zonas de océano abierto, mientras que la de la fracción <10 µm fue mayor en zonas de afloramiento costero.
- La cianobacteria unicelular *Cyanothece* sp. Miami BG 043511, cultivada en condiciones óptimas de luz y temperatura, exudó apenas ~1% del N<sub>2</sub> recientemente fijado en forma de DON, sugiriendo que las cianobacterias diazotróficas unicelulares marinas necesitan estar sometidas a algún tipo de estrés (lumínico, nutritivo, predatorio, bacterivoría, turbulencia) para exudar DON extracelularmente.
- Diazótrofos unicelulares (<10  $\mu$ m) en aguas oceánicas del Atlántico subtropical exudaron ~23% del N<sub>2</sub> fijado en forma de DON, mientras que la fracción >10  $\mu$ m exudó ~14%, confirmando que las poblaciones naturales de diazótrofos expuestas a distintos factores ambientales como la turbulencia, el exceso de luz o el estrés nutritivo, pueden exudar un porcentaje considerable del N<sub>2</sub> fijado extracelularmente. Sin embargo, las tasas de exudación de DON fueron constantes en ambas fracciones, no exhibieron un patrón geográfico determinado, ni se correlacionaron significativamente con ninguna de las variables consideradas (temperatura, salinidad, nutrientes, y AOD 550 nm).

- La deposición de polvo sahariano rico en hierro sobre las Islas Canarias, provocó efectos distintos en *Trichodesmium* y en diazótrofos unicelulares. Tras un evento de deposición, *Trichodesmium* pareció no verse afectado por la mayor disponibilidad de hierro y mantuvo tasas de fijación de N<sub>2</sub> similares. En cambio, las tasas de los diazótrofos unicelulares aumentaron ~90%. Tras el evento de deposición de polvo atmosférico, ~35% de los diazótrofos unicelulares aparecieron asociados a partículas. Esta asociación puede favorecer la ligación del hierro contenido en el polvo. *Trichodesmium* sin embargo se observó mayoritariamente en forma de tricomas libres, una disposición que no les permite "atrapar" las partículas de polvo atmosférico y aprovechar los materiales contenidos en éstas.
- Los análisis de biología molecular realizados (PCR-anidada) indicaron que la mayor parte de los organismos <10  $\mu$ m muestreados pertenecen al grupo UCYN-A. Análisis paralelos de TSA-FISH confirmaron estos datos, dada la gran abundancia de organismos <1  $\mu$ m.
- Las bajas tasas de fijación de N<sub>2</sub> medidas indicaron que su contribución a la producción nueva en el Atlántico Nordeste subtropical es muy baja (<1%). Es por tanto poco probable que la actividad diazotrófica produzca las señales de exceso de nitrógeno observadas frecuentemente en esta zona. Alternativamente, la deposición de nitrógeno atmosférico y/o la advección de DOM desde el afloramiento costero del Noroeste de África pueden contribuir significativamente al exceso de nitrógeno, pero sin embargo, las estimas actuales de estos flujos no lo explican en su totalidad.

### Futuras líneas de investigación

Inicialmente, se creía que los fijadores de N<sub>2</sub> marinos eran pocos (principalmente *Trichodesmium* y simbiontes de diatomeas), y que la actividad diazotrófica se restringía a las bandas tropicales de los océanos, donde las aguas son cálidas, oligotróficas y estratificadas. Tras casi dos décadas de investigación, estas premisas han cambiado ostensiblemente. Hoy en día sabemos que la fijación de N<sub>2</sub> puede darse a latitudes hasta ~50°N (Luo et al., 2012), a temperaturas <16°C (e.g. Sohm et al., 2011b), y en aguas turbulentas (e.g. Benavides et al., 2011).

Como indican investigaciones muy recientes, las UCYN-A (que tienen un metabolismo fotoheterotrófico) son las cianobacterias diazótrofas más abundantes del océano (Luo et al., 2012), y dependen de una fuente externa de materia orgánica para su nutrición, ya sea ésta el propio 'stock' de los océanos (Tripp et al., 2010), o como se ha visto hace muy poco, procedente de una relación simbiótica con otros organismos planctónicos (Thompson et al., 2012). También, otros estudios recientes indican que el nifH no-cianobacterial predomina sobre el perteneciente a cianobacterias a nivel global (Farnelid et al., 2011), y que la actividad diazotrófica heterotrófica predomina en las aguas del giro subtropical del Pacífico Sur (Halm et al., 2011). Por tanto, parece evidente que el estudio de la actividad diazotrófica heterotrófica ha de suponer toda una nueva era en la investigación del ciclo del nitrógeno oceánico. Debido a que los heterótrofos se nutren de materia orgánica, el siguiente paso lógico sería estudiar las interacciones de los diazótrofos heterótrofos con la materia orgánica en el océano, resolviendo cuestiones como si la composición de la materia orgánica favorece o desfavorece a determinados grupos de diazótrofos heterótrofos, o si éstos pueden presentar un modo de vida asociado a partículas bajo determinadas condiciones. Estudios anteriores indican que sólo un 10-15% de los heterótrofos de la columna de agua oceánica viven asociados a partículas (Turley and Mackie, 1994). Sin embargo, otros estudios más recientes sugieren que éste es el modo de vida preferido por los microheterótrofos (e.g. Baltar et al., 2009a). En el ambiente pelágico, las partículas suelen tener altas concentraciones de nutrientes y materia orgánica (véase la Tabla 5 en Simon et al., 2002), lo que propicia una intensa actividad microbiana. Por ejemplo, la colonización de estas partículas por bacterias puede estimular la oxidación de la materia orgánica, disminuyendo los niveles de oxígeno dentro y alrededor de la partícula, favoreciendo la fijación de N2 (Riemann et al., 2010).

Otra cuestión pendiente de ser resuelta es "quién está haciendo qué", es decir, qué organismos están detrás de cada proceso. En general, los estudios sobre actividad diazotrófica oceánica separan la actividad de la filogenia, i.e. se estiman tasas de fijación de  $N_2$  y se estudia la diversidad del gen *nifH*. Sin embargo, desconocemos cuánto  $N_2$  ha podido fijar cada uno de los diazótrofos presentes en una muestra determinada. Entre las técnicas más utilizadas actualmente está la qPCR -que cuantifica la cantidad de *nifH* presente en una muestra, pero su presencia no indica necesariamente que esté activo-, y la qRT-PCR -que cuantifica la expresión del gen *nifH*, y por tanto sí indica si éste estaba activo o no-. Sin embargo, ninguna de estas técnicas nos aclara **cuánto N<sub>2</sub> fija cada especie**. En un futuro próximo, esta cuestión puede resolverse mediante técnicas como el marcaje isotópico de ácidos nucleicos y el uso de tecnología nanoSIMS.

Por último, no debemos olvidar que **las tasas de fijación de N**<sub>2</sub> **publicadas anteriormente deben corregirse de acuerdo con el método mejorado de** <sup>15</sup>N<sub>2</sub> (Mohr et al., 2010). Tal y como Großkopf et al. (2012) señalaron, esta puede ser una empresa complicada, si no imposible, dada la gran cantidad de factores implicados en la subestimación de las tasas (tiempo de incubación, volumen de <sup>15</sup>N<sub>2</sub> inyectado, temperatura de la muestra, etc). Sin embargo, no debería abandonarse la posibilidad de **modelar la corrección de las tasas** para al menos llegar a una aproximación. La alternativa es aplicar el método mejorado por todas las zonas anteriormente muestreadas.

Basándose en la diazotrofía autotrófica, los estudios anteriores se centraron en la capa superficial del océano (generalmente <200 m). Dado que los límites de la actividad diazotrófica se han visto considerablemente aumentados en virtud de los últimos estudios, es necesario ahora muestrear a latitudes y profundidades mayores de lo que se ha venido haciendo hasta ahora, con objeto de reevaluar la **diversidad y actividad global de los diazótrofos marinos.** En concreto, existen regiones del océano que han recibido muy poca atención, como el Atlántico Sur y el Índico. Aumentando el rango espacial de estos estudios esperamos poder aumentar la tasa media de fijación de N<sub>2</sub> global lo suficiente como para compensar las pérdidas de nitrógeno fijado que se estiman actualmente, equilibrando el ciclo oceánico del nitrógeno.

PART V: References and annexes

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## Annex I: List of acronyms

AA Amino acids AES Atomic emission spectrophotometry **AF Azores Front** ALOHA A long time series habitat assessment ANCA-SL Automated nitrogen and carbon analyzer for solids and liquids AOD Aerosol optical depth ARA Acetylene reduction assay ATP Adenosine triphosphate AVHRR Advanced very high resolution radiometry AVISO Archiving, validation, and interpretation of satellite oceanographic remote sensing service BATS Bermuda Atlantic Time-series Study CAIBEX Shelf-ocean exchanges in the Canaries-Iberian large marine ecosystem CC Canary current CHN Carbon hydrogen nitrogen CTD Conductivity temperature depth CTZ Coastal transition zone CYFOD Cyanobacteria dominating the functioning of the ocean deserts DAPI 4',6-diamidino-2-phenylindole DCM Deep chlorophyll maximum DIC Dissolved inorganic carbon DIN Dissolved inorganic nitrogen DNA Deoxyribonucleic acid DOC Dissolved organic carbon DOCr Dissolved organic carbon release DOM Dissolved organic matter DON Dissolved organic nitrogen DONr Dissolved organic nitrogen release DOP Dissolved organic phosphorus EBUS Eastern boundary upwelling system EPS Extracellular polysaccharides exDOC Extracellular dissolved organic carbon exDON Extracellular dissolved organic nitrogen FID Flame ionization detector FITC Fluorescein isothiocyanate **GEOSECS** Geochemical Ocean Section Study GES DISC Goddard earth sciences data and information services center Giovanni GF/F Glass fiber filter HOT Hawaii Ocean Time-series HRP horseradish peroxidase HVS High volume sampler ICP-OES Inductively coupled plasma optical emission spectrometer

inDOC Intracellular dissolved organic carbon inDOCp Intracellular dissolved organic carbon production inDON Intracellular dissolved organic nitrogen inDONp Intracellular dissolved organic nitrogen production IPCC Intergovernmental Panel on Climate Change IRMS Isotope ratio mass spectrometry ITCZ Intertropical convergence zone JGOFS Joint Global Ocean Flux Study LUCIFER Lunar cycles and iron fertilization MIMS Membrane inlet mass spectrometry MLD Mixed layer depth MODIS Moderate resolution imaging spectroradiometer NASA National aeronautics and space administration NCEP National centers for environmental prediction **NE Northeast** NOAA National oceanic and atmospheric administration NW Northwest OMZ Oxygen minimum zone PAR Photosynthetically active radiance PBS phosphate-buffered saline PC Particulate carbon **PN** Particulate nitrogen Pnew New production POC Particulate organic carbon POM Particulate organic matter PON Particulate organic nitrogen Preg Regenerated production **RNA** Ribonucleic acid rRNA Ribosomal ribonucleic acid **RT** Room temperature SD standard deviation SPE Solid phase extraction SSHA Sea surface height anomaly SSS Sea surface salinity SST Sea surface temperature TDN Total dissolved nitrogen TSA-FISH Tyramide signal amplification - fluorescent in situ hybridization TSP Atmospheric total particulate matter UCYN Unicellular cyanobacteria UCYN-A Unicellular cyanobacteria of group A UCYN-B Unicellular cyanobacteria of group B UCYN-C Unicellular cyanobacteria of group C UNESCO United Nations educational, scientific and cultural organization WOCE World ocean circulation experiment

## Annex II: N<sub>2</sub> fixation rates summary

Table 1: Compilation of whole seawater  $N_2$  fixation rates measured in the subtropical Northeast Atlantic Ocean (between 23.5 – 40° N and 10 – 45°W).

Source: related article or investigator	Method	Latitude (ºN)	Longitude (ºW)	Depth (m)	N <sub>2</sub> fixation (nmol N L <sup>-1</sup> d <sup>-1</sup> )
Fernández et al. (2010)	<sup>15</sup> N <sub>2</sub> bubble	26.64	17.49	40	0.022
	<sup>15</sup> N <sub>2</sub> bubble	26.64	17.49	5	0.019
	<sup>15</sup> N <sub>2</sub> bubble	26.00	21.38	5	0.019
	<sup>15</sup> N <sub>2</sub> bubble	26.00	25.73	110	0.004
	<sup>15</sup> N <sub>2</sub> bubble	26.00	25.73	70	0.021
	<sup>15</sup> N <sub>2</sub> bubble	26.00	25.73	5	0.030
	<sup>15</sup> N <sub>2</sub> bubble	26.00	29.83	70	0.007
	<sup>15</sup> N <sub>2</sub> bubble	26.00	29.83	5	0.003
	<sup>15</sup> N <sub>2</sub> bubble	25.99	34.12	120	0.011
	<sup>15</sup> N <sub>2</sub> bubble	25.99	34.12	60	0.007
	<sup>15</sup> N <sub>2</sub> bubble	25.99	34.12	5	0.013
	<sup>15</sup> N <sub>2</sub> bubble	26.00	38.27	115	0.009
	<sup>15</sup> N <sub>2</sub> bubble	26.00	38.27	60	0.016
	<sup>15</sup> N <sub>2</sub> bubble	26.00	38.27	5	0.003
	<sup>15</sup> N <sub>2</sub> bubble	25.65	29.00	100	0.007
	<sup>15</sup> N <sub>2</sub> bubble	25.65	29.00	50	0.032
	<sup>15</sup> N <sub>2</sub> bubble	25.65	29.00	5	0.018
	<sup>15</sup> N <sub>2</sub> bubble	29.18	28.94	110	0.003
	<sup>15</sup> N <sub>2</sub> bubble	29.18	28.94	50	0.008
	<sup>15</sup> N <sub>2</sub> bubble	29.18	28.94	5	0.033
	<sup>15</sup> N <sub>2</sub> bubble	29.18	24.41	100	0.013
	<sup>15</sup> N <sub>2</sub> bubble	29.18	24.41	50	0.036
	<sup>15</sup> N <sub>2</sub> bubble	29.18	24.41	5	0.030
	<sup>15</sup> N <sub>2</sub> bubble	29.18	19.87	90	0.023
	<sup>15</sup> N <sub>2</sub> bubble	29.18	19.87	50	0.051
	<sup>15</sup> N <sub>2</sub> bubble	29.18	19.87	5	0.269
	<sup>15</sup> N <sub>2</sub> bubble	29.18	15.45	90	0.104
	<sup>15</sup> N <sub>2</sub> bubble	29.18	15.45	50	0.344
	<sup>15</sup> N <sub>2</sub> bubble	29.18	15.45	5	0.078
Moore et al. (2009)	<sup>15</sup> N <sub>2</sub> bubble	35.70	29.50	77	0.015
	<sup>15</sup> N <sub>2</sub> bubble	35.70	29.50	35	0.093
	<sup>15</sup> N <sub>2</sub> bubble	35.70	29.50	21	0.062
	<sup>15</sup> N <sub>2</sub> bubble	35.70	29.50	11	0.068
	<sup>15</sup> N <sub>2</sub> bubble	29.30	36.70	115	0.006
	<sup>15</sup> N <sub>2</sub> bubble	29.30	36.70	57	0.025
Source: related article or investigator	Method	Latitude (ºN)	Longitude (ºW)	Depth (m)	N2 fixation (nmol N L <sup>-1</sup> d <sup>-1</sup> )
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(continued)	<sup>15</sup> N <sub>2</sub> bubble	29.30	36.70	34	0.010
	<sup>15</sup> N <sub>2</sub> bubble	29.30	36.70	24	0.014
M. Mulholland (unpublished)	<sup>15</sup> N <sub>2</sub> bubble	37.86	38.64	2	9.000
	<sup>15</sup> N <sub>2</sub> bubble	37.86	38.64	2	8.356
	<sup>15</sup> N <sub>2</sub> bubble	37.86	38.64	6	7.791
	<sup>15</sup> N <sub>2</sub> bubble	37.86	38.64	6	3.760
	<sup>15</sup> N <sub>2</sub> bubble	37.86	38.64	29	1.388
	<sup>15</sup> N <sub>2</sub> bubble	37.86	38.64	29	2.090
	<sup>15</sup> N <sub>2</sub> bubble	27.80	23.35	15	0.221
	<sup>15</sup> N <sub>2</sub> bubble	27.80	23.35	25	0.359
	<sup>15</sup> N <sub>2</sub> bubble	27.80	23.35	40	0.100
	<sup>15</sup> N <sub>2</sub> bubble	27.80	23.33	15	0.367
	<sup>15</sup> N <sub>2</sub> bubble	27.80	23.33	25	0.259
	<sup>15</sup> N <sub>2</sub> bubble	27.80	23.33	40	0.200
	<sup>15</sup> N <sub>2</sub> bubble	27.75	23.40	15	0.425
	<sup>15</sup> N <sub>2</sub> bubble	27.75	23.40	25	0.300
	<sup>15</sup> N <sub>2</sub> bubble	27.75	23.40	40	0.500
	<sup>15</sup> N <sub>2</sub> bubble	27.51	23.22	15	0.360
	<sup>15</sup> N <sub>2</sub> bubble	27.51	23.22	25	1.200
	<sup>15</sup> N <sub>2</sub> bubble	27.51	23.22	40	0.700
	<sup>15</sup> N <sub>2</sub> bubble	27.54	22.57	15	0.424
	<sup>15</sup> N <sub>2</sub> bubble	27.54	22.57	25	0.300
	<sup>15</sup> N <sub>2</sub> bubble	27.54	22.57	40	0.500
	<sup>15</sup> N <sub>2</sub> bubble	27.57	22.55	15	0.432
	<sup>15</sup> N <sub>2</sub> bubble	27.57	22.55	25	0.400
	<sup>15</sup> N <sub>2</sub> bubble	27.57	22.55	40	0.500
	<sup>15</sup> N <sub>2</sub> bubble	27.65	22.39	15	0.269
	<sup>15</sup> N <sub>2</sub> bubble	27.65	22.39	25	0.193
	<sup>15</sup> N <sub>2</sub> bubble	27.65	22.39	40	0.100
	<sup>15</sup> N <sub>2</sub> bubble	25.92	35.24	3	0.716
	<sup>15</sup> N <sub>2</sub> bubble	25.92	35.24	18	0.155
	<sup>15</sup> N <sub>2</sub> bubble	29.30	36.70	3	0.337
	<sup>15</sup> N <sub>2</sub> bubble	29.30	36.70	17	1.215
	<sup>15</sup> N <sub>2</sub> bubble	38.53	20.35	3	0.138
	<sup>15</sup> N <sub>2</sub> bubble	38.53	20.35	12	0.120
	<sup>15</sup> N <sub>2</sub> bubble	35.05	20.85	3	0.055
	<sup>15</sup> N <sub>2</sub> bubble	35.05	20.85	16	0.101
	<sup>15</sup> N <sub>2</sub> bubble	31.15	20.72	4	0.151
	<sup>15</sup> N <sub>2</sub> bubble	31.15	20.72	17	0.071
Turk et al. (2011)	<sup>15</sup> N <sub>2</sub> bubble	26.11	24.00	1	1.123
	<sup>15</sup> N <sub>2</sub> bubble	26.11	24.00	9	0.428
	<sup>15</sup> N <sub>2</sub> bubble	26.11	24.00	16	0.269
	<sup>15</sup> N <sub>2</sub> bubble	26.11	24.00	23	0.317

Source: related article or investigator	Method	Latitude (ºN)	Longitude (ºW)	Depth (m)	N2 fixation (nmol N L <sup>-1</sup> d <sup>-1</sup> )
(continued)	<sup>15</sup> N <sub>2</sub> bubble	26.11	24.00	38	1.006
	<sup>15</sup> N <sub>2</sub> bubble	26.11	24.00	65	0.180
	<sup>15</sup> N <sub>2</sub> bubble	26.05	23.99	5	0.100
	<sup>15</sup> N <sub>2</sub> bubble	26.05	23.98	5	2.400
	<sup>15</sup> N <sub>2</sub> bubble	26.07	23.99	5	2.400
	<sup>15</sup> N <sub>2</sub> bubble	26.09	24.01	5	2.400
	<sup>15</sup> N <sub>2</sub> bubble	26.08	24.00	5	2.400
	<sup>15</sup> N <sub>2</sub> bubble	26.10	23.99	5	0.100
Großkopf et al. (2012)	<sup>15</sup> N <sub>2</sub> bubble	25.3	16.6	11	3.88
	Dissolved <sup>15</sup> N <sub>2</sub>	25.3	16.6	11	3.63
Benavides et al. (2011)	ARA	40.00	20.00	5	0.090
(Chapter 1)	ARA	37.98	20.01	5	0.107
	ARA	36.50	20.00	5	0.145
	ARA	34.99	19.97	5	0.127
	ARA	33.50	20.00	5	0.115
	ARA	31.50	20.00	5	0.107
	ARA	30.50	20.02	5	0.125
	ARA	29.85	19.19	5	0.145
	ARA	29.55	17.56	5	0.177
	ARA	29.25	15.93	5	0.031
		28.87	14.10	5	0.055
		31.00	10.01	5	0.072
	ARA	30.90	10.05	5	0.129
	ARA	31.00	10.91	5	0.980
	ARA	31.11	10.60	5	0.308
	ARA	31.01	11.31	5	0.094
	ARA	30.95	11.40	5	0.150
	ARA	30.89	11.45	5	0.159
	<sup>15</sup> N <sub>2</sub> bubble	40.00	20.00	5	0.072
	<sup>15</sup> N <sub>2</sub> bubble	37.98	20.01	5	0.038
	<sup>15</sup> N <sub>2</sub> bubble	34.99	19.97	5	0.035
	<sup>15</sup> N <sub>2</sub> bubble	33.50	20.00	5	0.017
	<sup>15</sup> N <sub>2</sub> bubble	29.85	19.19	5	0.053
	<sup>15</sup> N <sub>2</sub> bubble	29.55	17.56	5	0.055
	<sup>15</sup> N <sub>2</sub> bubble	29.25	15.93	5	0.018
	<sup>15</sup> N <sub>2</sub> bubble	28.87	14.10	5	0.023
	<sup>15</sup> N <sub>2</sub> bubble	31.00	10.01	5	0.039
	<sup>15</sup> N <sub>2</sub> bubble	30.74	10.65	5	0.028
	<sup>15</sup> N <sub>2</sub> bubble	30.90	10.80	5	0.014
	<sup>15</sup> N <sub>2</sub> bubble	31.00	10.91	5	0.381
	<sup>15</sup> N <sub>2</sub> bubble	31.11	10.60	5	0.174
	<sup>15</sup> N <sub>2</sub> bubble	31.01	11.31	5	0.057
	<sup>15</sup> N <sub>2</sub> bubble	30.95	11.40	5	0.084

Source: related article or investigator	Method	Latitude (ºN)	Longitude (ºW)	Depth (m)	N <sub>2</sub> fixation (nmol N L <sup>-1</sup> d <sup>-1</sup> )
(continued)	<sup>15</sup> N <sub>2</sub> bubble	30.89	11.45	5	0.097
Benavides et al. (submitted)	Dissolved <sup>15</sup> N <sub>2</sub>	27.78	13.34	5	8.640*
(Chapter 3)	Dissolved <sup>15</sup> N <sub>2</sub>	27.02	15.70	5	4.524*
	Dissolved <sup>15</sup> N <sub>2</sub>	26.02	18.76	5	3.156*
	Dissolved <sup>15</sup> N <sub>2</sub>	25.63	20.00	5	4.008*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.99	21.97	5	4.644*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.66	22.98	5	3.036*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	25.32	5	6.408*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	26.54	5	5.976*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	28.37	5	4.140*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	29.59	5	5.796*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	31.43	5	6.864*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	32.04	5	5.064*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	34.33	5	1.824*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	35.72	5	1.308*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	37.57	5	3.432*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	38.49	5	2.352*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	40.00	5	2.232*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	41.72	5	1.704*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	44.04	5	0.828*
	Dissolved <sup>15</sup> N <sub>2</sub>	24.50	44.50	5	3.156*
	<sup>15</sup> N <sub>2</sub> bubble	27.78	13.34	5	4.179
	<sup>15</sup> N <sub>2</sub> bubble	27.02	15.70	5	1.016
	<sup>15</sup> N <sub>2</sub> bubble	26.02	18.76	5	0.755
	<sup>15</sup> N <sub>2</sub> bubble	25.63	20.00	5	0.852
	<sup>15</sup> N <sub>2</sub> bubble	24.99	21.97	5	1.333
	<sup>15</sup> N <sub>2</sub> bubble	24.66	22.98	5	1.784
	<sup>15</sup> N <sub>2</sub> bubble	24.50	25.32	5	0.737
	<sup>15</sup> N <sub>2</sub> bubble	24.50	26.54	5	1.040
	<sup>15</sup> N <sub>2</sub> bubble	24.50	28.37	5	3.336
	<sup>15</sup> N <sub>2</sub> bubble	24.50	29.59	5	0.743
	<sup>15</sup> N <sub>2</sub> bubble	24.50	31.43	5	0.834
	<sup>15</sup> N <sub>2</sub> bubble	24.50	32.04	5	1.138
	<sup>15</sup> N <sub>2</sub> bubble	24.50	34.33	5	0.454
	<sup>15</sup> N <sub>2</sub> bubble	24.50	35.72	5	0.462
	<sup>15</sup> N <sub>2</sub> bubble	24.50	37.57	5	0.766
	<sup>15</sup> N <sub>2</sub> bubble	24.50	38.49	5	0.788
	<sup>15</sup> N <sub>2</sub> bubble	24.50	40.00	5	0.390
	<sup>15</sup> N <sub>2</sub> bubble	24.50	41.72	5	0.951
	<sup>15</sup> N <sub>2</sub> bubble	24.50	44.04	5	0.558
	<sup>15</sup> N <sub>2</sub> bubble	24.50	44.50	5	0.230
Benavides et al. (in press)	ARA	28.35	14.62	5	0.136
(Chapter 4)	ARA	28.35	14.62	5	0.089

Source: related article or investigator	Method	Latitude (ºN)	Longitude (ºW)	Depth (m)	$N_2$ fixation (nmol N L <sup>-1</sup> d <sup>-1</sup> )
(continued)	ARA	28.35	14.62	5	1.430
	ARA	28.35	14.62	5	1.177
	ARA	28.35	14.62	5	8.558
	ARA	28.35	14.62	5	5.517
	ARA	28.35	14.62	5	5.517
	ARA	28.35	14.62	5	1.578
	ARA	28.35	14.62	5	0.185
	ARA	28.35	14.62	5	1.011
	ARA	28.35	14.62	5	0.290
	ARA	28.35	14.62	5	3.095
	ARA	28.35	14.62	5	2.485
	ARA	28.35	14.62	5	2.485

\*Rates obtained by the sum of the >10  $\mu m$  and <10  $\mu m$  fractions  $N_2$  fixation rates.