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Microbial uptake and regeneration of inorganic nitrogen off the coastal Namibian upwelling system

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Abstract

We used 15N-labeled substrates to measure microbial nitrate (NO−3) and ammonium (NH4⁺) uptake, regeneration and associated dissolved organic nitrogen (DON) release in a coastal upwelling system off Namibia (Benguela Current) in the austral winter of 2011 with the aim of quantifying rates of new production (Pnew) and regenerated production (Preg). These measurements were made during four consecutive coastal-offshore transects. The water parcels sampled at the different stations over the transect were classified into three groups according to the time passed from the first contact of the water with the surface during coastal upwelling (‘pseudo-age’). The average Pnew was high in freshly upwelled waters with a pseudo-age <13 d (17.8 mmol N m⁻² h⁻¹), and decreased abruptly towards older waters (3.9 and 2.3 mmol N m⁻² h⁻¹ in waters with a pseudo-age of 13 to 55 d, and >55 d, respectively). Preg rates were similar in <13 d and 13–55 d waters (10.9 and 11.1 mmol N m⁻² h⁻¹, respectively), and decreased to 6.24 mmol N m⁻² h⁻¹ in waters with a pseudo-age >55 d. Measuring nitrogen regeneration and DON release fluxes allowed us to correct Pnew and Preg rates. NO−3 regeneration rates were low (<0.5 mmol N m⁻² h⁻¹), while NH4⁺ regeneration rates were in the range of NH4⁺ uptake rates (~2–5 mmol N m⁻² h⁻¹), thus influencing significantly Preg rates. Parallel studies presented in this volume indicate a relatively high abundance of dinoflagellates and mixotrophic microflagellates, which may be partly responsible for the high Preg rates observed. Our results suggest that nitrogen regeneration plays an important role in sustaining primary production in this upwelling system.

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1. Introduction

Eastern boundary upwelling systems (EBUS) are very productive areas created by the combined effect of equatorward wind stress and the Coriolis effect which give rise to Ekman offshore transport and the subsequent upwelling of nutrient-rich deep waters. Despite comprising a small percentage of the ocean’s total surface (~1%), EBUS provide ~2% of global ocean primary production, producing abundant fish landings (Carr and Kearns, 2003). This primary production is supported by dissolved inorganic nitrogen availability. This nitrogen may become from the deep upwelled waters which are rich in nitrate (NO−3), which is considered ‘new’ nitrogen (Dugdale and Goering, 1967), or it can be recycled and reused within the lit water column — such as ammonium (NH4⁺), which is considered ‘regenerated’ nitrogen.

The degree to which a system depends on new or regenerated nitrogen is estimated through the f-ratio, which measures the proportion of total production (new + regenerated production, i.e. NO−3 uptake + NH4⁺ uptake) attributable to new nitrogen uptake (Epplley and Peterson, 1979). The f-ratio is a proxy of the fraction of the total nitrogen that can be exported to the deep ocean or consumed by higher trophic levels, i.e. the more productive a given system is the higher f-ratio it will have and vice versa. This ratio has been amended in recent years by including other nitrogen fluxes such as NO−3 and NH4⁺ regeneration, dissolved organic nitrogen release (DONr), and atmospheric nitrogen (N₂) fixation. Excluding these fluxes may under or overestimate f-ratio values substantially (e.g. Fernández and Raimbault, 2007). New production (Pnew) rates are overestimated when NO−3 regeneration (i.e. nitrification) is not taken into account. The regeneration of NO−3 was thought to be inhibited by light, but it has been demonstrated that this process contributes importantly to NO−3 availability in well-lit surface waters of the ocean (Yool et al., 2007). Similarly, regenerated production (Preg) rates are underestimated when NH4⁺ regeneration is not measured, as this flux has been proven to occur at high rates in many marine systems (see review by Bronk and Steinberg, 2008). DONr resulting from either NO−3 or NH4⁺ uptake may also represent a considerable percentage of gross uptake (e.g. 30–40% in the central
North and South Atlantic; Varela et al., 2005), therefore obviating these fluxes underestimates Pnew and Preg rates. Measuring DON is also important given the recognized role of DON as a substrate for primary producers (Bronk et al., 2007) and as a promoter of export production in the open ocean (Letcher et al., 2013). Finally, N$_2$ fixation – which is thought to maintain ~50% of primary production in the oligotrophic open ocean (Capone et al., 2005) – can also contribute substantially to Pnew in coastal margins and other upwelling sites (Fernández et al., 2011; Raimbault and Garcia, 2008; Subramaniam et al., 2013). For example, Fernández et al. (2011) measured N$_2$ fixation rates generally <1 nmol L$^{-1}$ d$^{-1}$ in the Humboldt Current System, although values up to 14 nmol N L$^{-1}$ d$^{-1}$ were measured at specific locations. Similarly, Sohm et al. (2011) reported N$_2$ fixation rates up to 8 nmol L$^{-1}$ d$^{-1}$ in the Benguela Current System. These rates are comparable to those measured in the open ocean. Altogether, the accuracy of Pnew and Preg estimates depends on the inclusion of all these nitrogen fluxes in their calculation.

The MSM18/5 ‘Succession’ cruise was designed to track the evolution of physical, chemical and biological parameters along three stages of upwelled waters in the northern Benguela upwelling system off Namibia as proposed by Barlow (1982): (1) newly upwelled water, (2) maturing upwelled water, and (3) aged upwelled water. In our cruise, these three stages were established by grouping stations according to their ‘pseudo-age’, an indicator of the time passed since a water parcel first reached the surface when it upwelled near the coast (see Materials and methods). As in other companion papers of this volume, we use this classification to study the variability of Pnew and Preg rates along the above described water parcel continuum according to the in situ phytoplanktonic community and inorganic nutrient distributions.

2. Materials and methods

2.1. Sampling and hydrographic measurements

The MSM18/5 ‘Succession’ cruise was performed onboard the R/V Maria S. Merian from 23 August to 20 September 2011. The cruise consisted in a coastal to open ocean waters transect (perpendicular to the coast), which was repeated four times (trannsects 1, 2, 3 and 4 – T1, T2, T3 and T4, respectively; see Fig. 1). T1 took place from 27 to 30 August, T2 from 30 August to 2 September, T3 from 8 to 11 September, and T4 from 11 to 15 September 2011. Water samples were collected from the surface (2 m), 20 and 40 m depths with 10 L free flow bottles mounted in a Rosette sampler equipped with a SBE911+ conductivity sensors (WETlab FLRT-1754) oxygen (SBE43) and photosynthetic active radiance (PAR) (QP3 2530 by biospherical Instruments Inc.).

2.2. Station clustering approach

The objective of the ‘Succession’ cruise was to track changes in biological, physical and chemical parameters as waters separate from the coastal upwelling towards the open ocean. However, the intense mesoscale variability observed during our cruise (Mohrholz et al., 2014–in this volume) made it difficult to establish clear coastal to open ocean gradients of chemical and biological variables. For example, freshly upwelled waters may be found further offshore than it would be expected, transported from the coastal upwelling center through the filament. In order to classify the sampled water parcels according to the time passed from their first contact with the surface at the coastal upwelling until they reached oceanic waters further offshore, we use the ‘pseudo-age’ proxy. Related to the three stages of aging water described by Barlow (1982), the pseudo-age proxy is computed using temperature, oxygen and salinity data. The reader is referred to Mohrholz et al. (2014–in this volume) for further details on the calculation of the pseudo-age.

To summarize our nitrogen fluxes data, all stations were then operationally defined into three groups according to their pseudo-age and their biological and chemical properties (chlorophyll $a$ – Chl $a$ – and nutrient concentrations). These three groups are detailed in Table 1 of Hansen et al. (2014–in this volume): Stage 1 – waters with a pseudo-age $<13$ d, $13.5 \pm 0.4$ °C, $35.17 \pm 0.02$ salinity, $20.36 \pm 2.35$ μM NO$_3$ and 0.5–3.5 mg m$^{-3}$ Chl $a$ –. Stage 2 – waters with pseudo-age 13–55 d, $14.5 \pm 0.5$ °C, $35.27 \pm 0.06$ salinity, $14.85 \pm 2.58$ μM NO$_3$ and 1.5–7 mg m$^{-3}$ Chl $a$ –, and finally Stage 3 – waters with pseudo-age >55 d, $16.2 \pm 0.3$ °C, $35.48 \pm 0.05$ salinity, $7.75 \pm 4.03$ μM NO$_3$ and <1 mg m$^{-3}$ Chl $a$ –.

2.3. Incubations with $^{15}$N-labeled substrates

Water for $^{15}$NO$_3$ and $^{15}$NH$_4^+$ uptake, regeneration and release experiments was collected at 3 stations during T1 (stations 1, 14 and 18), 2 stations during T2 (stations 4 and 14), 3 stations during T3 (stations 1, 7 and 17), and 5 stations during T4 (stations 1, 7, 17, 21 and 25), at the surface (2 m), 20 m and 40 m depth, making a total of 39 samples for each type of nitrogen flux measurement (i.e. for each uptake, release or regeneration flux). Seawater was directly transferred from the sampling bottles of the Rosette into acid-cleaned 2 L transparent polycarbonate bottles (Nalgene) using silicone tubing. Trace additions of $^{15}$N-labeled substrates were added to the incubation bottles as 1 mL of K$^{15}$NO$_3$ (200 μM; 99 at.%; 0.1$\mu$M $^{15}$N final concentration), or 1 mL of $^{15}$NH$_4$Cl (20 μM; 99 at.%; 0.01$\mu$M $^{15}$N final concentration) (Sigma-Aldrich). Two bottles were used per each type of isotope addition. One of the two bottles was immediately filtered onto substrate to determine the initial $^{15}$N at.% enrichment of particulate organic nitrogen (PON) in the samples. All other bottles were incubated for 3–4 h on in-deck incubators cooled with surface seawater and shaded with mesh to mimic in situ photosynthetic active radiation (PAR) levels at the relevant depths. The light intensity of on-deck incubators was checked with a PAR sensor. The resulting PAR attenuation was 5–10% for 2 m, 15–20% for 20 m and 25% for 40 m. The incubations were usually performed between 10:00 and 13:00 UTC, coinciding with the local solar zenith, which occurs around 11:00 UTC at this time of the year. Isotope
enrichments were on average 11 and 21% for NO$_3^-$ and NH$_4^+$, respectively. After the incubation period, the two replicates were filtered onto precombusted GF/F filters, which were stored in sterile 1.5 mL centrifuge microtubes (Eppendorf) at −20 °C until isotope ratio analysis ashore. These samples were used to calculate NO$_3^-$ and NH$_4^+$ uptake.

For $^{15}$NO$_3^-$ and $^{15}$NH$_4^+$ regeneration and associated release of DON, the filtrates were kept to analyze final NO$_3^-$, NO$_2^-$, NH$_4^+$ and DON concentrations (see below), and the $^{15}$N-enrichment of the final NO$_3^-$, NH$_4^+$ and DON pools using the protocol proposed by Slaywick and Raimbault (1995) and Raimbault et al. (1999). For the latter, 80 mL of filtrate were stored in 100 mL borosilicate flask (VWR) and poisoned with 1% HgCl$_2$ (Sigma-Aldrich) to stop microbial activity. The extraction protocol consisted of three steps: (1) NO$_3^-$ and nitrite (NO$_2^-$) were reduced and stripped off together with initial NH$_4^+$ as ammonium sulfate, (2) the remaining dissolved nitrogen (i.e. the DON pool) was oxidized to NO$_2^+$ by persulfate oxidation (Valderrama, 1981), and (3) step (1) was repeated to strip off DON as ammonium sulfate.

Before analysis, GF/F filters were dried in an oven at 60 °C for 24 h and stored inside a desiccator until analysis. The $^{15}$N at.% enrichment of $^{15}$NO$_3^-$ and $^{15}$NH$_4^+$-amended samples was determined using an EA1108 Carlo Erba elemental analyzer connected to a MAT253 Thermo Finnigan isotope-ratio mass spectrometer (IRMS).

$^{15}$N$_2$ uptake experiments were conducted at 2 stations during T1 (NAM001, NAM014) and 5 stations during T4 (NAM01, NAM007, NAM017, NAM021, NAM025). In order to prevent a significant underestimation of N$_2$ fixation rates associated with labeling water samples with gaseous $^{15}$N$_2$ (Mohr et al., 2010), we pre-dissolved 1 mL of high purity $^{15}$N$_2$ (99.2 at.%, CAMPRO Scientific Berlin) in a septum-capped glass bottle containing 110 mL of seawater (from the same depth as the sample to be incubated) previously filtered under 0.2 μm polycarbonate filters. The $^{15}$N$_2$ was injected through the septum using a Pressure-Lok A-2 gas-tight syringe (VICI Precision Sampling Inc.). The glass bottles were vigorously shaken five times for 1 min within 2 h in order to intensify the dissolution of the $^{15}$N$_2$ bubble. Then, 50 μl aliquots of this enriched seawater were added to 535 mL glass incubation bottles. According to the ideal gas law and Weiss (1970) equations, this results in a final concentration of ~378.38 μM $^{15}$N per incubation bottle. The bottles were filled with sample seawater from 2 m, 20 m and 40 m and incubated in on-deck incubators connected to the flow-through system of the ship, and shaded with neutral density filters to reproduce different light intensities found throughout the lit water column (100%, and incubations were terminated after ~6 h (varying between 10:00 am and 07:30 pm) by filtration onto precombusted GF/F filters. These were treated equally to filters from $^{15}$NO$_3^-$ and $^{15}$NH$_4^+$ incubations before analysis. The $^{15}$N at.% enrichment of these samples was measured using a continuous-flow Finnigan Delta S IRMS. N$_2$ fixation rates were calculated using the mass-balance approach by Montoya et al. (1996). The $^{15}$N at.% enrichment of the enriched seawater added to the samples was not checked by membrane-introduction mass spectrometry (MIMS) and therefore the theoretical complete dissolution of the bubble was assumed (as in Montoya et al., 1996). Thus, the N$_2$ fixation rates presented here can be slightly underestimated (Großkopf et al., 2012; Mohr et al., 2010) and should be interpreted cautiously.

2.4. New and regenerated production rate calculations

To avoid under or overestimations of nitrogen fluxes, NO$_3^-$ and NH$_4^+$ regeneration and DONr associated with uptake and N$_2$ fixation were included in the calculation of Pnew and Preg rates as follows (Fernández et al., 2009; Raimbault and Garcia, 2008):

\[
P_{\text{new}} = \text{NO}_3^- \text{uptake} + \text{DONr associated with NO}_3^- \text{uptake} - \text{NO}_3^- \text{regeneration} + \text{N}_2 \text{fixation}
\]

\[
P_{\text{reg}} = \text{NH}_4^+ \text{uptake} + \text{DONr associated with NH}_4^+ \text{uptake} + \text{NH}_4^+ \text{regeneration} + \text{NO}_3^- \text{regeneration}.
\]

Short incubation times may lead to an overestimation of nitrogen fluxes (Lipschultz, 2008). However, short incubations (2–6 h) are usually recommended to minimize the recycling of $^{15}$NH$_4^+$ (e.g. Bronk and Ward, 2000). Given the length of the incubations used here (3–4 h), the nitrogen uptake fluxes measured are likely better represented as ‘gross uptake’ (inorganic nitrogen uptake into cellular biomass plus inorganic nitrogen fixed to DON) rather than as ‘net uptake’ (only uptake into biomass) (Bronk et al., 1994). Gross NO$_3^-$ and NH$_4^+$ uptake rates are then calculated as follows (Diaz and Raimbault, 2000; Raimbault and Garcia, 2008):

\[
\text{Gross NO}_3^- \text{uptake} = \text{NO}_3^- \text{uptake} + \text{DONr associated with NO}_3^- \text{uptake}
\]

\[
\text{Gross NH}_4^+ \text{uptake} = \text{NH}_4^+ \text{uptake} + \text{DONr associated with NH}_4^+ \text{uptake}.
\]

The gross f-ratio ($f_g$) is then expressed as:

\[
f_g = \frac{P_{\text{new}}}{(P_{\text{new}} + P_{\text{reg}})}.
\]

The contribution of N$_2$ fixation to Pnew could only be determined during T1 and T4, since this parameter was not measured during the other transects. The equations used to calculate each of the abovementioned rates are summarized in Table 1.

2.5. Dissolved inorganic and organic nutrient analysis

In situ (pre-incubation) NO$_3^-$, NO$_2^-$, NH$_4^+$, phosphate (PO$_4^{3-}$) and silicate (SiO$_4^{4-}$) concentrations were measured directly onboard. NH$_4^+$ was measured photometrically using a Shimadzu UVmini 1240 spectrophotometer at 630 nm. NO$_3^-$ and NO$_2^-$ were determined based on the method described by Grasshoff et al. (1983) and Bendschneider and Robinson (1952), respectively. PO$_4^{3-}$ was determined colorimetrically at 885 nm (Murphy and Riley, 1962). Silicate content was determined colorimetrically at 810 nm, (Carlberg, 1972). Post-incubation NH$_4^+$ concentrations were also measured onboard using spectrophotometry.

After the incubation period, water samples filtrated through precombusted GF/F filters were stored in 15 mL polyethylene vials (VWR) and stored frozen at −20 °C for analysis of NO$_3^-$, NO$_2^-$ and total dissolved nitrogen (TDN) ashore. These concentrations were measured using a AA3 Bran + Luebbe autoanalyzer with detection limits of 0.01 and 0.03 μM for NO$_3^-$ and NO$_2^-$, respectively. TDN concentrations were measured by oxidizing TDN to NO$_3^-$ using the Valderrama method (Valderrama, 1981). DON concentrations were estimated by subtraction of NO$_3^+$ + NO$_2^-$ + NH$_4^+$ (dissolved inorganic nitrogen − DIN) concentrations from TDN concentrations. Propagation of error calculations were used to estimate errors associated with DON concentrations (Bronk et al., 2000).

2.6. Chlorophyll a analysis

Chl a concentrations were estimated fluorometrically by means of an AU-10 Turner Designs bench fluorometer, previously calibrated with pure chlorophyll a (Sigma), according to Holm-Hansen et al. (1965). Seawater samples (500 mL) were filtered through Whatman GF/F filters. Pigments were extracted in cold acetone (90% v/v) for 24 h. The regression expression between CTD fluorescence (F) values and chlorophyll a concentrations was: Chl a = 0.233 × F + 0.361, with Chl a expressed in mg m$^{-3}$. The correlation coefficient was $r^2 = 0.71$. 
3. Results

In order to suppress daily variability caused by differences between the repeated transects (T1 to T4) and mesoscale variability introduced by filaments and eddies, the nitrogen fluxes presented here have been integrated in depth (surface to 40 m), and averaged into the three stages previously mentioned (i.e. Stage 1 = <13 d, Stage 2 = 13–55 d, and Stage 3 = >55 d; see Table 2).

Average gross NO$_3^-$ uptake was high in Stage 1 waters and decreased abruptly (one order of magnitude) in Stages 2 and 3, configuring a decreasing gradient from freshly upwelled to aged waters (Table 2). The regeneration of NO$_3^-$ was one to two orders of magnitude lower than gross NO$_3^-$ uptake (<0.5 mmol N m$^{-2}$ h$^{-1}$). Gross NH$_4^+$ uptake was significantly lower than gross NO$_3^-$ uptake in Stage 1 waters (t-test, p < 0.05), but not significantly different in Stages 2 and 3 (t-test, p > 0.05). Gross NH$_4^+$ uptake rates were not significantly different to NH$_4^+$ regeneration in Stages 1 and 3 (t-test, p > 0.05), but significantly higher than NH$_4^+$ regeneration rates in Stage 2 (t-test, p < 0.05). The maximum integrated N$_2$ fixation rate was 1.2 × 10$^{-3}$ mmol N m$^{-2}$ h$^{-1}$ (Table 2). These rates were too low to influence Pnew significantly (N$_2$ fixation contributed <0.05% to Pnew; Table 2).

Pnew decreased from Stage 1 to Stations 2 and 3 stations by an order of magnitude (Table 2). Indeed, Pnew was statistically different between the three Stages (one-way ANOVA, p < 0.05). Preg was similar at Stages 1 and 2 stations, decreasing to about half at Stage 3 stations. No statistical difference was found between Preg at different Stages (one-way ANOVA, p > 0.05). Preg was higher than Pnew at Stages 2 and 3 stations, although no statistical difference was found between Pnew and Preg in any of the three Stages (t-test, p > 0.05). Preg was strongly influenced by NH$_4^+$ regeneration (~30–60%; Table 2). The resulting f$_4$-ratios indicate the predominance of Pnew in freshly upwelled waters (Stage 1), and the importance of Preg in sustaining primary production as waters become older (Stages 2 and 3).

4. Discussion

4.1. Nitrogen fluxes associated to aging water parcels and the in situ planktonic community

Grouping stations according to their pseudo-age allowed us to follow nitrogen metabolism changes according to the increasing age of water parcels and the concomitant loss of nutrients and changes in the phytoplankton community (Hansen et al., 2014–in this volume). Average gross NO$_3^-$ uptake was fairly high in Stage 1 waters (pseudo-age <13 d), coinciding with low Chl a concentrations and high inorganic nutrient concentrations (see Table 1 in Hansen et al., 2014–in this volume). This lack of phytoplankton biomass accumulation indicates that the uptake of nutrients is rapid once they become available through upwelling, but the growth of phytoplankton cells takes place later according to the nutrients stored previously. The accumulation of phytoplankton biomass is therefore expected to occur in older waters (Barlow, 1982). In agreement with this, during our study the highest Pnew rates were observed in Stage 1 (Table 2). The high NH$_4^+$ regeneration measured in Stage 1 was likely driven by dinoflagellates and mixotrophic microflagellates, which were found in high abundances (Hansen et al., 2014–in this volume).

The consumption of nutrients from Stage 1 to Stage 2 (NO$_3^-$ concentrations dropped by ~5 μM, see Table 1 in Hansen et al., 2014–in this volume).
volume) caused an abrupt decrease in Pnew rates (Table 2), while an accumulation of phytoplanktonic biomass and a high abundance of diatoms was observed (see Fig. 7 in Hansen et al., 2014–in this volume). The dramatic decrease in SiO$_2$ availability from Stage 1 to Stage 3 stations (10–31 to <2 µM), and the increased temperature of the seawater (from ~13 to 16 °C) restricted the growth of diatoms at Stage 3, where a higher abundance of microflagellates and dinoflagellates was observed, accompanied by considerably high Preg rates (Table 2).

### 4.2. The importance of NH$_4^+$ regeneration

Similarly to other studies measuring inorganic nitrogen fluxes in coastal upwelling systems (e.g. Benavides et al., 2013), the results presented here (Preg rates ranging from 6.24 to 11.08 mmol N m$^{-2}$ h$^{-1}$; Table 2) corroborate the recently recognized importance of nitrogen regeneration in sustaining primary production in upwelling systems, stressing the importance of heterotrophic and mixotrophic plankton activity.

In general terms, the magnitude of the rates of gross NO$_3^-$ and NH$_4^+$ uptake and regeneration observed during our study are comparable to those observed in other upwelling systems. Gross NO$_3^-$ and NH$_4^+$ uptake were in the range of those measured in the Canary Current System: 5.04 and 5 mmol N m$^{-2}$ h$^{-1}$, respectively (Benavides et al., 2013). NO$_3^-$ regeneration rates were slightly higher than those measured in the Humboldt Current System (0.08 mmol N m$^{-2}$ h$^{-1}$) and in the Canary Current System (0.16 mmol N m$^{-2}$ h$^{-1}$) by Raimbault and Garcia (2008) and Benavides et al. (2013), respectively. However, NH$_4^+$ regeneration (ranging between ~41 and 62 mmol N m$^{-2}$ d$^{-1}$, considering that regeneration processes take place during 12 h per day) was higher than in other EBUS (e.g. 19 mmol N m$^{-2}$ d$^{-1}$ and ~8 mmol N m$^{-2}$ d$^{-1}$ in the Humboldt and Canary Current Systems, considering also 12 h of active NH$_4^+$ regeneration, respectively) (Benavides et al., 2013; Raimbault and Garcia, 2008). Indeed, NH$_4^+$ regeneration rates were in the same range as gross NH$_4^+$ uptake rates, affecting the final value of Preg significantly and indicating that Preg may be severely underestimated if NH$_4^+$ regeneration is not taken into account.

The length of incubations must be taken into account when comparing NH$_4^+$ regeneration rates across studies and ecosystems. The compilation of published average NH$_4^+$ regeneration rates points towards higher rates associated with shorter incubation times and vice versa. For example, studies using 12–24 h reported NH$_4^+$ regeneration rates ranging from 0.12 to 1.4 mmol N m$^{-2}$ h$^{-1}$—considering an average integration depth of 40 m (Díaz and Raimbault, 2000; Fernández and Raimbault, 2007; Fernández et al., 2009; Raimbault and García, 2008), while studies using 2–6 h incubation periods reported NH$_4^+$ regeneration rates ranging from 3.2 to 14.8 mmol N m$^{-2}$ h$^{-1}$ (Benavides et al., 2013; Dickson and Wheeler, 1995; Varela et al., 2003, 2005). Given the range of average NH$_4^+$ regeneration values for long (12–24 h) and short (2–6 h) incubation periods, we estimate that using short incubations can overestimate NH$_4^+$ regeneration rates by ~4 to 10%.

It is also worth noting that GF/F filters retain variable amounts of heterotrophic bacteria (Morán et al., 1999) and therefore their use may underestimate NH$_4^+$ uptake rates, if the uptake of regenerated nitrogen sources by heterotrophic bacteria is significant (Fawcett et al., 2011; Raimbault and García, 2008).

The regeneration of NH$_4^+$ may occur as a result of (1) protozoan's grazing pressure on phytoplankton, (2) NH$_4^+$ released during bacterial remineralization of organic matter, (3) as an excretory product of zoo-plankton, and (4) as a result of photochemical breakdown of dissolved organic matter (Bronk and Steinberg, 2008). While we do not have the data to discuss the possible importance of (4), the NH$_4^+$ regeneration rates measured in this study likely correspond to a combination of (1) and (2). The contribution of (3) can be disregarded since the volume of seawater enclosed for incubations with $^{15}$NH$_4^+$ (2 L, see Materials and methods) is not enough to represent sufficiently the in situ
concentration of zooplanktonic biomass (Harris et al., 2000) and thus NH$_4^+$ regeneration caused by zooplankton grazing is likely not well represented in our samples. Zooplankton-derived NH$_4^+$ regeneration rates for this cruise may be found elsewhere (Fernández-Urruzola et al., 2014–in this volume). In the case of (1), the change from a predominance of diatoms to a higher abundance of microflagellates and dinoflagellates in Stage 3 stations (Hansen et al., 2014–in this volume) supports the possibility of a higher grazing pressure on phytoplankton, leading to lower large phytoplankton biomass and higher NH$_4^+$ regeneration rates. The role of bacterial remineralization of organic matter (2) in driving the NH$_4^+$ regeneration rates observed may be questionable given the low bacterial protein production rates reported for this cruise (Bergen et al., in prep.).

Another nitrogen flux only recently added to f-ratio calculations is the inclusion of N$_2$ fixation as a source of Pnew (e.g. Raimbault and García, 2008). The maximum integrated N$_2$ fixation rate during our cruise was $1.2 \times 10^{-3}$ mmol N m$^{-2}$ h$^{-1}$ (Table 2), which is at the lower end of the previous N$_2$ fixation measurements in other EBUS such as the Humboldt Current System (e.g. 0.63–16 $\times 10^{-3}$ mmol N m$^{-2}$ h$^{-1}$; Fernández et al., 2011), and the Benguela Current System (0.0 to 7 $\times 10^{-3}$ mmol N m$^{-2}$ h$^{-1}$; Sohm et al., 2011). Sohm et al. (2011) suggested that N$_2$ fixation may be widespread in the Benguela Current System, and tentatively calculated a total contribution of 0.077 Tg N y$^{-1}$, which represents 5.3% of the N$_2$ fixation-basin-wide rate estimated for the Southern Atlantic Ocean by Großkopf et al. (2012) (data not shown). During our study gross NO$_3^-$ uptake rates were generally two to three orders of magnitude higher than N$_2$ fixation, resulting in a negligible contribution of N$_2$ fixation to Pnew (<0.05%; Table 2). Nonetheless, we must note that the N$_2$ fixation rates presented here may be somewhat underestimated. Our failure to totally dissolve the added $^{15}$N$_2$ into the sample seawater impedes us calculating accurate N$_2$ fixation rates (see Materials and methods). Furthermore, the incubations for N$_2$ fixation measurements were performed during the daylight (10:00 am to 07:30 pm), implying a poor contribution of unicellular diazotrophic cyanobacteria, which fix N$_2$ in the dark to avoid the photosynthesis-derived oxygen deactivation of the nitrogenase enzyme. The predominant N$_2$ fixation activity of these small (<10 µm) diazotrophs has been reported in all the four major coastal upwelling systems of the world (Benavides et al., 2011; Hamersley et al., 2011; Raimbault and García, 2008; Sohm et al., 2011), indicating that daytime incubations lead to an underestimation of the N$_2$ fixation potential of these waters. Despite that our N$_2$ fixation rates are subject to some methodological uncertainties, not even the maximum N$_2$ fixation rates reported by Sohm et al. (2011) would have contributed significantly to Pnew (0.04, 0.18 and 0.31% for Stages 1, 2 and 3, respectively, data not shown). This low contribution of N$_2$ fixation to Pnew is similar to what was previously found in the Canary Current System (Benavides et al., 2013).

In summary, the f$_r$-ratios (corrected for nitrogen regeneration and N$_2$ fixation — although not significantly influenced by the latter) were most importantly affected by NH$_4^+$ regeneration. The f$_r$-ratio only surpassed 0.5 in freshly upwelled waters (Stage 1) coinciding with high gross NO$_3^-$ uptake rates and high nutrient concentrations. In Stages 2 and 3, the f$_r$-ratio was almost equal (~0.3), which is similar to previous measurements in EBUS (e.g. 0.28 in the Humboldt Current System; Raimbault and García, 2008).

Our results indicate that Preg contributes substantially to primary production in the upwelling system off Namibia. This fact reinforces the need to measure NH$_4^+$ regeneration routinely when $^{15}$N uptake experiments are performed in the field, and to reconsider the role of Preg in coastal upwelling systems.

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