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# Nitrogen fixation in the Southern Ocean: a case of study of the Fe-fertilized Kerguelen region (KEOPS II cruise)

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# Abstract

 $N_2$  fixation rates were measured during the KEOPS2 cruise in the HNLC area of Southern Ocean and in naturally iron-fertilized waters (Kerguelen Island 49.25° S, 69.58° E) using the <sup>15</sup>N isotopic technique. We detected  $N_2$  fixation within the mixed layer at all stations, from the surface to 140 m depth. The data shows high variability with rates ranging between 0.42 and 20.11 nmol N L<sup>-1</sup> d<sup>-1</sup>. The highest rates were concentrated in the euphotic layer and maximum values were obtained north of polar front (station F-L), which coincide with a positive N<sup>\*</sup> ([NO<sub>3</sub>]–16[PO<sub>4</sub>]), high chlorophyll concentration

- and dissolved iron. N<sub>2</sub> fixation rates were also obtained in stations with moderate (A3-2;
- <sup>10</sup> E-4W) and also low (R-2) iron levels as well as Chl *a*, suggesting that beside the microbial biomass, its composition/structure is a driving factor controlling N<sub>2</sub> fixation activities. Molecular analysis showed a diazotrophic community dominated by heterotrophic bacterioplankton. Size fractioned experiments indicated that most of N<sub>2</sub> fixating activities came from < 5  $\mu$ m community and it was on line with molecular analysis revealing
- a low diversity diazotrophic community dominated by heterotrophic bacterioplankton. This study shows for first time N<sub>2</sub> fixation is occurring in the Southern Ocean, at rates exceeding previous reports for high latitudes. Our findings suggest an indirect role of dFe in the regulation of N<sub>2</sub> fixation through the enhancement of regenerated primary production and the availability of phytoplankton-derived dissolved organic matter, which
   in turn may stimulate heterotrophic bacterioplankton.
  - 1 Introduction

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Molecular nitrogen ( $N_2$ ) fixation, carried out by diazotrophs, adds nitrogen to the ocean and effectively contributes to the input of new N for new primary production (Falkowski, 1997) and exported production (Gruber and Sarmiento, 1997). Although it has been suggested that  $N_2$  fixation could balance N losses derived from anaerobic processes such as denitrification (Codispoti, 1995) and anammox (Dalsgaard et al., 2003) at the



scale of ocean circulation (1000 years), the N cycle in the current ocean seems to be out of balance. Part of the uncertainty precluding a better estimation of N inputs and outputs comes from early assumptions of  $N_2$  fixation only occurring in chronically oligotrophic conditions.

- It is currently accepted that N<sub>2</sub> fixation is not limited to photosynthetic organisms such as *Trichodesmiun* sp. (Capone et al., 2005) and other diazotrophic coccoid cyanobacteria (Zehr et al., 1998; Montoya et al., 2004). Molecular tools have allowed observing diazotrophic activity in unicellular cyanobacteria as well as non-photosynthetic bacterioplankton (Church et al., 2005; Falcón et al., 2004; Moisander et al., 2008; Riemann
- et al., 2010; Zehr et al., 2008, 1998, 2001). The genes for N<sub>2</sub> fixation have been observed in many aquatic habitats, even when biogeochemical assays have failed to detect N<sub>2</sub> fixation (Moisander et al., 2006). Although the abundance of N<sub>2</sub>-fixing bacteria is relatively low compared to non N<sub>2</sub>-fixing populations (Zehr and Kudela, 2011), the diversity of diazotrophs is increasingly important and it currently includes groups
   within alpha and gamma-proteobacteria and archaea (Loescher et al., 2014; Mehta
  - and Baross, 2006; Short et al., 2004).

There is a variety of strategies for diazotrophic communities to cope with oxygen (nitrogen fixation is an anaerobic process) as well as nutrient limitation. Specialized structures such as heterocysts in *Anabaena* and diazocyst in *Trichodesmiun* (Bergman

- and Carpenter, 1991) help diazotrophic organisms to prevent oxygen inhibition. Some diazotrophs also live in symbiosis with diatoms and dinoflagellates (i.e. the cianobacterium *Richelia*). Anaerobic microniches in aggregates of organic matter can also host significant N<sub>2</sub> fixation, especially in post bloom periods when bacterial abundance in organic aggregates increases (Riemann et al., 2000). Another possibility is to alternate
- <sup>25</sup> overnight N<sub>2</sub> fixation with oxygenic photosynthesis during the day (Berman-Frank et al., 2007) or using photofermentative metabolisms allowing fixing N<sub>2</sub> during 24 h such as UCYN-A (Tripp et al., 2010; Zehr et al., 2008).

 $N_2$  fixation can be limited by iron (Fe), which is an essential cofactor required in large quantities for this process (Raven, 1988; Falkowski, 1997). Nutrients such as phos-



phate ( $PO_4^{3-}$ ), has been suggested to co-limit diazotrophy along with Fe (Mills et al., 2004). The presence of inorganic nitrogen ( $NH_4^+$  and  $NO_3^-$ ) in high concentrations could also inhibit this process. However, there is substantial disagreement on this last point, because of contradictory results with cultivated strains and diazotrophic activity reports in environments with high concentrations of DIN (Mehta et al., 2003) and in productive coastal areas with  $NO_3^-$  concentrations higher than 5 µmol L<sup>-1</sup> (Voss et al., 2004; Fernandez et al., 2011; Riemann et al., 2010; Bird et al., 2005). These facts suggest that N<sub>2</sub> fixation requires relatively large inputs of energy, reducing power, auxiliary antioxidant enzymes and metallic co-factors such as iron and molybdenum in order to function (Kurdles et al., 2002).

<sup>10</sup> function (Kustka et al., 2003; Raven, 1988).

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Around 20 % of the global ocean shows High Nutrients Low Chlorophyl (HNLC) conditions (Blain et al., 2008a; Goeyens et al., 1998). Although the Southern Ocean is one of the largest HNLC areas of the world, natural and massive phytoplankton blooms occur annually in South Georgia, Crozet and Kerguelen Islands (Blain et al., 2007, 2008b;

- Pollard et al., 2007) due to natural iron fertilization. The Kerguelen area in particular has a deep reservoir of Fe coming from sediment contribution, biogenic particles regeneration and possibly lithogenic Fe dissolution (Chever et al., 2010). Natural Fe-fertilization efficiency can be 10 to 150 times higher than artificial Fe-fertilization with respect to atmospheric CO<sub>2</sub> sequestration bellow 200 m (Blain et al., 2007). This area provides
- a natural laboratory for studying scientific questions currently pressing the knowledge of the regulation of marine N<sub>2</sub> fixation. Temperature and physical forcing (particularly mesoscale activity) as a limiting factor needs to be better understood, as it is the occurrence of N<sub>2</sub> fixation in environments with micro molar concentrations of DIN and DIP. More importantly, its regulation via Fe availability and P and the effect of ammonium production via diazotrophic activity on the local N cycle need to be explored.

This study aims to investigate the occurrence of diazotrophic activity in naturally fertilized waters off the Kerguelen plateau and its relationship with Fe availability in natural fertilization conditions. The study area has attracted attention in the past, such as the project KEOPS1 that performed a detailed study of two contrasting sites, one located in



iron-fertilized waters above the plateau, and one located in offshore HNLC waters. The data for the present study were obtained from the KEOPS2 project that extended its study region to different sites located in iron fertilized waters east of Kerguelen Island.

## 2 Materials and methods

# 5 2.1 Sampling

The KEOPSII cruise was conducted between October and November 2011 (R/V *Marion Dufresne*) in the region of Kerguelen Islands in the Southern Ocean (49.25° S, 69.58° E; Fig. 1). The sampling strategy included two transects and four "process" stations illustrated in Fig. 1: St. R-2 in HNLC waters as a reference station, A3-2 above
the Kerguelen plateau, E-4W at the break of the plateau and F-L north of the Polar Front. A pseudo-Lagrangian study was also carried out in a stationary meander of the polar front characterized by strong mesoscale activity, hereafter referred as station E (Zhou et al., 2014). Four visits were carried out over one month time (E-1, E-3, E-4E, and E-5; Blain et al., 2014).

## 15 2.2 N<sub>2</sub> fixation rates

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Experiments were done at all process stations and at the pseudo-Lagrangian stations "E".

Physical-chemical parameters were obtained with a CTDO rosette (SBE 32) equipped with  $24 \times 10$  L Niskin bottles. Samples were taken from the niskin bottles at 7 depth levels between the surface and the base of the euphotic zone.

Natural abundances of N isotopes  $({}^{15}N/{}^{14}N)$  in particulate organic matter (POM) were determined at the same stations and depth levels as N<sub>2</sub> fixation experiments were carried out. The isotopic composition expressed as  $\delta^{15}N$  is used as a reference natural enrichment in  ${}^{15}N$  relative to  ${}^{14}N$  that will be compared to the corresponding



<sup>15</sup>N signal in N<sub>2</sub> fixation calculations, therefore avoiding the use of literature values (Montoya et al., 1996). For doing so, seawater samples (1 L in duplicate) were filtered using a vacuum pump system at each depth through 0.7 μm precombusted glass fiber filters (450°C – 12 h). Filters were then dried at 60°C and stored in similar conditions
 <sup>5</sup> until laboratory analysis by mass spectrometry (Termo Finnigan delta Plus).

Profiles of N<sub>2</sub> fixation were obtained at the 4 process stations described above and also during visits to pseudo-Lagrangian station "E" (Fig. 1, 8 sampling stations in total). Water samples (prefiltered by 25 µm) were taken in 1 L Nalgene bottles with septum caps, which were enriched with 2 mL of gas  ${}^{15}N_2$  (Montoya et al., 1996) injected with a gas tight syringe. After agitation, samples were incubated for 24 h using on deck 10 incubators that simulated light attenuation at different depth (black, 0.01, 0.3, 1, 4, 16, 25, 45, 75%, Lee filters<sup>®</sup>). Temperature was controlled by circulating surface seawater. Incubations were finished by filtration immediately after enrichment (T0) and after 24 h (Tf). Additionally, incubations were done for 2 size fractions (total and  $< 5 \,\mu m$ ) with an intermediate sampling time after 12 h of incubation.  $^{15}N_2$  incubations were filtered 15 through a peristaltic system using precombusted glass fiber filters (GF/F) of 0.7 µm pore (450°C for 12 h). Filters were recovered and dried at 60°C for 24 h. Subsequently, filters were kept at 60 °C until laboratory analysis through the IRMS (Isotope Ratio Mass Spectrometer Delta Plus, Finnigan). Rates were estimated according to previous studies (Fernandez et al., 2011; Montoya et al., 1996) and will be expressed as daily 20 rates as  $nmol L^{-1} d^{-1}$ .

## 2.3 Nutrients and picoplankton abundance

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Nutrient samples (10 mL) for the determination of  $NO_3^-$ ,  $NO_2^-$ , and  $PO_4^{3-}$  were filtered through 0.7 µm (GF/F) and analyzed on board with a Scalar autoanalyzer (Blain et al., 2014).

Samples for picoplankton abundance were collected on 2 mL cryotubes (1350  $\mu$ L seawater and 150  $\mu$ L of 1 % gluteraldehyde) and were frozen in liquid nitrogen. Sam-



ples were then stored at -80 °C for subsequent analysis using a BD FACSCalibur flow cytometer (Christaki et al., 2014; Marie et al., 2000; Vaulot et al., 1989).

# 2.4 Community composition of diazotrophs

Water samples were collected at station E-1 from 20 m depth for molecular characterization of the diazotroph community. In spite of an extended sampling effort (1 L water filtered at each process station and depth), amplification was only effective at station E-1 using large volume sampling filters. The following protocol corresponds to this effective sampling.

Between 15 and 20 L of prefiltered seawater (200 and 5 µm) was filtered onto 0.2 µm
SuporPlus membranes (142 mm diameter) and stored at -80 °C. All nucleic acid extractions where performed in triplicates by dividing the filters as described in a parallel study (Beier et al., 2014). Cellular lysis was performed with Proteinase K (2h incubation, 56 °C) and followed by mechanical disruption using low binding zirconium beads (OPS Diagnostics, Lebanon, NJ, USA). DNA concentration was measured by
Quant-it Picogreen dsDNA Assay (Invitrogen Life Technologies, Carlsbad, CA, USA) at 485 nm excitation wavelength and 535 nm emission wavelength in a Victor3 1420 Multilabel counter (PerkinEl, emr, Waltham, MA, USA) run with Wallac 1420 3. Genomic DNA extracts were stored at -20 °C until further processing. To amplify nifH genes, a nested Polymerase Chain Reaction (nested-PCR) was performed using re-

- 20 ported PCR primers (Zehr and McReynolds, 1989). The PCR mixture contained Go TAQ Green Master Mix 2x (Promega), 1 μL of 25 μM nifH3 (5'-ATR TTR TTN GCN GCR TA-3') and nifH4 (5'-TTY TAY GGN AAR GGN GG-3'), 10–40 ng μL<sup>-1</sup> of DNA extract and the volume was adjusted with water to obtain a 25 μL reaction. Thermal cycling for the first PCR was performed as follows: 5 min at 95 °C, 30 cycles of 1 min
- <sup>25</sup> at 95 °C, 1 min at 50 °C, and 1 min at 72 °C, followed by a final extension step of 7 min at 72 °C. The second PCR was carry out by same reagent mixture as above with internal nifH primers [50] 1  $\mu$ L of 25  $\mu$ M nifH1 (TGYGAYCCNAARGCNGA) and 1  $\mu$ L of 25  $\mu$ M nifH2 (ADNGCCATCATYTCNCC), however, the template volume was reduced



by 50 %. Second-round reactions were amplified with one denaturation step of 5 min at 95 °C, 30 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, and a final 7 min extension cycle at 72 °C. Several replicates of these reactions (n = 3) were combined and concentrated using the E.Z.N.A. Cycle-Pure Kit (Omega Bio-Tek). In both PCRs, negative and positive control using water and *Trichodesmiun* sp. DNA extract, respec-

- tively, were run together with the samples. The purified PCR products were cloned using the pGEM-T Easy vector systems according to the manufacturer's instructions (Promega). Sequencing was done by Macrogen Inc. (Korea).
- Phylogenetic analyses were performed using 34 valid sequences. The sequences
   were first vector trimmed. Low quality sequences where removed using SeqMan DNAstar software. Curated sequences were then aligned in the nucleotide space with MUSCLE (Edgar, 2004) and similarities were computed using Bosque software (Ramirez-Flandes and Ulloa, 2008). One operational taxonomic units (OTUs) were estimated using > 98 % of nucleotide similarity. Phylogenetic trees were constructed us ing a maximum-likelihood algorithm by Fast Tree (Price et al., 2010) (Bosque software, Ramirez-Flandes and Ulloa, 2008) using the OTU retrieved from the study area together with cultured representatives having nifH and similar environmental sequences retrieved from NCBI Blast. Sequence of the representative OTU was deposited in the

#### 20 3 Results

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## 3.1 Hydrographic conditions

GenBank database under accession number KM242081.

The study area holds a complex structure of circulation, water masses and fronts. The stations located north of the polar front (TNS1, 2, TEW1, 2, 7, 8 and F-L) are influenced by AASW (Antarctic Surface Water) and AAIW (Antarctic Intermediate Water) reflected in high temperatures (4°C), low salinity (< 34) and low density (< 27 kg m<sup>-3</sup>) in the upper 200 m (Fig. 2a and c). The stations south of the polar front however (TNS3-10,



A3-2, TEW3-6 and "E" stations) are mainly influenced by Winter Waters (WW) characterized by low temperatures (< 2 °C), lower salinity (> 34) and density between 27 and 27.5 kg m<sup>-3</sup> in the upper 200 m (Fig. 2a and c). The "E" stations showed a temperature increment (2.5 to > 3.5 °C) and a density decrease (> 27 to < 26.95 kg m<sup>-3</sup>) in the first 100 m over time (from E-1 to E-5; Fig. 3a).

The Mixed Layer Depth (MLD) showed high variability among stations (Park et al., 2014). Values of MLD in the HNLC area (R-2 station) and above the plateau (A3-2 station) were deeper (123 m) than the F-L station which showed a strong stratification and a shallow MLD (40 m; Table 1). The Photic Zone was defined as the depth where Photosynthetically Active Radiation (PAR) reaches 1 % and was also deeper at R-2 (92 m) than A3-2 (38 m) compared to F-L (26 m; Table 1). For the Lagrangian stations "E", MLD values were variable, with a maximum depth at the fourth visit E-4E (80 m) and minimum at the final sampling E-5 (38 m). On the contrary, Ze values were lower at E-4E (48 m) compared to E-5 (68 m; Table 1).

#### 15 3.2 Nutrients and fluorescence distribution

The general distribution of NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, and PO<sub>4</sub><sup>3-</sup> as well as stoichiometric conditions in the study area are reported in detail in this issue (Blain et al., 2014). The distribution of N\* (Gruber and Sarmiento, 1997; Michaels et al., 1996) showed a slight increment from 0 to 2 µmol L<sup>-1</sup> south of the TNS transect over 150 m and a remarkable increment over the MLD (0 to 8 µmol L<sup>-1</sup>) at stations north of the polar front (TEW7, 8 and F-L stations; Fig. 2b and d). This indicates an excess of N (probably NO<sub>3</sub><sup>-</sup>) with respect to PO<sub>4</sub><sup>3-</sup> in an area where a large diatom bloom developed during this study and high rates of nitrification were also detected (Cavagna et al., 2014; Lasbleiz et al., 2014). Stations "E" showed a decreasing evolution of N\* values over time (Fig. 3b) which match the increase in fluorescence (Fig. 3c) and nutrient utilization as the blooms develops. The vertical distribution of nutrients for all process stations is reported in Fig. 4. The reference station R-2 showed constant values of NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup> and PO<sub>4</sub><sup>3-</sup> concentrations in



the first 200 m (25 and  $2 \mu mol L^{-1}$  respectively). NH<sub>4</sub><sup>+</sup> remained at 0.2  $\mu mol L^{-1}$  values in the MLD (120 m) and decreased rapidly to near 0 values at 200 m (Fig. 4e). Station A3-2 showed  $NO_3^- + NO_2^-$  concentrations near 25 µmol L<sup>-1</sup> in surface waters and close to 30  $\mu$ mol L<sup>-1</sup> at 150 m. PO<sub>4</sub><sup>3-</sup> concentrations were constantly around 2  $\mu$ mol L<sup>-1</sup> in the MLD while high values of  $NH_{4}^{+}$  were observed in surface (> 0.2  $\mu$ mol L<sup>-1</sup> down to 80 m depth) as well as deeper layers (0.1  $\mu$ mol L<sup>-1</sup> at 150 m depth, Fig. 4f). Station E-4W showed high levels of  $NO_3^- + NO_2^-$  and  $PO_4^{3-}$  distributed homogeneously in the MLD  $(25-27 \text{ and } 1.5-2 \mu \text{mol L}^{-1} \text{ respectively, Fig. 4g})$ . However ammonium concentrations were close to 0 in surface waters but showed a dramatic increase to  $0.35 \,\mu\text{mol}\,\text{L}^{-1}$  over 100 m (Fig. 4g). Interestingly, station F-L showed the lowest surface concentrations of  $NO_3^- + NO_2^-$  (20 µmol L<sup>-1</sup>), which increased in subsurface layers to values exceeding 30  $\mu$ mol L<sup>-1</sup>. PO<sub>4</sub><sup>3-</sup> concentrations were also below average values (1.2  $\mu$ mol L<sup>-1</sup>) in surface waters that increased to  $2 \mu mol L^{-1}$  at 200 m. Ammonium concentrations showed a distinct subsurface peak of 0.5 µmol L<sup>-1</sup> at 60-80 m depth. Deeper values however were close to detection limit and reached 0 at 200 m (Fig. 4h). Concerning 15 station "E", a decrease in macronutrients was observed with time while the nutricline deepened from surface at E-1 to 100 m depth during the last visit (E-5). A NH<sup>+</sup><sub>4</sub> subsurface maximum was detected (0.4 to 0.65  $\mu$ mol L<sup>-1</sup>) which was twice as high as surface values (0.2 to 0.3 µmol L<sup>-1</sup>) suggesting an increase in remineralization in subsurface waters (Blain et al., 2014). 20

In general, a phytoplankton bloom with fluctuating biomass in time and space was developed at all stations, except for station R-2 (Fig. 4a) located in a permanent HNLC area (Blain et al., 2014; Lasbleiz et al., 2014). Station A3-2 showed surface fluorescence values > 2 μg L<sup>-1</sup> (Fig. 4b) with a main contribution of microphytoplankton with respect to picophytoplankton (Lasbleiz et al., 2014). Moreover, bacterioplankton was consistently present throughout the water column and characterized by a high relative abundance of HNA (High Nucleid Acid) bacteria at A3-2 (Fig. 4b). The highest fluorescence values (> 4 μg L<sup>-1</sup>) were found north of the polar front (F-L station; Fig. 4d),



with a main contribution of large diatoms (Lasbleiz et al., 2014). Also at F-L, bacterial abundance was high in the MLD (Fig. 4d) and included a high percentage of HNA and nanoeukaryotes which coincide with the distribution of Chlorophyll *a* (Christaki et al., 2014). For "E" stations fluorescence showed an increment from E-1 to E-5 (< 0.75 to > 1  $\mu$ g L<sup>-1</sup>) and a deepening of its vertical distribution (0.75  $\mu$ g L<sup>-1</sup> isoline) following the MLD (Fig. 3; Table 1). The contribution of picoeukariotes and nanoeukariotes were maximum at the beginning and at the end of sampling at the "E" stations and the microphytoplankton community. Bacterioplankton showed a maximum abundance (> 400 × 10<sup>3</sup> cells mL<sup>-1</sup>) at E-4 over 50 m which is consistent with the distribution of fluorescence (Fig. 3).

#### 3.3 Nitrogen fixation rates

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 $N_2$  fixation was detected at all the sampled stations with rates ranging between 0.4 (E-1, 129 m and 0% incident light) and 20.1 nmol NL<sup>-1</sup> d<sup>-1</sup> (F-L, 2 m and 75% incident light), with high spatial and temporal variability.

The reference station R-2 and station A3-2 showed similar average rates  $(4.45 \pm 2.6 \text{ and } 3.2 \pm 2.8 \text{ nmol}\text{NL}^{-1}\text{d}^{-1}$ , respectively) over the MLD (Table 1). N<sub>2</sub> fixation at these stations was concentrated mainly in the photic zone with values ranging between 1.05 and 7.9 nmolNL<sup>-1</sup>d<sup>-1</sup> at R-2 and between 2.09 and 6.49 nmolNL<sup>-1</sup>d<sup>-1</sup> at A3-2. However, rates at R-2 station increased (1.05 to 3.13 nmolNL<sup>-1</sup>d<sup>-1</sup>) under 1% light which also coincide with a dFe increment (Quéroué et al., 2014). Bacterial abundance and fluorescence for R-2 are constant and low in the MLD (< 300 × 10<sup>3</sup> cells mL<sup>-1</sup> and < 0.5 µgL<sup>-1</sup> respectively) as well as nutrients (Fig. 4a and e). But in A3-2, higher bacterioplankton and fluorescence values were observed (300–400 × 10<sup>3</sup> cells mL<sup>-1</sup> and 2 µgL<sup>-1</sup>, respectively) as well as a slight NH<sup>4</sup> subsurface peak (Fig. 4b and f).

Station E-4W station was located west of the meander and in the break of plateau. Average  $N_2$  fixation rates reached 5.41±2.2 nmol NL<sup>-1</sup> d<sup>-1</sup> over the MLD (Table 1), with



high values (8.56 nmolNL<sup>-1</sup>d<sup>-1</sup>) at 12 m and 16 % incident light (Fig. 4c). The highest N<sub>2</sub> fixation rates in this study were detected at station F-L, with an average rate of 10.27 ± 7.5 nmolNL<sup>-1</sup>d<sup>-1</sup> over the MLD (Table 1), the maximum values were obtained at surface (20.1 nmolNL<sup>-1</sup>d<sup>-1</sup> at 2 m and 75 % incident light) and decreased with depth (2.2 nmolNL<sup>-1</sup>d<sup>-1</sup> at 20 m and 4 % incident light, Fig. 4d). Fluorescence values at both stations were high compared to R-2 and A3-2, with values between 2 and 4 µgL<sup>-1</sup> for E-4W and F-L respectively, which was consistent with bacterial abundance (> 600 × 10<sup>3</sup> cellsL<sup>-1</sup>) over MLD (Fig. 4c and d). The vertical nutrient distribution showed DIN utilization in surface waters and a rapid increase in concentrations under the MLD.
<sup>10</sup> Also, at both stations a subsurface NH<sup>4</sup><sub>4</sub> peak (0.35 and 0.42 µmolL<sup>-1</sup> respectively) was observed (Fig. 4g and h). It should be noted that the MLD and photic layer were

shallow compared to R-2 and A3-2 (Fig. 4a-c and d, Table 1).

During successive measurements at station "E", N<sub>2</sub> fixation rates coincided with decreasing values of  $\delta^{15}$ N in particulate organic matter (POM; Fig. 5a and b), which is consistent with the isotopic fractionation associated to POM created via N<sub>2</sub> fix-

ation (Zahn et al., 1994). The highest rates were detected at the beginning and end of the time series (average over the MLD 3.5±2.8 nmolNL<sup>-1</sup> d<sup>-1</sup> for E-1 and 4.8±1.2 nmolNL<sup>-1</sup> d<sup>-1</sup> for E-5; Table 1) which coincided with the fluorescence distribution (Fig. 3), particularly with picoeukaryotes and nanoeukaryotes. Moreover, the highest rates were observed within the photic layer (Table 1) with values between 1.8–7.6 nmolNL<sup>-1</sup> d<sup>-1</sup>, while subsurface rates reached 1.1 nmolNL<sup>-1</sup> d<sup>-1</sup> in average down to 140 m (Fig. 5a)

A multivariate Principal Components Analysis (PCA) indicated that the variables that mainly influenced the physical-chemical conditions at the contrasting stations sampled were  $PO_4^{3-}$ ,  $NO_3^{-}$ , density, temperature and Chlorophyll *a*. Station F-L was associated mainly with temperature, Chlorophyll *a*, dFe (data reported in Queroué et al., 2014) and NH<sub>4</sub><sup>+</sup>. Stations A3-2, E-3 and E-4W on the other hand were associated mainly with  $PO_4^{3-}$ ,  $NO_3^{-}$  and density, while R-2 and E-5 were negatively related to these variables. This coincides with the influence of Antarctic Surface Waters (AASW) and Winter Wa-



ters (WW), as well as with the nutrient distribution and utilization at each zone. The correlation between PC1 and N<sub>2</sub> fixation rates over the MLD was significant (p = 0.01) with a negative correlation coefficient (r = -0.9). Therefore N<sub>2</sub> fixation seems overall correlated with Chlorophyll *a*, dFe, NH<sub>4</sub><sup>+</sup> and temperature.

#### **5 3.3.1 Size fraction and time course experiments**

During time course experiments of N<sub>2</sub> fixation, rates were obtained at 12 and 24 h incubation times (Fig. 6a and b), with different incident light levels (75, 25 and 1%) and size fractionation (total community and < 5 µm). At st R-2 there were no significant differences between treatments although an increase in total community rates was observed  $(1.19 \pm 0.8 \text{ nmol N L}^{-1} \text{ d}^{-1} \text{ at } 12 \text{ h to } 3.8 \pm 0.9 \text{ nmol N L}^{-1} \text{ d}^{-1} \text{ at } 24 \text{ h})$  with respect to the lower size fraction at 24 h (Fig. 6a). At st A3-2, a significant difference was observed over time for 75 and 25% light levels (*p* = 0.002, Tukey test: 0.0016 and 0.027, respectively) with maximum rates at 12 h (3.39 ± 0 and 4.80 ± 2.4 nmol N L<sup>-1</sup> d<sup>-1</sup>, respectively) and a decline at 24 h (2.55 ± 0.7 and 2.02 ± 1.5 nmol N L<sup>-1</sup> d<sup>-1</sup>, respectively)

- tively). No significant differences between light intensities were found, but the decrease was less significant at 75% (Fig. 6b). At st E-3 no significant differences were observed, but the total community incubated at 75% light had a positive evolution over time  $(3.65 \pm 0 \text{ nmol N L}^{-1} \text{ d}^{-1}$  at 12 h to  $5.1 \pm 1.4 \text{ nmol N L}^{-1} \text{ d}^{-1}$  at 24 h) while the total community incubated at 1% light showed decreasing rates at 24 h. Finally, at F-L sta-
- <sup>20</sup> tion the highest rates were observed for both size fraction and both light intensities. Significant differences between both size fractions were obtained (*t* test *p* = 0.039), where the maximum rates were at 12 h, with a decay at 24 h for the total community. Also rates from the total community incubated at 75 and 25 % light has a significant increase between 0 and 12 h (ANOVA, *p* < 0.001 Tukey test, *p* < 0.001) reaching to
- <sup>25</sup>  $9.07 \pm 0$  and  $7.31 \pm 0.3$  nmol N L<sup>-1</sup> d<sup>-1</sup> respectively. Overall, the lack of significant differences between size fraction suggest a dominant role of < 5 µm diazotrophs in this area.



## 3.4 Community composition of diazotrophs

We detected the nifH gene only at 20 m in E-1 station. The community structure was characterized by low diversity represented by 1 Operational Taxonomic Unit (OTU), which included 34 sequences with a > 98 % similarity in the nucleotide space. The phylogenetic analysis showed that all sequences are affiliated to Cluster I and to the

<sup>5</sup> phylogenetic analysis showed that an sequences are annated to cluster 1 and to the subcluster 1J, 1U and 1K previously reported by (Zehr et al., 2003), which are associated to heterotrophic bacteria. The OTU obtained in this study was similar to sequences recovery from diverse ecosystems (Fig. 7) such as the Eastern Tropical South Pacific (Fernandez et al., 2011), North Pacific (Zehr et al., 2001), Mediterranean Sea
 <sup>10</sup> (Man-Aharonovich et al., 2007) and some sequences derived from symbionts of marine organisms such as dinoflagellates (Farnelid et al., 2010). On the other hand, the sequences obtained were not related to possible contaminants previously reported (Turk-Kubo et al., 2013).

4 Discussion

<sup>15</sup> This study provides the first evidence of high N<sub>2</sub> fixation rates for the Southern Ocean, the largest HNLC zone in the global ocean. It shows the occurrence of this process in a variety of Fe-fertilized conditions, including HNLC and iron fertilized waters at the polar front.

Temperature has been suggested as a limitation factor for the distribution of diazotrophic communities, since the highest rates reported to date are associated to temperate waters (Karl et al., 2002; LaRoche and Breitbarth, 2005). However through this study we demonstrated that N<sub>2</sub> fixation can also occur at low temperatures (< 4 °C), which is in concordance with recent reports of N<sub>2</sub> fixation in the Artic zone (Blais et al., 2012). We therefore support the idea that N<sub>2</sub> fixation is an ubiquitous process.

Although we obtained high  $N_2$  fixation rates, we also observed high spacial and temporal variability. The  $N_2$  fixation rates detected in the Southern Ocean, in general,



were higher than in other HNLC and oligotrophic areas, (up to 5 times higher; Table 2) considering integrated values. Moreover our average integrated rate ( $265 \mu mol m^{-2} d^{-1}$ ) for the study area are two orders of magnitude higher than rates reported for the Arctic (Blais et al., 2012) and Mediterranean sea (Yogev et al., 2011). High temporal and spatial variability has indeed been reported for other marine ecosystems e.g. Eastern

- <sup>5</sup> spatial variability has indeed been reported for other marine ecosystems e.g. Eastern Tropical South Pacific including the Oxygen Minimum Zone (Dekaezemacker et al., 2013; Fernandez et al., 2011). Field experiments suggest that this variability can be linked to nutrients availability (mainly DIP and Fe) and environmental conditions (e.g. ENSO).
- The role of Fe in controlling N<sub>2</sub> fixation is centered in its importance as a co-factor for the nitrogenase enzyme (Mills et al., 2004; Dekaezemacker et al., 2013; Kustka et al., 2002). Several studies have been carried out in recent years, which included addition of different amounts of Fe to microcosms experiments and measurements of N<sub>2</sub> fixation rates over time. Results have been highly variable and unconclusive on the ultimate regulation factor for this process (Sohm et al., 2011). However, a model study at global
- regulation factor for this process (Sohm et al., 2011). However, a model study at global scale showed that Fe (as dust) is not the main controlling factor of this process (Luo et al., 2014). The KEOPS2 cruise allowed exploring the link between Fe and N<sub>2</sub> fixation in naturally fertilized conditions, where the control was a permanent HNLC condition area, and stations with different Fe concentrations were sampled.

According to our PCA results, N<sub>2</sub> fixation could be co-regulated by dFe and primary productivity off the Kerguelen plateau. Higher rates coincided with high levels of primary production and phytoplankton standing stock (Cavagna et al., 2014) which in turn is stimulated by Iron fertilization. Indeed, the strongest average rates found at F-L over the MLD (10.27 nmol L<sup>-1</sup> d<sup>-1</sup>) could be related to a high dFe supply (Quéroué et al., 2014) which is the back to be the back to be a high dFe supply (Quéroué et al., 2014).

<sup>25</sup> 2014) which in turn favors high phytoplanktonic biomass (>  $4\mu$ gL<sup>-1</sup>) related with diatoms (Lasbleiz et al., 2014). Besides, in this station as well as the others, maximum rates coincided with high incident light (e.g. 20.1 nmolL<sup>-1</sup> found at 75% light for F-L) and a shallow MLD (Table 1). Remarkably, the highest N<sup>\*</sup> values were also found at F-L (~  $8\mu$ molL<sup>-1</sup>) and could be partially explained by the active N<sub>2</sub> fixation although its



contribution is surely combined with that of other major processes such as nitrification (Cavagna et al., 2014). Stations E-4W and A3-2 showed moderate N<sub>2</sub> fixation rates (5.41 and 3.27 nmol L<sup>-1</sup> d<sup>-1</sup>, respectively, see Table 1). These rates also coincided with high phytoplankton stocks (Lasbleiz et al., 2014) although lower than F-L, and presented a different community structure of mainly large diatoms compared to F-L and R-2.

Station R-2 showed a low phytoplankton biomass (<  $1\mu g L^{-1}$ ) and low dFe supply, thus we expected low N<sub>2</sub> fixation rates. However, rates within the MLD (both station with similar MLD) were moderated and exceeded rates obtained at A3-2 (4.45 and 3.27 nmol L<sup>-1</sup> d<sup>-1</sup> average, respectively). This could be related to the phytoplankton community supported mainly by nano and picoplankton, and also by the deep MLD at both stations.

In parallel, the evolution observed at stations "E" showed higher rates at the last visit, E-5, which coincided with fluorescence distribution (Fig. 4), changes in phytoplankton community structure (Lasbleiz et al., 2014) and the <sup>15</sup>N isotopic fractionation of POM (according to which low  $\delta^{15}$ N values were found in POM at E-5 compared to E-1).

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These records show that the magnitude and time variability of  $N_2$  fixation rates could be related with primary production and/or with phytoplankton community structure, and therefore with the availability and origin of dissolved organic matter in the system. This

- hypothesis is consistent with findings suggesting a relationship between N<sub>2</sub> fixation rates in the Eastern and Tropical South Pacific and the availability (Dekaezemacker et al., 2013; Bonnet et al., 2013) and quality of DOM (Fernandez et al., 2014) derived from phytoplankton (although the rates found at the tropical HNLC area in the ETSP are 5 times lower than rates found in our study). The diazotrophic community was available to the study with a study with the study of the study of the study of the study.
- <sup>25</sup> was evaluated in this study using nifH gene, which was successfully amplified just for E-1 station (at the beginning of the bloom). The phylogenetic analysis showed one OTU corresponding to 33 sequences all within Cluster I and associated to subcluster 1J, 1U and 1K, which are related to heterotrophic bacteria, which would support the idea of an important role of organic matter in the regulation of N<sub>2</sub> fixation processes. These also



coincide with other phylogenetic studies mainly at South Pacific area where N<sub>2</sub> fixation is dominated by heterotrophic diazotrophs (Fernandez et al., 2011; Halm et al., 2012; Dekaezemacker et al., 2013; Bonnet et al., 2013; Loescher et al., 2014). Considering the low diversity found we cannot dismiss the possibility of other diazotrophic organ-

- <sup>5</sup> isms such as cyanobacteria and/or symbionts. This suggestion is supported based on the heterogeneity observed in the Kerguelen bloom and the differential response of the diazotrophic community during the size-fractionation experiments which showed that larger fractions are enhanced during long incubations at some stations and light incidences including HNLC conditions (Fig. 6).
- <sup>10</sup> Although the limitation of N<sub>2</sub> fixation by Fe and co-limitation by Fe and  $PO_4^{3-}$  e.g. (Mills et al., 2004; Dekaezemacker et al., 2013) is generally accepted, the occurrence of diazotrophy in HNLC conditions and in Fe fertilized water suggest that the role of Fe in the N<sub>2</sub> fixation processes is indirect, mainly stimulating primary production and consequently diazotrophic activity by providing DOM from associated phytoplankton
- <sup>15</sup> communities. Moreover the Fe limitation for diazotrophy is known to control diazotrophic cyanobacteria such as *Trichodesmiun* (Bonnet et al., 2009), which were not detected in this study. On the other hand, the dFe supply and the consequent phytoplankton bloom development were heterogeneous in magnitude, space and time during the KEOPS2 cruise (Quéroué et al., 2014). This variability mirrors the spatial and time variability
- of N<sub>2</sub> fixation in the Kerguelen region. However, it is also possible that diazotrophic communities remained limited by dFe during the bloom in spite of DOM availability as heterotrophic bacterioplankton (the only diazotrophic group identified in this study) seems to be outcompeted for dFe by higher trophic levels during the fertilization event (Fourquez et al., 2014). It is evident that further efforts are necessary to achieve a better characterization of diazotrophic activity in the Southern Ocean.

The biological production at the HNLC area in the KEOPS study region (st R-2) is mainly supported by regenerated production, which means that the N source for primary producers is primarily  $NH_4^+$  compared to  $NO_3^-$  uptake (Cavagna et al., 2014). The N<sub>2</sub> fixation rates found at R-2 (where the dFe supply is low and limits



primary production) were in the same range of the A3-2 and "E" stations (meander area), which was unexpected. Overall, this N source should be a new source for primary producers in all conditions; therefore new production during the bloom could be underestimated and regenerated production overestimated. Considering an average NO<sub>3</sub> uptake rate of 0.94 umol L<sup>-1</sup> d<sup>-1</sup>, N<sub>2</sub> fixation could add an additional 0.5% (0.005 umol L<sup>-1</sup> d<sup>-1</sup>) to new primary production (corrected by nitrification using an average rate of 0.23 umol L<sup>-1</sup> d<sup>-1</sup>, Fernandez unpublished data). Moreover, N<sub>2</sub> fixation is likely to co-occur with nitrification, which was found over 200 m in the water column with high rates (Cavagna et al., 2014; Dehairs et al., 2014; Fernandez et al. unpublished data). Ammonium by diazotrophy could support 20% of ammonium oxidation in the study area considered. In this context, if the entire N fixed by diazotrophy is transformed to NO<sub>3</sub>, this process could contribute to the excess of N observed at the end of the bloom (KEOPS1)

# 5 Conclusions

- <sup>15</sup> This is the first record of N<sub>2</sub> fixation and a first phylogenetic approach of diazotrophic community for the Southern Ocean in a naturally Fe fertilized area. This contribution, combined to others studies confirm that diazotrophy is a ubiquitous processes. In addition, dFe concentrations had an indirect effect on N<sub>2</sub> fixation through primary production, since higher rates were observed in surface waters of areas with major phy-
- <sup>20</sup> toplankton stocks. The diazotrophic community composition was mainly heterotrophic, which complement the idea of N<sub>2</sub> fixation is related to DOM availability mainly release by phytoplankton and as a consequence related with community composition of phytoplankton (DOM quality). Therefore the regulation of N<sub>2</sub> fixation by nutrient and Fe availability should be reconsidered, and further studies should include Fe availability as
- $_{\rm 25}\,$  well as phytoplankton and the diazotroph community composition. Finally, the spatial coverage of our study is limited, but considering that N<sub>2</sub> fixation can occur in Fe limited areas as well as other Fe fertilized zones in the Southern Ocean, the significance of



this process at the basin scale is underestimated by our data. This has implications for the assessment of the discordance between input and outputs of N to the global ocean.

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<b>Discussion</b> Pape	-	<b>BGD</b> 11, 17151–17185, 2014						
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**Table 1.** Sampling stations visited during the KEOPS2 cruise and rates of  $N_2$  fixation, ammonium and nitrate assimilation obtained.

Statio	n Latitude (° S)	Longitude (° E)	MLD (m)	PZ (m)	$N_2$ fixation rate (nmol L <sup>-1</sup> d <sup>-1</sup> )	$ ho NH_4^+$ (µmol L <sup>-1</sup> d <sup>-1</sup> )*	$ ho NO_3^-$ (µmol L <sup>-1</sup> d <sup>-1</sup> )*
R-2	50.3834	66.6835	123	92	$4.45 \pm 2.6$	$0.44 \pm 0.01$	$0.029 \pm 0.01$
A3-2	50.6168	72.0501	123	38	$3.27 \pm 2.8$	$0.6 \pm 0.02$	$0.55 \pm 0.36$
E-4W	48.7503	71.4168	64	31	$5.41 \pm 2.2$	$0.004 \pm 0.001$	$0.8 \pm 0.53$
F-L	48.5169	74.6501	47	29	$10.27 \pm 7.5$	$0.33 \pm 0.3$	$1.9 \pm 1.4$
E-1	48.4501	72.1834	133	64	$3.48 \pm 2.8$	$0.041 \pm 0.02$	$0.11 \pm 0.08$
E-3	48.7000	71.9667	40	68	$2.59 \pm 0.5$	$0.076 \pm 0.04$	$0.153 \pm 0.03$
E-4E	48.7003	72.5502	80	48	$2.82 \pm 1.2$	$0.10 \pm 0.03$	$0.26 \pm 0.17$
E-5	48.4002	71.8836	38	54	$4.82 \pm 1.1$	$0.08 \pm 0.04$	$0.18 \pm 0.14$

\* Data reported in Cavagna et al. (2014).

**Table 2.** Comparative table of areal N<sub>2</sub> fixation rates of different regions of the global ocean. HNLC (high Nutrient Low Chlorophyll), ETSP (Easter Tropical South Pacific), SPG (South Pacific Gyre), WEP (Western Equatorial Pacific), NPO (North Pacific Ocean), TNA (Tropical North Atlantic) and STNA (SubTropical North Atlantic).

$N_2$ fixation rate (µmol m <sup>-2</sup> d <sup>-1</sup> )	Region	Source
4-53 94 6.5 48 2.6 18 10 37-47 4	HNLC zone ETSP SPG Arctic Zone HNLC zone ETSP Mediterranean Sea HNLC zone WEP Oligotrophic NPO TNA STNA	(Dekaezemacker et al., 2013) (Halm et al., 2012) (Blais et al., 2012) (Fernandez et al., 2011) (Yogev et al., 2011) (Bonnet et al., 2009) (Needoba et al., 2007) (Falcón et al., 2004)





**Figure 1.** Location of sampling stations in Kerguelen Island. Black dots represent the location of R-2 station (HNLC area). Red dots are location of the processes stations A3-2, E-4W and FL. Green dots represent the location of "E" stations with a Lagrangian framework.





**Figure 2.** Sectional distribution of density and N<sup>\*</sup> for both transects. (a) Density  $(kgm^{-3})$  and (b) N<sup>\*</sup> for the north to south transect (TNS), (c) density  $(kgm^{-3})$  and (d) N<sup>\*</sup> for the west to east transect (TWE).





**Figure 3.** Sectional distribution of density  $(kgm^{-3})$ , N<sup>\*</sup>, fluorescence  $(\mu gL^{-1})$  and bacterial abundance  $(10^3 \text{ cells mL}^{-1})$  for "E" stations. **(a)** Density, **(b)** N<sup>\*</sup>, **(c)** fluorescence, and **(d)** bacterial abundance.





















**Figure 7.** Phylogenetic tree including 34 nucleotide nifH sequences obtained from the study site (E-1 station, 20 m), the sequences are represented in one OUT and sequences form uncultured and cultures diazotrophic groups. The values represent the number of boostrap in base of 1000 replicates from mayor cluster to subclusters. The subcluster indicated at right side of the tree it was obtained from previous reports (Zehr et al., 2003; Turk-Kubo et al., 2013).

