Effects of penguin guano and moisture on nitrogen biological fixation in maritime Antarctic soils.

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Abstract

Biological nitrogen fixation (BNF) is a high energy-demanding process that may be inhibited by penguin guano. We tested this hypothesis in Ardley Island by measuring BNF in biological soil crusts (BSC) directly within a Penguin Colony and in sites unaffected by penguins. We also explored the effect of adding guano to BSCs in sites free of the influence of penguins. Water availability is also one of the most limiting element for life in the Antarctica and we expected that a wetter growing season would stimulate BNF. To evaluate the effect of moisture on BNF we added water to BSCs under laboratory conditions and estimated BNF by means of the acetylene reduction assay during three growing seasons (2012, 2013 and 2014), with contrasting temperature and precipitation conditions. The results reveal an almost complete inhibition of N fixation in the BSCs of the Penguin Colony. In sites free of ammonium and phosphate in rainwater, BNF rates reached up to 3 kg N ha^{-1} y^{-1} during warmer and wetter years. The addition of guano to BSCs significantly inhibited the rates of BNF. In laboratory incubations, the addition of water significantly stimulated rates of BNF during the warmer growing season with more sunshine hours. The likely increases in soil moisture levels due to climate change and glacier melting in the Antarctic Peninsula may enhance the rates of BNF. However, this may be constrained by accompanying changes in the distribution of penguin colonies.

Key words: Biological nitrogen fixation, Ardley Island, Penguin Colony, palaeo-beaches

Introduction

Several palaeo-beaches along the shoreline of the South Shetland Islands provide evidence for isostatic uplift during the Holocene, as the land rebounded after glacial melting (Fretwell et al. 2010). As the raised beaches emerged and formed part of the terrestrial landscape, weathering, *i.e.* biological, chemical and physical pedogenetic processes occurred at the surface. In recently exposed substrates, the biogenic elements of soils such as carbon (C) and nitrogen (N) are virtually absent. However, phosphorus (P) may be highly available in P rich minerals. Biological nitrogen fixation (BNF) is a key process incorporating new N to ecosystems and is favoured under N depletion and P abundance. Therefore, the beginning of ecosystems is completely dependent on the performance of diazotrophic bacteria that feed on di-nitrogen and perform this key ecosystem service. Diazotrophic bacteria (e.g. *Nostoc* spp) accomplish N fixation using the nitrogenase enzyme, that catalyzes the breaking of the triple bond of elemental N and transform it to ammonium in an energy costly reducing process. The nitrogenase enzyme is inhibited by high N availability (Zuberer 1998), which in turn reduces the costs of incorporating new N to the ecosystem. Free living or symbiotic diazotrophs in cryptogamic flora assist in the formation of biological soil crusts (BSCs) which are the first assemblages of organisms to become established on denuded soil (Chapin et al. 2002), initiating ecosystem development of N and C stocks after large landscape disturbances. The bacteria that live symbiotically with the BSC forming cryptogams provide nearly half of the N inputs via BNF to terrestrial ecosystems (Ellbert et al. 2012).

Studies in maritime Antarctica report that soil N and P levels are extraordinarily high, with C/N ratios even lower than in temperate regions (Beyer et al. 2000 a, b). The authors attributed the N and P enrichment to seabird droppings and aerosols transported long distances. In situ activity of birds, such as penguins, has led to the development of ornithogenic soils formed by the strong weathering on loams and gravels promoted by guano, and in parallel increasing the rates of net N mineralization, soil respiration and soil enzymatic activity (Tscherko et al. 2003). This suggests that N is not a limiting factor for the development of vegetation (Beyer et al. 2000 a, b). It is well documented that birds affect the composition of cryptogamic flora and its distribution in Antarctica and the accumulation of C and P in soils (Tatur 2002; Simas et al. 2007; Michel et al. 2010). How N accumulates in non-ornithogenic soils is less well understood. For maritime Antarctica it has been postulated that N is not a limiting factor for plant physiological processes as N is abundantly provided from penguin colonies (Robinson et al. 2003). Recent studies have reported long distant transport of N and P emitted from penguin guano (Crittenden et al. 2015; Zhu et al. 2014), which can increase N and P availability in soils even in sites located far distant in the wind direction from the penguin colonies. Even under low N availability in the cold deserts of Antarctica endolithic cryptogamic communities are not N-limited because of its low N requirements for photosynthesis (Johnston and Vestal 1991). These findings raise questions about why rates of BNF in BSCs are limited in areas of guano deposition and in areas relatively free of bird droppings.

We argue that in non-ornithogenic ecosystems, BSCs that fix N from the atmosphere play a major role in the accumulation of N in soils. We also postulate that bird droppings, either direct via guano deposition and further N mineralization from urea or indirect by long-distance transport via deposition of ammonium, would inhibit nitrogenase activity in BSCs, in a negative feedback mechanism, but enhance the denitrification rates of soils, in a positive feedback mechanism.

Additionally, as water is one of the principal limiting factors of biological activity in the Antarctic we also hypothesise that increased moisture levels during wetter growing seasons enhance the rates of BNF. It is anticipated that rates of BNF would be higher in the BSCs on the older sites than on the younger sites because the limiting elements, such as C and P, that control BNF in Sub-Antarctic glacier forelands accumulate through time with the development of soils (Arróniz-Crespo et al. 2014; Pérez et al. 2014).

The main objective of this study was to test the following specific hypotheses: i) BNF is inhibited in the BSCs at the Penguin Colony site and the addition of guano inhibits BNF in BSCs in sites located in the upwind direction and ii) the addition of water to BSCs under laboratory conditions increases BNF. Furthermore, from the high N and P emission rates reported for ornithogenic soils in the study site (Sun et al. 2002; Zhu et al. 2008; Zhu et al. 2014), we expect a corresponding high ammonia and phosphate concentrations in rainwater and soil and high denitrification rates. Our findings have advanced our understanding of how BNF and, therefore, how primary productivity in maritime Antarctica will respond under increasing warming, following the trend observed during the last five decades, together with higher moisture levels in soils due to increases in precipitation and glacier melting (Vaughan 2006).

Study Area and Methods

Study Sites

Ardley Island is a 1.2 km^2 ice-free land surface that lies off the south east coast of Fildes Peninsula, at the extreme south of King George Island, the largest of the South Shetland Islands (Fig.1). Six study sites were selected along an east-to-west transect that crosses the Ardley Island from a Penguin Colony at the easternmost site, to an area upwind of the

Penguin Colony, on the western coast free of influence from the penguins (Fig.1). Ardley Island is one of the Antarctic Specially Protected Areas and represents a natural laboratory to test the hypotheses as the landscape is shaped by a chronosequence of palaeo-beaches located upwind of a colony of Gentoo Penguin (*Pygoscelis papua*). Data from the Bellingshausen Meteorological Station located in the close vicinity of the study sites indicate a mean annual precipitation of ca. 700 mm y^{-1}, a mean summer temperature of 1.5°C (January/February) and a mean winter temperature of -6.5°C (July/August). Strong winds predominantly come from the west with speeds that commonly exceed 100 km h^{-1} (Peter et al. 2008). Sites were sampled during three field campaigns in February 2012, 2013 and 2014, during which contrasting temperature and humidity data were recorded at the Bellingshausen Meteorological Station. February 2012 had the highest maximum temperature, precipitation and hours of sunshine (Table 1). February 2013 had similar temperatures to 2012, but less than the half of sunshine hours than the preceding year. February 2014 had the lowest maximum and mean temperatures, but similar amount of precipitation to 2013, which were also lower than 2012 (Table 1). Soil types of the study area belong to Histic Ornithic Crysols and Folic Crysols (Michel et al. 2010).

A site where the BSC is dominated by the mosses *Sanionia uncinata* and *S. georgicouncinata,* and the algae *Prasiola crispa* was selected at the margins of the penguin colony (Penguin Colony site) located at the north-eastern shore of the island (Fig. 1). A second site not occupied by penguins and with the BSC dominated by the mosses *Sanionia uncinata*, *Chorizodontium aciphyllum* and the lichens *Usnea* spp. was identified about 400 m upwind from the Penguin Colony at a plateau about 40 m above sea level, showing polygonal soil pattering evidencing an active process of cryoturbation. This site is called “Patterned Ground”. A third site is located ~ 1100 m westward from the Penguin Colony

(Fig. 1), where the soil is covered by a BSC dominated by lichens *Usnea* spp, *Psoroma hypnorum*, *Pannaria hookeri* and the moss *Chorizodontium aciphyllum*. This site is called the Reference Site, as it does not belong to the adjacent chronosequences of palaeobeaches described below and, therefore, it is considered to be a control for the time variable. A chronosequence of palaeo-beaches is located on the south-western shore of the island and about 1200 m away from, and upwind of the Penguin Colony (Fig. 1). Three continuous palaeo-beaches (PB) were selected; PB 7, is the oldest and estimated to have formed ca. 7200 cal. yrs BP, an intermediate stage PB 5 estimated to between 2500-4400 cal. yrs BP and the youngest, PB 2 estimated to have formed between 650-200 cal. y BP (Fretwell et al. 2010). BSCs on PB 7 and PB 5 are dominated by the lichens *Usnea* spp., *Sphaerophorus globosus*, *Ochrolechia frigida, Psoroma hypnorum* and *Pannaria hookeri,* and the bryophytes *Chorizodontium aciphyllum*, *Barbilophozia hatcheii* and *Herzogobryum teres*. PB 2 is the closest to the western shore and the farthest from the Penguin Colony and the BSC is dominated by *Usnea* spp. and also importantly by the crustose lichens, such as *Buelia coniops* and *Lecidea cremonicolor*. The dominant mosses on PB 2 are *Lophozia excisa* and *Sanionia unciniata*.

Soil and rainwater sampling and chemical analysis

Six random samples of surface soils (0-10 cm), separated more than 10 cm each other and following the elevation contour line of the terrain, from directly beneath the BSCs were taken with a shovel from each site (the approximate length of the study area was 60 m). Soils were sieved using a 2 mm mesh size prior to chemical analysis. Plant available inorganic N (N_{in}) was extracted in a 0.021 mol L^{-1} $KAl(SO_4)_2$ solution (1:4) and the determination of available N as ammonium and nitrate was by means of fractionated steam distillation (Pérez et al. 1998). Plant available P (P_a) was extracted through lactation using the CAL (Calcium-Acetate-Lactate) method and determined colorimetrically using the molybdenum blue method (Steubing and Fangmeier 1992). Water content of soil samples was determined gravimetrically. Soil reaction was determined with a pH electrode in a 1:2 soil:water suspension. The dry samples of soil were ground for the determination of total N and C by means of flash combustion using an NA2500 Carlo Erba Element Analyzer. P from ground soil material was extracted with concentrated sulfuric acid together with a water peroxide solution in a Hach Digesdahl digester and determined by colorimetric molybdenum-blue method.

Rainwater was collected in 60 ml narrow-mouth Nalgene bottles connected to a 10 cm diameter funnel (acid washed) fixed by a pole at ~ 0.8 m from the ground on each one of the three palaeo-beaches, one at the Patterned Ground, two in the Penguin Colony and two directly in the *guanera*, which is a place conformed only of guano deposit with no BSC covering the soil. Rain samples were collected during three precipitation events in February 2012 and 2013 and two events in February 2014. Rainwater samples from the Patterned Ground were taken only during 2013. The concentration of ammonium, nitrate and phosphate in the rain samples were determined by ion chromatography using an 861 Advanced Compact Metrohm IC.

Biological nitrogen fixation

At each site, six random samples of BSCs were obtained, separated more than 10 m apart along transects following the elevation contour lines (the approximate length of the study area was 60 m), and incubated in the field and in the laboratory during three consecutive years; February 2012, 2013, 2014. The acetylene reduction technique was used to estimate symbiotic N fixation rates (Myrold et al. 1999). This method is based on the fact that the diazotrophs are also able to reduce acetylene to ethylene (Hardy et al. 1968). In the field, samples of BSCs (from 3-15 g dry weight) were deposited inside 130 ml glass jars, hermetically closed and incubated in a mixture of air and acetylene at 10% v/v for up to two days. In parallel, at each sampling point a sample of BSC (a mass within the range as controls) was incubated with thoroughly mixed penguin guano. An additional sample (a mass within the range above) was incubated without acetylene as a control for samples with and without guano. Three gas samples per jar were taken periodically up to 48 h and injected in 4 ml BD vacutainers® and transported to the Biogeochemistry Laboratory at the Pontificia Universidad Católica de Chile for analysis. Ethylene concentrations in the gas samples were measured using a GC 8A Schimadzu gas chromatograph equipped with a Porapack column and FID detector. Ethylene concentrations were determined from a calibration curve by diluting a 100 ppm ethylene standard balanced of helium (Scotty® Analyzed Gases). Acetylene Reduction Activity (ARA) was estimated from the slope of the linear fit of the ethylene production during incubation within a 130 ml headspace. Linearity in acetylene reduction rates was obtained within the incubation period. Plant samples were dried at 70ºC and after 48 hrs weighed. In order to scale up the rates of acetylene reduction activity obtained in the field to the complete growing season (GS: from December to March), we assumed no significant changes in wind direction that could bring ammonia-enriched rainfall from penguin colonies. We also assumed that moisture levels in BSCs are representative values for the complete GS, as air relative humidity (86-89%) had a coefficient of variation within each GS during the three consecutive years of 5-1%. In order to obtain an estimation of the N fixation rate per unit of area, the biomass of the BSCs was sampled using a 10 x10 cm pvc frame and dried in the oven at 70°C for >48 hrs. According to theoretical stoichiometry, one mol of N_2 is fixed per three moles of acetylene reduced to ethylene (Hardy et al. 1968). Thus the rates of BNF performed by BSC was expressed in kg N ha^{-1} GS^{-1}. In situ experiments adding guano were performed for three consecutive years (2012-2014) for site PB7, and for 2013 and 2014 across the rest of the sites. During each field incubation, the temperature in the BSCs was measured using a soil thermometer.

Mixed samples of BSCs from each site were taken to the laboratory at the Escudero Base on the Fildes Peninsula, King George Island, and incubated with the addition of deionized water (n=6) and controls (n=6) with the field moisture content and following the same procedures as in the field.

Potential rates of denitrification

Following the same transect line and points for the samples taken for BNF, six intact soil cores were taken from each study site and incubated under laboratory conditions at the Escudero Base. Denitrification rates were determined using the acetylene blocking assay (Groffman et al. 1999). This method is based on the inhibition of nitrous oxide (N_2O) reductase by acetylene, allowing the accumulation of nitrous oxide in an acetylene atmosphere, which can be measured by gas chromatography. Soil samples were placed inside 130 ml hermetic glass jars and incubated for 6 hours under a 10% v/v acetylene atmosphere. Gas samples were taken at two and six hours and injected into 4 ml BD vacutainers® for analysis. The N_2O concentration in the gas samples were determined using a GC 8A Shimadzu gas chromatograph equipped with a Porapack column Q 80/100 and electron capture detector. A calibration curve was prepared by diluting a 1 ppm nitrous oxide balance of nitrogen (Scotty® Analyzed Gases). As the top soils have a relatively high carbon content and low C/N ratio we assumed that the acetylene treatment would not affect denitrification rates. Denitrification rates were estimated from the differences in N_2O-N concentrations between 6 hrs and 2 hrs and referred to an area basis. All chemical analyses were conducted at the Biogeochemistry Laboratory at the Pontificia Universidad Católica de Chile, Santiago.

Statistical analysis

To evaluate the effect of each site on the chemical parameters of the soils, either one way ANOVA or Kruskall-Wallis tests were applied, depending on Levene's tests for the equality of variances. To evaluate the effect of each site and the sample year on BNF and denitrification rates a one way ANOVA for repeated measurements was applied. In order to evaluate the effect of each site and the addition of water each year on ARA in laboratory incubations a two factor ANOVA was applied. In order to evaluate the effect of guano on BNF a one way ANOVA was applied per site and per year. A-posteriori Tukey tests or Multiple Comparisons test were applied in order to detect the differences among cases. A statistical significance was accepted at the $p<0.05$ significance level. Because ARA, BNF and denitrification data are inherently skewed they were also box-cox transformed before statistical analysis. All tests were performed using Statistica 7.0 software.

Results

Chemical analysis of soils and rainwater

There was no significant difference in the chemical parameters of soils according to the ages of the palaeo-beaches, in neither in the Reference nor the Patterned Ground sites (Table 2). The Penguin Colony has significantly higher contents of available inorganic N ($F_{5,30} = 3.16$, $p=0.02$) than PB 7 and PB 5 and highest available P ($F_{5,30} = 88.86$, $p<0.0001$)

and the lowest pH ($F_{5,30} = 50.63$, $p<0.0001$) than all sites. Soils at the Penguin Colony also presented higher moisture levels than the PB 7, Reference and Patterned Ground sites ($F_{5,30} = 4.64$, $p=0.003$). Soils at the Penguin Colony also have higher concentrations of total N ($\chi^2_{5,30} =14.67$, $p=0.012$) and total C ($F_{5,30} =3.31$, $p=0.017$) than at PB 7. Both the soils of PB 7 and the Penguin Colony had the highest total P content ($F_{5,30} = 26.44$, $p<0.0001$). Soils at the Reference Site presented lower C/N ratios than PB 5 and PB 2 ($\chi^2_{5,30}=14.66$, $p=0.012$).

Ammonia (Fig. 2a) and phosphate (Fig. 2b) concentrations in rainwater evidenced an upward trend directly in the *guanera* and declining towards the margins of the Penguin Colony during the three years of the study. Nitrate concentration in rainwater was similar in the different localities and showed little variation during the three years of the study (Fig. 2c).

Biological nitrogen fixation

In relation to the inter-annual variation of BNF in field assays, there was a significant effect from the year ($F_{5,52} = 7.75$, $p<0.0001$) and each site ($F_{5,26} = 9.39$, $p<0.0001$) on BNF, with the highest rates during the year 2012 reaching up to 3 kg of N ha^{-1} growing season^{-1} at PB 7 (Fig. 3). The temperatures of the BSCs during field incubation reached their highest values during 2012 (Table 3). BNF in BSCs in the Penguin Colony was significantly lower than at the PB 7, Reference and Patterned Ground sites (Fig. 3). The Reference Site presented significantly higher BNF rates than PB 2. During the three years of the study there was a trend to higher BNF from PB 2 to PB 7, however differences among these sites were not statistically significant (Fig. 3).

In relation to the effect of guano on BNF in field assays, at PB 7 the addition of guano significantly inhibited BNF during 2012 ($F_{1,9} = 8.46$, $p=0.02$) and 2013 ($F_{1,9} =$

16.711, p=0.003) (Fig. 4a). During 2013 the addition of guano significantly inhibited rates of BNF at the PB 5 (Fig. 4b) ($F_{1,7}$ = 30.41, p<0.0001), Reference (Fig. 4c) ($F_{1,10}$ = 49.06, p<0.0001) and Patterned Ground sites (Fig. 4d) ($F_{1,10}$ = 12.59, p=0.005). During 2014 there was a significant inhibition of BNF only at the Reference Site ($F_{1,7}$ = 34.189, p<0.0001) (Fig. 4c). There was no significant effect from the addition of guano at PB 2 in any year (Fig. 4e).

In relation to the effect of moisture on BNF in laboratory incubations, during 2012 the addition of water significantly stimulated ARA ($F_{1,59}$ = 193.6, p<0.0001) in almost all sites except PB 2 and the Penguin Colony (Fig. 5a). During this year, the water content of control samples in BSCs were on average 30% (Table 3). There was also a site effect ($F_{5,59}$ = 20.64, p<0.0001), where the Penguin Colony presented the lowest ARA and the Reference Site higher rates than PB 5, PB 2 and Patterned Ground (Fig. 5a). During 2013 there was no effect from the addition of water ($F_{1,56}$ = 0.35, p=0.56) (Fig. 5b), when the water content of control samples was on average 62.5% (Table 3). During 2013, there was a site effect ($F_{5,56}$ = 21.93, p<0.0001) with the lowest rates in the Penguin Colony. Highest rates presented the Patterned Ground, Reference Site and PB 5 (Fig. 5b). During 2014 there was a significant effect from the addition of water ($F_{1,54}$ = 17.67, p<0.0001) which stimulated ARA in PB 7 and the Reference Site (Fig. 5c). There was also a site effect ($F_{5,54}$ = 20.1, p<0.0001), with lowest rates in Penguin Colony and PB 2 and higher rates in the Patterned Ground site than at PB 5 and PB 7 (Fig. 5c).

Potential denitrification

There was a significant year ($F_{2,60}$ = 48.58, p<0.0001) and site effect ($F_{5,30}$ = 10.14, p<0.0001) on denitrification rates with the highest rates during 2012. Statistically significant higher rates of denitrification were reached in the soils of the Penguin Colony across years (Fig.6). During year 2013 the site Patterned Ground next to the Penguin Colony presented high rates of denitrification as well, however it was not statistically different from the other sites (Fig. 6).

Discussion

The effect of guano on N and P in soil and rainwater.

The chemical parameters of the soils are very similar amongst the study sites except for the Penguin Colony, which as expected, had the highest contents of available inorganic N and P and the lowest pH. Similar N and P enrichment in soils under bird influence are reported in maritime (Tatur 2002; Tscherko et al. 2003; Simas et al. 2007) and continental Antarctica as well (Cocks et al. 1998; Cannone et al. 2008; Ball et al. 2015). The main source of these elements are penguin excreta, mainly from uric acid (Lindeboom 1984). Uric acid mineralizes and can either follow the pathway of ammonia volatilization and/or nitrification or denitrification. High concentrations of ammonium in rainwater and high rates of denitrification provide evidence that these two pathways are occurring at the Penguin Colony study site. In the present study, estimated input of inorganic nitrogen via wet deposition is 0.2 kg N $ha^{-1}year^{-1}$ in the palaeo-beaches and 1.36 kg N ha^{-1} $year^{-1}$ in the Penguin Colony, where 73% is NO_3-N in the former while it is only 25% in the latter. This suggests that nitrate is an important form of reactive nitrogen incorporated to ecosystems via wet deposition in areas located distant from the penguin colonies. The mean nitrate concentration of 2.1 μmol L^{-1} in rainwater of palaeo-beaches is within the range of those reported for the South Pacific Ocean and Coast of Chile, which range from 0.2-2.9 μmol L^{-1} (Jung et al. 2011). Among the possible sources of nitrate documented to occur to the pristine troposphere of the maritime Antarctica are: i) N-fixation by lightning, ii) re-emissions from snow, firn layer and soils, iii) oxidation of ammonia, and iv) anthropogenic emissions from the surrounding bases, among others (Wagenbach et al. 1998, Jones et al. 2000, Savarino et al 2007). As no clear pattern was observed in our study sites, all these sources may be contributing to nitrate in precipitation in Ardley Island. Dry deposition of nitrogen was not measured in the present study; however, model simulations indicate that it may play a minor role in total deposition of reactive nitrogen in maritime Antarctica, reaching ca. 20-30% in the South Shetlands region (Li et al. 2010).

In relation to P, a gaseous reduced form of P called phosphine is emitted at extraordinary high rates at the Penguin Colony on Ardley Island (Zhu et al. 2014), which can be oxidized to water soluble phosphate in precipitation and, therefore, be transported long distances. A similar pattern of phosphate concentration in the rainwater was identified by this study and which were much higher directly in the *guanera* and declining towards the margins of the Penguin Colony and Patterned Ground sites. Similarly to ammonium, the effect of phosphate was at a small scale and did not reach the paleo-beaches located upwind from the Penguin Colony. It has been documented that the main source of N to plants in Sub-Antarctic islands is N originating from guano (Erskine et al. 1998; Crittenden et al. 2015) and may even be sourced from abandoned-ancient penguin rookeries (Wasley et al. 2006). Because the sites are located in the upwind direction of the Penguin Colony, its effect on BNF performed by BSCs is on a small scale on Ardley Island.

The effect of guano, moisture and temperature on BNF.

In this study the rates of BNF performed by BSCs are enhanced during warmer and wetter years in the maritime Antarctic. Similar results are reported in the High Arctic and Sub-

Artic regions, where moisture and temperature are found the main factors controlling BNF in soil and vegetation (Zielke et al. 2005, Sorensen et al. 2006). However, BNF is completely inhibited even during favourable years at the Penguin Colony with high N availability. Moreover, we report that enhanced inputs of N via guano have a significant negative effect on BNF performed by BSCs which cover soils with lower N availability than in the Penguin Colony and distant from the effect of guano aerosols in rainwater. A similar inhibition of BNF was reported for soils under the influence of breeding snow petrels (*Pagodroma nivea*), in Dronning Maud Land, continental Antarctica (Cocks et al. 1998). These findings suggests that the ultimate control on BNF in northern maritime Antarctica under the effect of penguin colonies and in regions of continental Antarctica under bird influence is N availability.

In the laboratory the addition of water increased the rates of diazotrophic activity in almost all study sites during the warmer growing season 2012, with lower mean water content of BSCs, except for the Penguin Colony and PB 2. The lower water content in BSCs during 2012 could be the effect of higher evapotranspiration in spite of higher precipitation, leading to water limitation of BNF.

During the three years of the study, we found a trend towards higher rates of BNF from the youngest PB 2 to the Reference Site. Similar trends of higher BNF in soils towards older sites more distant to the glacier were also found on Anvers Island, maritime Antarctica (Strauss et al. 2012). The trend to higher BNF observed in older sites (e.g. PB 7 and Reference Site) distant to the Penguin Colony is likely to be linked to changes in microbial community structure as it has been recently reported for the study area, where the N-fixing cyanobacteria *Chamaesiphon* is two hundred folds more abundant in pristine soils of Fildes Peninsula than in the Penguin Colony of Ardley Island (Wang et al. 2015).

Another factor that may also control rates of BNF but was not considered in the present study is light intensity (Belnap 2001; Paerl and Priscu 1998; Sorensen et al. 2006). This could explain the high rates of BNF detected in the BSCs at the Patterned Ground during the coldest growing season, located at a higher altitude than the other study sites (Table 2).

In the present study, we report that during wetter and warmer years, BNF can reach higher levels of up to 3 kg N ha^{-1} y^{-1} on the oldest paleo-beach PB 7, which is fifteen times higher than N inputs via wet deposition. Similar estimations of BNF on Signy Island (maritime Antarctica) have reported levels of 0.46 and 1.92 kg N ha^{-1} y^{-1} for dry turf and wet carpets respectively (Vincent 2000). Ranges of symbiotic N fixation reported from other types of cold biomes such as the moist and alpine tundra are 1 - 4.9 kg N ha^{-1} y^{-1} (Reed et al. 2011) and up to 1.3 kg N ha^{-1} y^{-1} in the Arctic tundra of northern Alaska (Hobara et al. 2006). However, even higher rates have been reported for soils crusts in a low Arctic tundra landscape of Canada and in the Sub-Arctic region of Sweden of up to 11 kg N ha^{-1} y^{-1} (Stewart et al. 2011; Sorensen et al. 2006). Together all these data suggest that even in the colder high latitude biomes such as maritime Antarctica and Arctic, “hot spots” of BNF can be present where free of the influence of sea bird guano.

Potential denitrification rates.

Gaseous losses of N were associated with higher N availability and water content in soils at the Penguin Colony. Both factors enhance the reduction of nitrate under aerobic conditions by denitrifiers, which has been documented to be highly diverse in microbial mats within maritime Antarctica (Alcántara et al. 2014). Our results suggests that ornithogenic soils within penguin colonies are an important source of nitrous oxide, which may have become very active under favourable laboratory conditions during the colder growing season of

2014. Conversely, we found no trend along the chronosequence, as the young and oldest palaeo-beach did not present significant differences in denitrification rates. In other studies it has been found that there was a small increase in potential denitrification rates with time since glacier retreat in amended (with potassium nitrate and dextrose) soils on Anvers Island, linked to an increase in nitrate content in the soils, although at very low rates < 1 µmol N m^{-2} h^{-1} (Strauss et al. 2009, 2012). Our mean values for the palaeo-beaches of 0.21 µmol N m^{-2} h^{-1} are in the lower ranges in comparison to the Anvers Island chronosequence. The highest denitrification rates within the Penguin Colony (12 µmol N m^{-2} h^{-1}) during 2014 is one order of magnitude higher than potential denitrification in the amended and older soils on Anvers Island. However, even higher emission rates of N_2O-N in field assays that varied from 53-194 µmol N m^{-2} h^{-1} have been estimated from the Penguin Colony on Ardley Island (Zhu et al. 2008).

Conclusions

The strong inhibition of BNF through the addition guano could explain the shaping of the composition of BSCs on Ardley Island, where the cyanolichens, amongst others, *Psoroma hypnorum* and *Pannaria hokkeri* or the moss *Chorizodontium aciphyllum* and the liverwort *Herzogobrym teres* (both with positive ARA, data not presented here) are excluded from the Penguin Colony. Values of BNF on the oldest PB 7 and Reference Site during wetter and warmer years are above the ranges reported from other studies in the Antarctica.

Our results suggest that increases in moisture levels by both increases in surface fluxes by glacier melting or by increases in precipitation could positively affect the rates of

BNF. However, consequent changes in the distributional pattern of the penguin colonies could have drastic effects on the BSCs that are able to fix N from the atmosphere.

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Figure Legends

Figure 1. Location of study Sites on Ardley Island, indicating its relative position to Fildes Peninsula, King George Island, and South Shetland Islands.

Figure 2. Mean ammonium, phosphate and nitrate content in rainwater samples taken on an event basis during February 2012 (*n*= 3), 2013 (*n*=3) and 2014 (*n*=2). The study site Patterned Ground was sampled only during 2013. Bars indicate *SD*.

Figure 3. In situ nitrogen fixation rates in biological soil crusts of Ardley Island during the growing season (GS) of 2012, 2013 and 2014. Bars indicate *SE* of mean values (*n*=6). Different letters among sites indicate statistically significant differences according to a-posteriori Tukey tests ($p < 0.05$).

Figure 4. Effect of the addition of guano on in situ nitrogen fixation in biological soil crusts during the growing season (GS) 2012, 2013 and 2014 in PB 7 (a) and 2013 and 2014 for PB 5 (b), Reference Site (c), Patterned Ground (d) and PB 2 (e). The asterisks indicate significant differences among treatments (Tukey tests $p<0.05$). Bars indicate *SE* of mean values (*n*=6).

Figure 5. The effect of the addition of water on acetylene reduction activity in laboratory incubation of biological soil crusts in Ardley Island during February 2012 (a), 2013 (b) and 2014 (c). The asterisks indicate significant differences among treatments (Tukey tests $p<0.05$). Bars indicate *SE* of mean values (*n*=6). Different letters among sites indicate statistically significant differences according to a-posteriori Tukey tests ($p < 0.05$).

Figure 6. Potential denitrification rates in surface soils of Ardley Island during February 2012, 2013 and 2014. Bars indicate *SE* of mean values (*n*=6). Different letters among sites indicate statistically significant differences according to a-posteriori Tukey tests ($p < 0.05$).

Figure 1

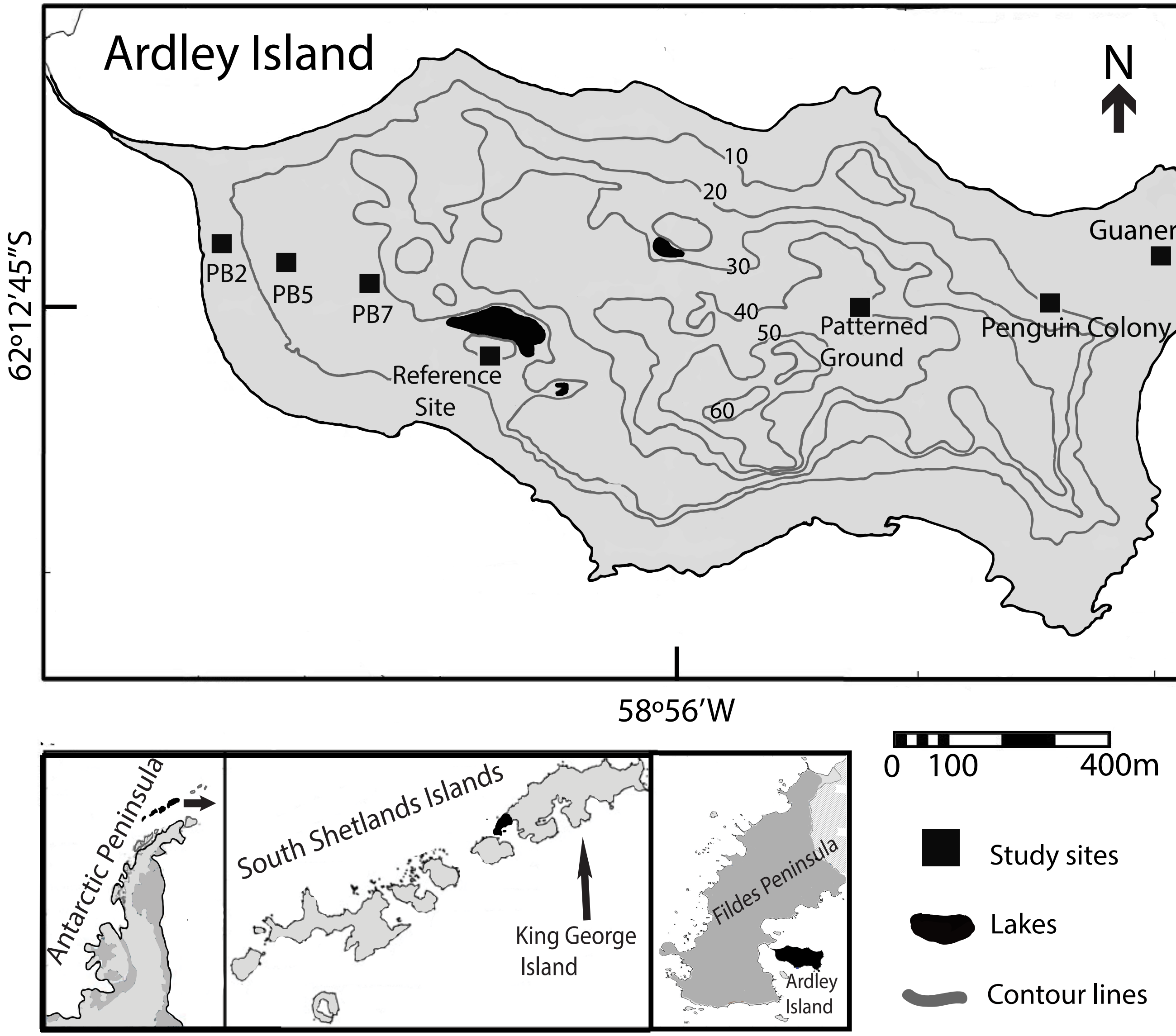
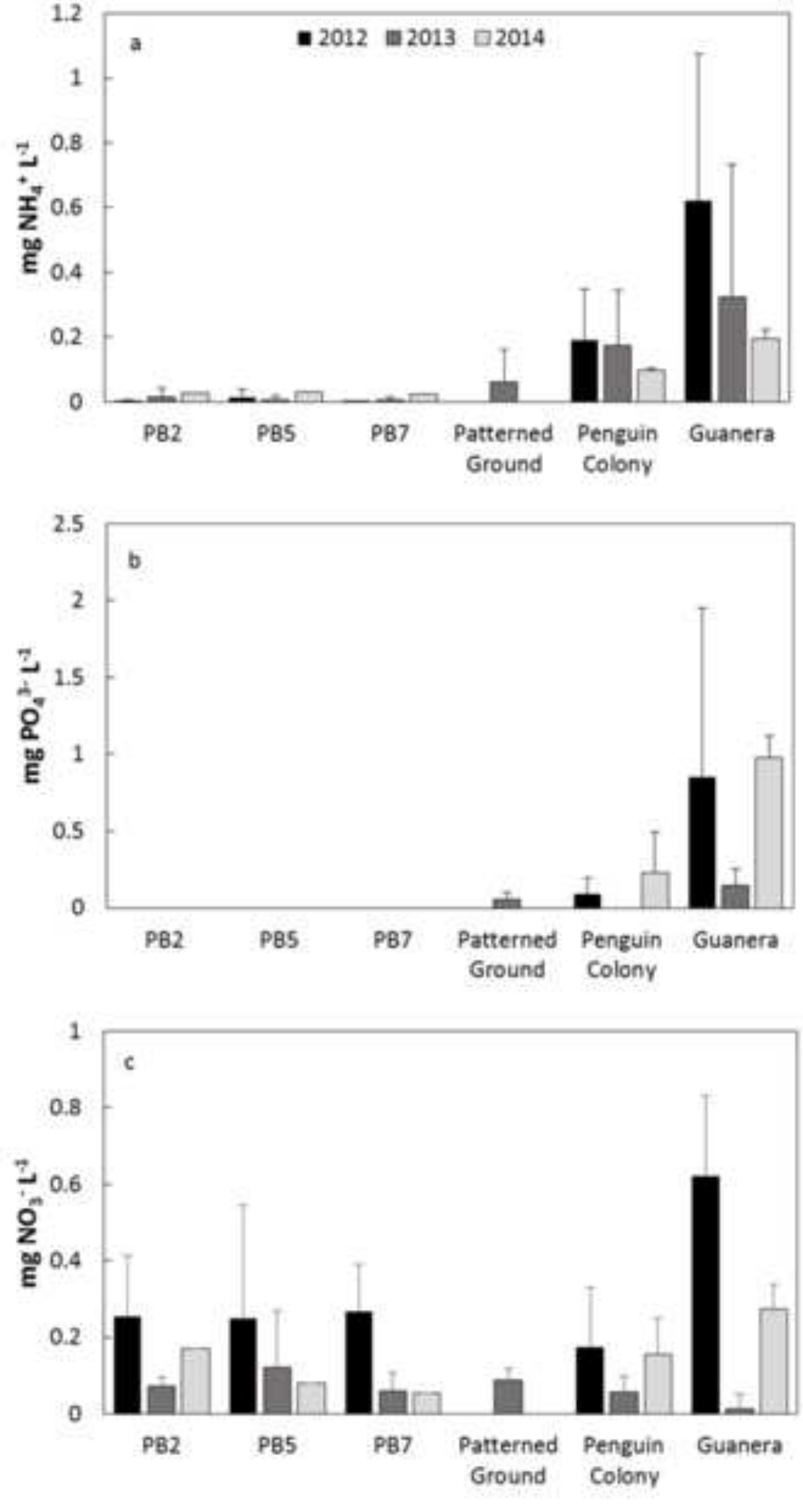

Ardley Island
N
10
20
30
40
50
60
PB2
PB5
PB7
Reference Site
Patterned Ground
Penguin Colony
Guanera
62°12'45"S
58°56'W
0
100
400m
Antarctic Peninsula
South Shetlands Islands
King George Island
Fildes Peninsula
Ardley Island
Study sites
Lakes
Contour lines

Figure 2



a
■ 2012 ■ 2013 □ 2014
mg NH4+ L-1
1.2
1
0.8
0.6
0.4
0.2
0
PB2
PB5
PB7
Patterned Ground
Penguin Colony
Guanera
b
mg PO43- L-1
2.5
2
1.5
1
0.5
0
PB2
PB5
PB7
Patterned Ground
Penguin Colony
Guanera
c
mg NO3- L-1
1
0.8
0.6
0.4
0.2
0
PB2
PB5
PB7
Patterned Ground
Penguin Colony
Guanera

Figure 3

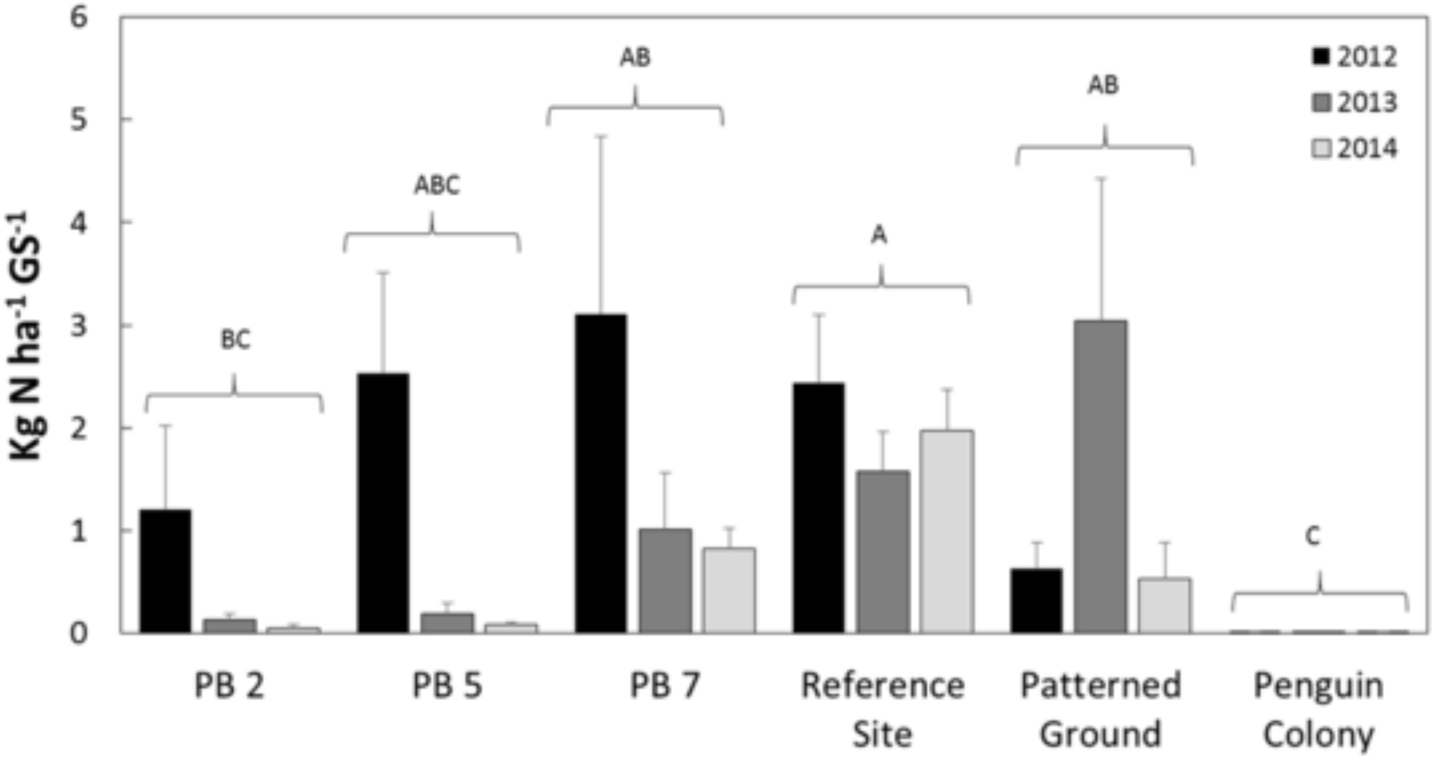

6
5
4
3
2
1
0
Kg N ha^{-1} GS^{-1}
■ 2012
■ 2013
□ 2014
BC
ABC
AB
A
AB
C
PB 2
PB 5
PB 7
Reference Site
Patterned Ground
Penguin Colony

Figure 4

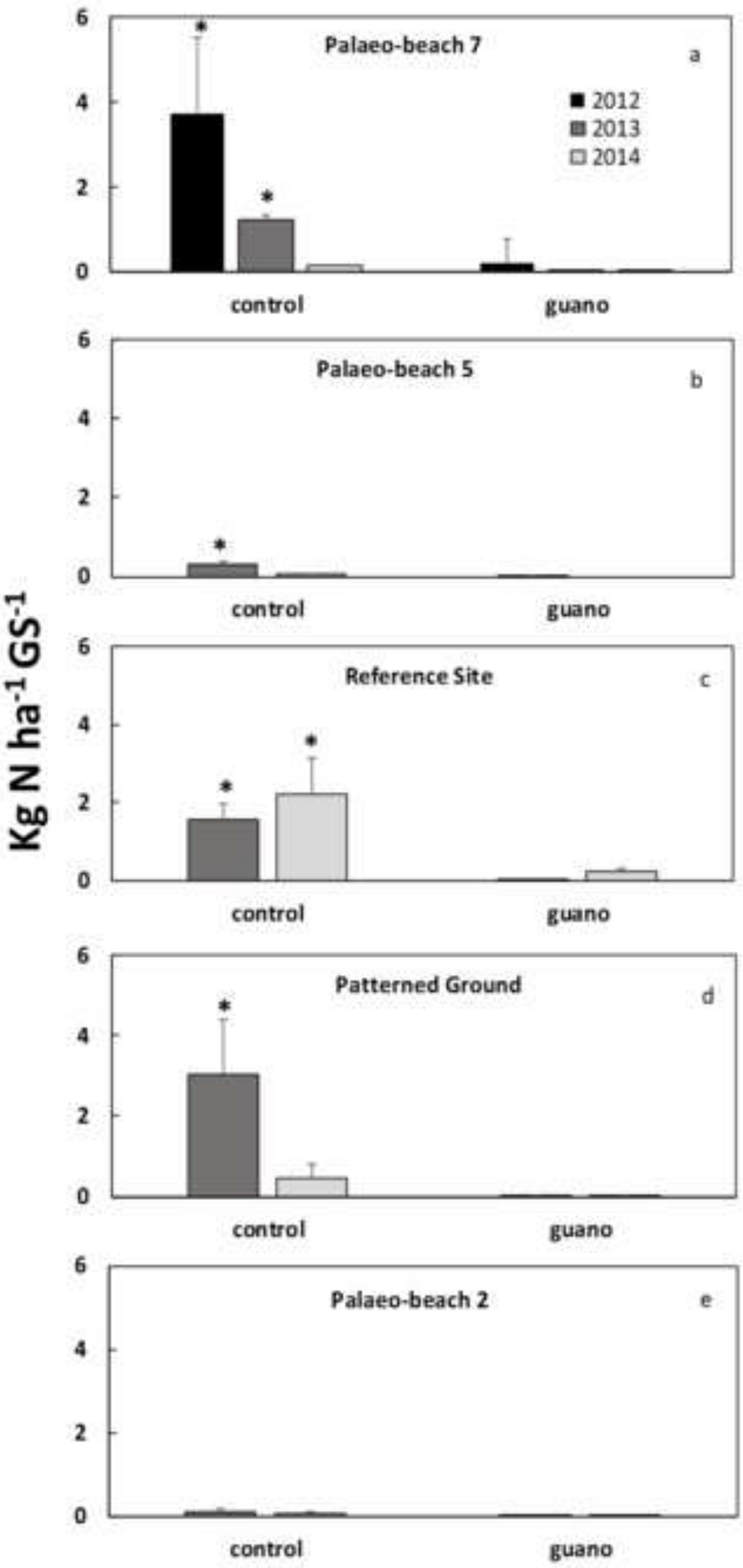
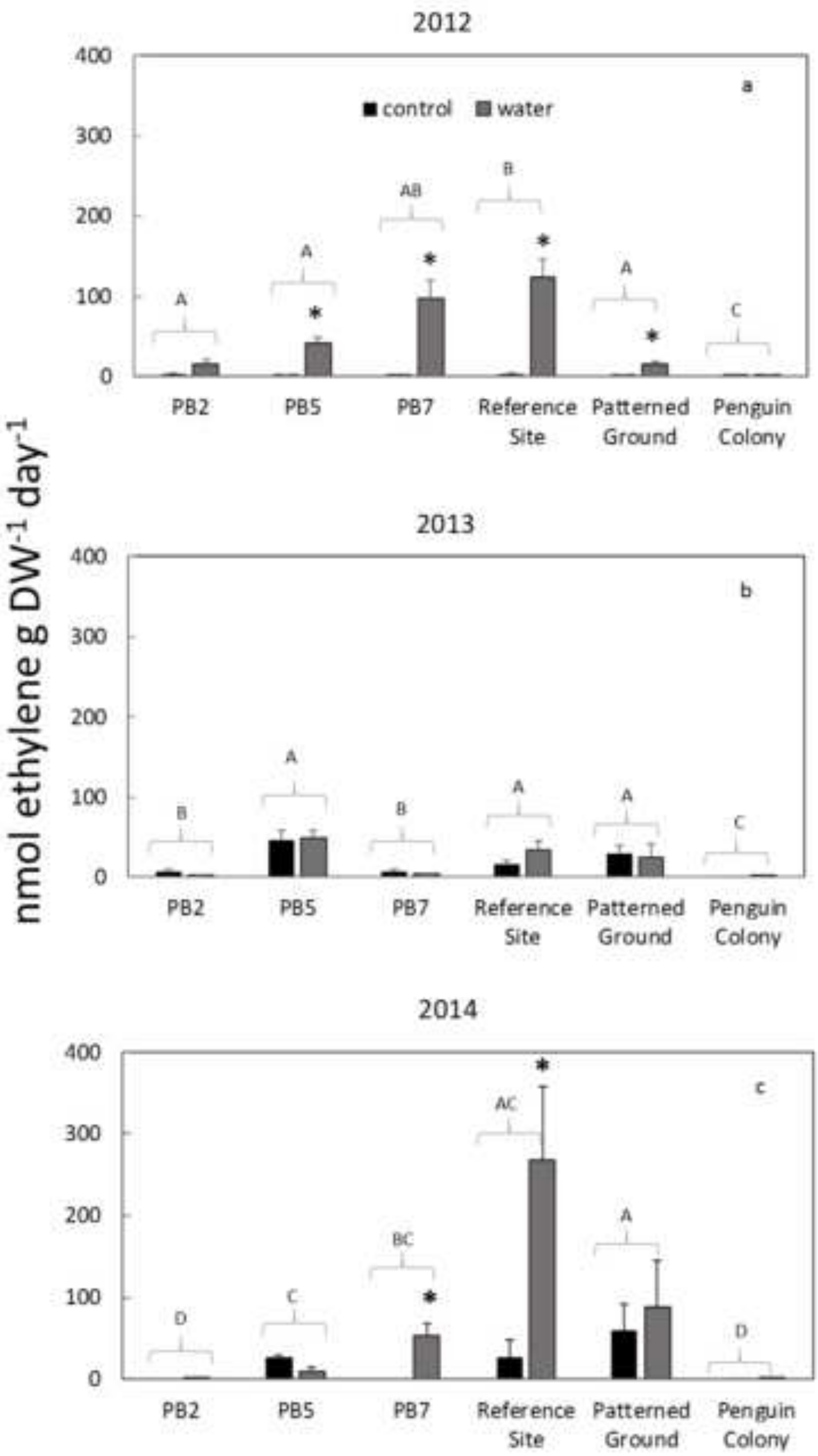
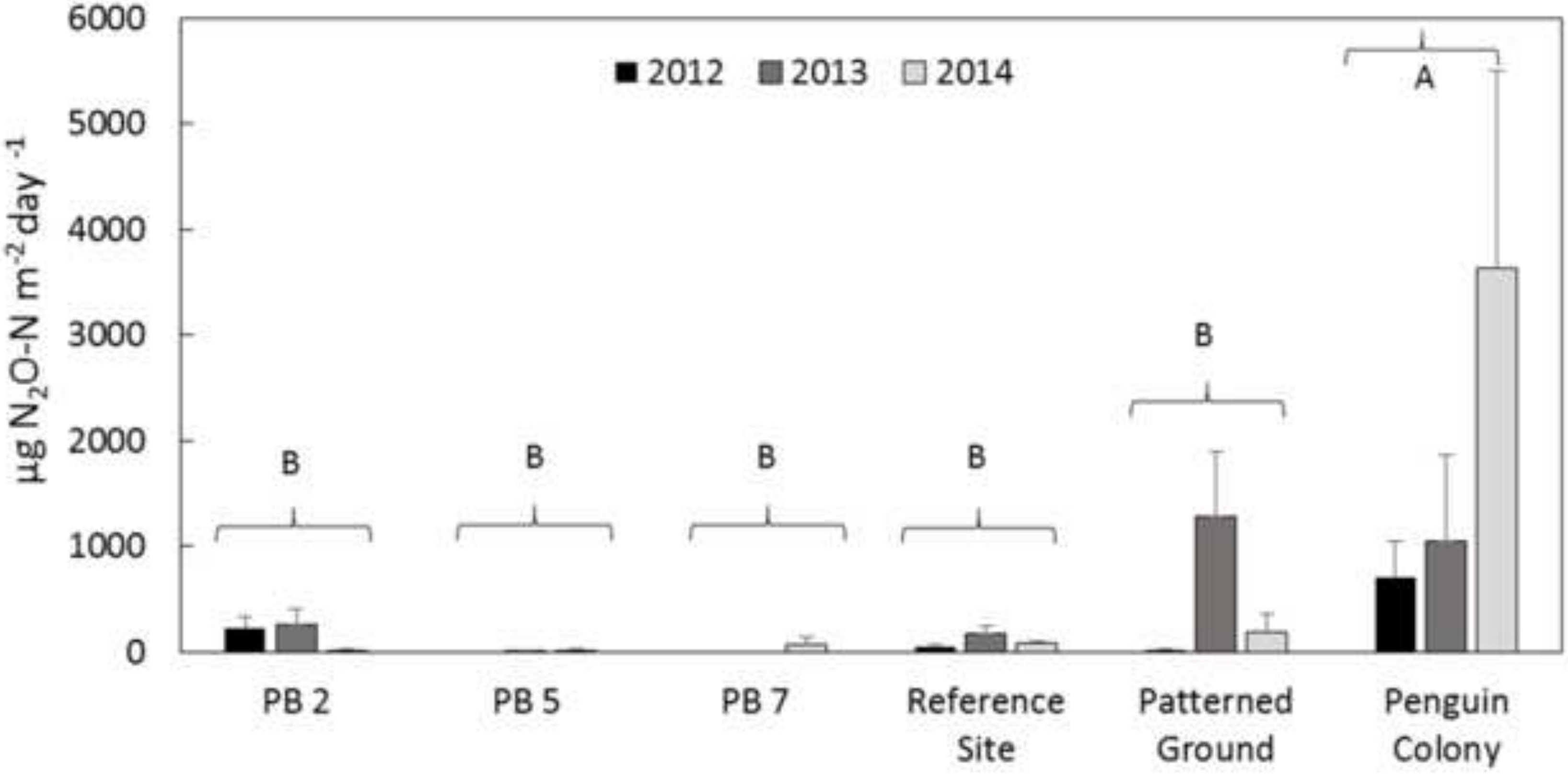

Kg N ha^{-1} GS^{-1}
Palaeo-beach 7
a
2012
2013
2014
Palaeo-beach 5
b
Reference Site
c
Patterned Ground
d
Palaeo-beach 2
e
6
4
2
0
*
control
guano

Figure 5



2012
a
■ control ■ water
400
300
200
100
0
A
A
AB
B
A
C
*
*
*
*
PB2
PB5
PB7
Reference Site
Patterned Ground
Penguin Colony
nmol ethylene g DW-1 day-1
2013
b
400
300
200
100
0
B
A
B
A
A
C
PB2
PB5
PB7
Reference Site
Patterned Ground
Penguin Colony
2014
c
400
300
200
100
0
D
C
BC
AC
A
D
*
*
PB2
PB5
PB7
Reference Site
Patterned Ground
Penguin Colony

Figure 6



■ 2012 ■ 2013 □ 2014
μg N_2O-N m^{-2} day^{-1}
6000
5000
4000
3000
2000
1000
0
B
B
B
B
B
A
PB 2
PB 5
PB 7
Reference Site
Patterned Ground
Penguin Colony

Table 1: Climatic data for the month of February for the three consecutive years of the study (source Bellingshausen Station, Fildes Peninsula, King George Island).

	2012	**2013**	**2014**
Mean Air Temperature (°C)	0.8	0.7	-0.1
Maximum air temperature (°C)	2.5	2.3	1.3
Precipitation (mm)	61.7	49.5	46.6
Sunshine hours	127.9	47.4	54.0

Table 2: Chemical characteristics of surface soils, Ardley Island. In parenthesis is the *SD*, *n*=6. Different letters indicate significant differences among treatments according to Tukey tests or multiple comparisons ($p < 0.05$).

	N_{in} (mg kg^{-1})	**P_a (mg kg^{-1})**	**pH (H_2O)**	**Water content (%)**	**%N**	**%C**	**%P**	**C/N**
Palaeo-beach 2 (PB 2)	29.57ab (3.87)	22.96a (2.22)	5.54a (0.05)	54.47ab (2.9)	2.23ab (0.12)	27.61ab (2.11)	0.04b (0.0)	12.29ac (0.38)
Palaeo-beach 5 (PB 5)	25.03a (3.67)	24.25a (1.08)	5.30ab (0.05)	50.86ab (2.4)	2.27ab (0.2)	28.29ab (2.7)	0.04b (0.01)	12.44c (0.18)
Palaeo-beach 7 (PB 7)	25.00a (3.39)	26.60a (0.65)	5.45a (0.06)	45.99a (2.16)	1.80a (0.1)	20.97a (1.16)	0.14a (0.02)	11.64abc (0.1)
Reference Site	30.93ab (6.05)	24.54a (1.07)	5.13b (0.06)	46.73a (1.93)	2.29ab (0.12)	25.41ab (1.13)	0.06b (0.01)	11.12b (0.15)
Patterned Ground	36.32ab (9.53)	25.94a (1.82)	5.41a (0.07)	45.47a (2.23)	1.97ab (0.23)	23.13ab (2.31)	0.05b (0.01)	11.70abc (0.22)
Penguin Colony	62.21b (14.42)	77.55b (2.91)	4.40c (0.05)	57.54b (2.16)	2.57b (0.10)	29.28b (1.02)	0.17a (0.02)	11.42abc (0.13)

Table 3: Mean temperature and water content of BSCs taken during in situ incubations. In parenthesis is the *SD*, *n* = 6.

	2012		**2013**		**2014**	
	Temperature (°C)	Water content (%)	Temperature (°C)	Water content (%)	Temperature (°C)	Water content (%)
Palaeo-beach 2 (PB 2)	6.5 (1.4)	48.2 (3.8)	4.1 (2.5)	67.9 (2.0)	3.5 (4.0)	53.6 (4.0)
Palaeo-beach 5 (PB 5)	9.3 (2.9)	35.2 (6.0)	4.6 (2.2)	61.9 (2.2)	2.7 (3.2)	48.4 (5.1)
Palaeo-beach 7 (PB 7)	6.2 (1.1)	31.7 (6.9)	4.9 (1.9)	47.7 (6.9)	1.9 (2.9)	50.5 (4.7)
Reference Site	7.6 (1.4)	26.4 (3.1)	3.8 (0.6)	64.4 (1.9)	2.0 (3.3)	56.6 (8.1)
Patterned Ground	7.2 (1.1)	21.1 (3.9)	3.3 (1.8)	55.3 (4.1)	1.1 (1.4)	41.8 (6.0)
Penguin Colony	7.7 (4.8)	20.4 (3.9)	2.9 (2.2)	77.9 (2.3)	0.0 (0.7)	60.8 (5.5)
Mean	7.4 (2.1)	30.5 (4.6)	3.9 (1.9)	62.5 (3.2)	1.9 (2.6)	52.0 (5.6)