

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

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25 Abstract

26 Biological nitrogen fixation (BNF) is a high energy-demanding process that may be
27 inhibited by penguin guano. We tested this hypothesis in Ardley Island by measuring BNF
28 in biological soil crusts (BSC) directly within a Penguin Colony and in sites unaffected by
29 penguins. We also explored the effect of adding guano to BSCs in sites free of the influence
30 of penguins. Water availability is also one of the most limiting element for life in the
31 Antarctica and we expected that a wetter growing season would stimulate BNF. To evaluate
32 the effect of moisture on BNF we added water to BSCs under laboratory conditions and
33 estimated BNF by means of the acetylene reduction assay during three growing seasons
34 (2012, 2013 and 2014), with contrasting temperature and precipitation conditions. The
35 results reveal an almost complete inhibition of N fixation in the BSCs of the Penguin
36 Colony. In sites free of ammonium and phosphate in rainwater, BNF rates reached up to 3
37 kg N ha⁻¹ y⁻¹ during warmer and wetter years. The addition of guano to BSCs significantly
38 inhibited the rates of BNF. In laboratory incubations, the addition of water significantly
39 stimulated rates of BNF during the warmer growing season with more sunshine hours. The
40 likely increases in soil moisture levels due to climate change and glacier melting in the
41 Antarctic Peninsula may enhance the rates of BNF. However, this may be constrained by
42 accompanying changes in the distribution of penguin colonies.

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

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49 Introduction

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

70 Studies in maritime Antarctica report that soil N and P levels are extraordinarily
71 high, with C/N ratios even lower than in temperate regions (Beyer et al. 2000 a, b). The
72 authors attributed the N and P enrichment to seabird droppings and aerosols transported

73 long distances. In situ activity of birds, such as penguins, has led to the development of
74 ornithogenic soils formed by the strong weathering on loams and gravels promoted by
75 guano, and in parallel increasing the rates of net N mineralization, soil respiration and soil
76 enzymatic activity (Tscherko et al. 2003). This suggests that N is not a limiting factor for
77 the development of vegetation (Beyer et al. 2000 a, b). It is well documented that birds
78 affect the composition of cryptogamic flora and its distribution in Antarctica and the
79 accumulation of C and P in soils (Tatur 2002; Simas et al. 2007; Michel et al. 2010). How
80 N accumulates in non-ornithogenic soils is less well understood. For maritime Antarctica it
81 has been postulated that N is not a limiting factor for plant physiological processes as N is
82 abundantly provided from penguin colonies (Robinson et al. 2003). Recent studies have
83 reported long distant transport of N and P emitted from penguin guano (Crittenden et al.
84 2015; Zhu et al. 2014), which can increase N and P availability in soils even in sites
85 located far distant in the wind direction from the penguin colonies. Even under low N
86 availability in the cold deserts of Antarctica endolithic cryptogamic communities are not N-
87 limited because of its low N requirements for photosynthesis (Johnston and Vestal 1991).
88 These findings raise questions about why rates of BNF in BSCs are limited in areas of
89 guano deposition and in areas relatively free of bird droppings.

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

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

102 The main objective of this study was to test the following specific hypotheses:

103 i) BNF is inhibited in the BSCs at the Penguin Colony site and the addition of guano
104 inhibits BNF in BSCs in sites located in the upwind direction and ii) the addition of water
105 to BSCs under laboratory conditions increases BNF. Furthermore, from the high N and P
106 emission rates reported for ornithogenic soils in the study site (Sun et al. 2002; Zhu et al.
107 2008; Zhu et al. 2014), we expect a corresponding high ammonia and phosphate
108 concentrations in rainwater and soil and high denitrification rates. Our findings have
109 advanced our understanding of how BNF and, therefore, how primary productivity in
110 maritime Antarctica will respond under increasing warming, following the trend observed
111 during the last five decades, together with higher moisture levels in soils due to increases in
112 precipitation and glacier melting (Vaughan 2006).

113

114 Study Area and Methods

115 Study Sites

116 Ardley Island is a 1.2 km² ice-free land surface that lies off the south east coast of Fildes
117 Peninsula, at the extreme south of King George Island, the largest of the South Shetland
118 Islands (Fig.1). Six study sites were selected along an east-to-west transect that crosses the
119 Ardley Island from a Penguin Colony at the easternmost site, to an area upwind of the

120 Penguin Colony, on the western coast free of influence from the penguins (Fig.1). Ardley
121 Island is one of the Antarctic Specially Protected Areas and represents a natural laboratory
122 to test the hypotheses as the landscape is shaped by a chronosequence of palaeo-beaches
123 located upwind of a colony of Gentoo Penguin (*Pygoscelis papua*). Data from the
124 Bellingshausen Meteorological Station located in the close vicinity of the study sites
125 indicate a mean annual precipitation of ca. 700 mm y⁻¹, a mean summer temperature of
126 1.5°C (January/February) and a mean winter temperature of -6.5°C (July/August). Strong
127 winds predominantly come from the west with speeds that commonly exceed 100 km h⁻¹
128 (Peter et al. 2008). Sites were sampled during three field campaigns in February 2012, 2013
129 and 2014, during which contrasting temperature and humidity data were recorded at the
130 Bellingshausen Meteorological Station. February 2012 had the highest maximum
131 temperature, precipitation and hours of sunshine (Table 1). February 2013 had similar
132 temperatures to 2012, but less than the half of sunshine hours than the preceding year.
133 February 2014 had the lowest maximum and mean temperatures, but similar amount of
134 precipitation to 2013, which were also lower than 2012 (Table 1). Soil types of the study
135 area belong to Histic Ornithic Crysol and Follic Crysol (Michel et al. 2010).

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

144 (Fig. 1), where the soil is covered by a BSC dominated by lichens *Usnea* spp, *Psoroma*
145 *hypnorum*, *Pannaria hookeri* and the moss *Chorizodontium aciphyllum*. This site is called
146 the Reference Site, as it does not belong to the adjacent chronosequences of palaeobeaches
147 described below and, therefore, it is considered to be a control for the time variable. A
148 chronosequence of palaeo-beaches is located on the south-western shore of the island and
149 about 1200 m away from, and upwind of the Penguin Colony (Fig. 1). Three continuous
150 palaeo-beaches (PB) were selected; PB 7, is the oldest and estimated to have formed ca.
151 7200 cal. yrs BP, an intermediate stage PB 5 estimated to between 2500-4400 cal. yrs BP
152 and the youngest, PB 2 estimated to have formed between 650-200 cal. y BP (Fretwell et al.
153 2010). BSCs on PB 7 and PB 5 are dominated by the lichens *Usnea* spp., *Sphaerophorus*
154 *globosus*, *Ochrolechia frigida*, *Psoroma hypnorum* and *Pannaria hookeri*, and the
155 bryophytes *Chorizodontium aciphyllum*, *Barbilophozia hatcheii* and *Herzogobryum teres*.
156 PB 2 is the closest to the western shore and the farthest from the Penguin Colony and the
157 BSC is dominated by *Usnea* spp. and also importantly by the crustose lichens, such as
158 *Buelia coniops* and *Lecidea cremonicolor*. The dominant mosses on PB 2 are *Lophozia*
159 *excisa* and *Sanionia unciniata*.

160

161 Soil and rainwater sampling and chemical analysis

162 Six random samples of surface soils (0-10 cm), separated more than 10 cm each other and
163 following the elevation contour line of the terrain, from directly beneath the BSCs were
164 taken with a shovel from each site (the approximate length of the study area was 60 m).

165 Soils were sieved using a 2 mm mesh size prior to chemical analysis. Plant available
166 inorganic N (N_{in}) was extracted in a 0.021 mol L⁻¹ KAl(SO₄)₂ solution (1:4) and the
167 determination of available N as ammonium and nitrate was by means of fractionated steam

168 distillation (Pérez et al. 1998). Plant available P (P_a) was extracted through lactation using
169 the CAL (Calcium-Acetate-Lactate) method and determined colorimetrically using the
170 molybdenum blue method (Steubing and Fangmeier 1992). Water content of soil samples
171 was determined gravimetrically. Soil reaction was determined with a pH electrode in a 1:2
172 soil:water suspension. The dry samples of soil were ground for the determination of total N
173 and C by means of flash combustion using an NA2500 Carlo Erba Element Analyzer. P
174 from ground soil material was extracted with concentrated sulfuric acid together with a
175 water peroxide solution in a Hach Digesdahl digester and determined by colorimetric
176 molybdenum-blue method.

177 Rainwater was collected in 60 ml narrow-mouth Nalgene bottles connected to a 10
178 cm diameter funnel (acid washed) fixed by a pole at ~ 0.8 m from the ground on each one
179 of the three palaeo-beaches, one at the Patterned Ground, two in the Penguin Colony and
180 two directly in the *guanera*, which is a place conformed only of guano deposit with no BSC
181 covering the soil. Rain samples were collected during three precipitation events in February
182 2012 and 2013 and two events in February 2014. Rainwater samples from the Patterned
183 Ground were taken only during 2013. The concentration of ammonium, nitrate and
184 phosphate in the rain samples were determined by ion chromatography using an 861
185 Advanced Compact Metrohm IC.

186

187 Biological nitrogen fixation

188 At each site, six random samples of BSCs were obtained, separated more than 10 m apart
189 along transects following the elevation contour lines (the approximate length of the study
190 area was 60 m), and incubated in the field and in the laboratory during three consecutive
191 years; February 2012, 2013, 2014. The acetylene reduction technique was used to estimate

192 symbiotic N fixation rates (Myrold et al. 1999). This method is based on the fact that the
193 diazotrophs are also able to reduce acetylene to ethylene (Hardy et al. 1968). In the field,
194 samples of BSCs (from 3-15 g dry weight) were deposited inside 130 ml glass jars,
195 hermetically closed and incubated in a mixture of air and acetylene at 10% v/v for up to
196 two days. In parallel, at each sampling point a sample of BSC (a mass within the range as
197 controls) was incubated with thoroughly mixed penguin guano. An additional sample (a
198 mass within the range above) was incubated without acetylene as a control for samples with
199 and without guano. Three gas samples per jar were taken periodically up to 48 h and
200 injected in 4 ml BD vacutainers® and transported to the Biogeochemistry Laboratory at
201 the Pontificia Universidad Católica de Chile for analysis. Ethylene concentrations in the gas
202 samples were measured using a GC 8A Shimadzu gas chromatograph equipped with a
203 Porapak column and FID detector. Ethylene concentrations were determined from a
204 calibration curve by diluting a 100 ppm ethylene standard balanced of helium (Scotty®
205 Analyzed Gases). Acetylene Reduction Activity (ARA) was estimated from the slope of the
206 linear fit of the ethylene production during incubation within a 130 ml headspace. Linearity
207 in acetylene reduction rates was obtained within the incubation period. Plant samples were
208 dried at 70°C and after 48 hrs weighed. In order to scale up the rates of acetylene reduction
209 activity obtained in the field to the complete growing season (GS: from December to
210 March), we assumed no significant changes in wind direction that could bring ammonia-
211 enriched rainfall from penguin colonies. We also assumed that moisture levels in BSCs are
212 representative values for the complete GS, as air relative humidity (86-89%) had a
213 coefficient of variation within each GS during the three consecutive years of 5-1%. In order
214 to obtain an estimation of the N fixation rate per unit of area, the biomass of the BSCs was
215 sampled using a 10 x10 cm pvc frame and dried in the oven at 70°C for >48 hrs. According

216 to theoretical stoichiometry, one mol of N₂ is fixed per three moles of acetylene reduced to
217 ethylene (Hardy et al. 1968). Thus the rates of BNF performed by BSC was expressed in kg
218 N ha⁻¹ GS⁻¹. In situ experiments adding guano were performed for three consecutive years
219 (2012-2014) for site PB7, and for 2013 and 2014 across the rest of the sites. During each
220 field incubation, the temperature in the BSCs was measured using a soil thermometer.

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

225

226 Potential rates of denitrification

227 Following the same transect line and points for the samples taken for BNF, six intact soil
228 cores were taken from each study site and incubated under laboratory conditions at the
229 Escudero Base. Denitrification rates were determined using the acetylene blocking assay
230 (Groffman et al. 1999). This method is based on the inhibition of nitrous oxide (N₂O)
231 reductase by acetylene, allowing the accumulation of nitrous oxide in an acetylene
232 atmosphere, which can be measured by gas chromatography. Soil samples were placed
233 inside 130 ml hermetic glass jars and incubated for 6 hours under a 10% v/v acetylene
234 atmosphere. Gas samples were taken at two and six hours and injected into 4 ml BD
235 vacutainers® for analysis. The N₂O concentration in the gas samples were determined
236 using a GC 8A Shimadzu gas chromatograph equipped with a Porapak column Q 80/100
237 and electron capture detector. A calibration curve was prepared by diluting a 1 ppm nitrous
238 oxide balance of nitrogen (Scotty® Analyzed Gases). As the top soils have a relatively high
239 carbon content and low C/N ratio we assumed that the acetylene treatment would not affect

240 denitrification rates. Denitrification rates were estimated from the differences in N₂O-N
241 concentrations between 6 hrs and 2 hrs and referred to an area basis. All chemical analyses
242 were conducted at the Biogeochemistry Laboratory at the Pontificia Universidad Católica
243 de Chile, Santiago.

244

245 Statistical analysis

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

257

258 Results

259 Chemical analysis of soils and rainwater

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

264 and the lowest pH ($F_{5,30} = 50.63, p < 0.0001$) than all sites. Soils at the Penguin Colony also
265 presented higher moisture levels than the PB 7, Reference and Patterned Ground sites ($F_{5,30}$
266 $= 4.64, p = 0.003$). Soils at the Penguin Colony also have higher concentrations of total N (χ^2
267 $_{5,30} = 14.67, p = 0.012$) and total C ($F_{5,30} = 3.31, p = 0.017$) than at PB 7. Both the soils of PB 7
268 and the Penguin Colony had the highest total P content ($F_{5,30} = 26.44, p < 0.0001$). Soils at
269 the Reference Site presented lower C/N ratios than PB 5 and PB 2 ($\chi^2_{5,30} = 14.66, p = 0.012$).

270 Ammonia (Fig. 2a) and phosphate (Fig. 2b) concentrations in rainwater evidenced
271 an upward trend directly in the *guanera* and declining towards the margins of the Penguin
272 Colony during the three years of the study. Nitrate concentration in rainwater was similar in
273 the different localities and showed little variation during the three years of the study (Fig.
274 2c).

275

276 Biological nitrogen fixation

277 In relation to the inter-annual variation of BNF in field assays, there was a significant effect
278 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
279 the highest rates during the year 2012 reaching up to 3 kg of N ha⁻¹ growing season⁻¹ at
280 PB 7 (Fig. 3). The temperatures of the BSCs during field incubation reached their highest
281 values during 2012 (Table 3). BNF in BSCs in the Penguin Colony was significantly lower
282 than at the PB 7, Reference and Patterned Ground sites (Fig. 3). The Reference Site
283 presented significantly higher BNF rates than PB 2. During the three years of the study
284 there was a trend to higher BNF from PB 2 to PB 7, however differences among these sites
285 were not statistically significant (Fig. 3).

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

288 16.711, $p=0.003$) (Fig. 4a). During 2013 the addition of guano significantly inhibited rates
289 of BNF at the PB 5 (Fig. 4b) ($F_{1,7} = 30.41$, $p<0.0001$), Reference (Fig. 4c) ($F_{1,10} = 49.06$,
290 $p<0.0001$) and Patterned Ground sites (Fig. 4d) ($F_{1,10} = 12.59$, $p=0.005$). During 2014 there
291 was a significant inhibition of BNF only at the Reference Site ($F_{1,7} = 34.189$, $p<0.0001$)
292 (Fig. 4c). There was no significant effect from the addition of guano at PB 2 in any year
293 (Fig. 4e).

294 In relation to the effect of moisture on BNF in laboratory incubations, during 2012
295 the addition of water significantly stimulated ARA ($F_{1,59} = 193.6$, $p<0.0001$) in almost all
296 sites except PB 2 and the Penguin Colony (Fig. 5a). During this year, the water content of
297 control samples in BSCs were on average 30% (Table 3). There was also a site effect ($F_{5,59}$
298 $= 20.64$, $p<0.0001$), where the Penguin Colony presented the lowest ARA and the
299 Reference Site higher rates than PB 5, PB 2 and Patterned Ground (Fig. 5a). During 2013
300 there was no effect from the addition of water ($F_{1,56} = 0.35$, $p=0.56$) (Fig. 5b), when the
301 water content of control samples was on average 62.5% (Table 3). During 2013, there was a
302 site effect ($F_{5,56} = 21.93$, $p<0.0001$) with the lowest rates in the Penguin Colony. Highest
303 rates presented the Patterned Ground, Reference Site and PB 5 (Fig. 5b). During 2014 there
304 was a significant effect from the addition of water ($F_{1,54} = 17.67$, $p<0.0001$) which
305 stimulated ARA in PB 7 and the Reference Site (Fig. 5c). There was also a site effect ($F_{5,54}$
306 $= 20.1$, $p<0.0001$), with lowest rates in Penguin Colony and PB 2 and higher rates in the
307 Patterned Ground site than at PB 5 and PB 7 (Fig. 5c).

308

309 Potential denitrification

310 There was a significant year ($F_{2,60} = 48.58$, $p<0.0001$) and site effect ($F_{5,30} = 10.14$,
311 $p<0.0001$) on denitrification rates with the highest rates during 2012. Statistically

312 significant higher rates of denitrification were reached in the soils of the Penguin Colony
313 across years (Fig.6). During year 2013 the site Patterned Ground next to the Penguin
314 Colony presented high rates of denitrification as well, however it was not statistically
315 different from the other sites (Fig. 6).

316

317 Discussion

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

319 The chemical parameters of the soils are very similar amongst the study sites except for the
320 Penguin Colony, which as expected, had the highest contents of available inorganic N and
321 P and the lowest pH. Similar N and P enrichment in soils under bird influence are reported
322 in maritime (Tatur 2002; Tscherko et al. 2003; Simas et al. 2007) and continental
323 Antarctica as well (Cocks et al. 1998; Cannone et al. 2008; Ball et al. 2015). The main
324 source of these elements are penguin excreta, mainly from uric acid (Lindeboom 1984).
325 Uric acid mineralizes and can either follow the pathway of ammonia volatilization and/or
326 nitrification or denitrification. High concentrations of ammonium in rainwater and high
327 rates of denitrification provide evidence that these two pathways are occurring at the
328 Penguin Colony study site. In the present study, estimated input of inorganic nitrogen via
329 wet deposition is $0.2 \text{ kg N ha}^{-1}\text{year}^{-1}$ in the palaeo-beaches and $1.36 \text{ kg N ha}^{-1} \text{ year}^{-1}$ in the
330 Penguin Colony, where 73% is $\text{NO}_3\text{-N}$ in the former while it is only 25% in the latter. This
331 suggests that nitrate is an important form of reactive nitrogen incorporated to ecosystems
332 via wet deposition in areas located distant from the penguin colonies. The mean nitrate
333 concentration of $2.1 \mu\text{mol L}^{-1}$ in rainwater of palaeo-beaches is within the range of those
334 reported for the South Pacific Ocean and Coast of Chile, which range from $0.2\text{-}2.9 \mu\text{mol L}^{-1}$
335 (Jung et al. 2011). Among the possible sources of nitrate documented to occur to the

336 pristine troposphere of the maritime Antarctica are: i) N-fixation by lightning, ii) re-
337 emissions from snow, firn layer and soils, iii) oxidation of ammonia, and iv) anthropogenic
338 emissions from the surrounding bases, among others (Wagenbach et al. 1998, Jones et al.
339 2000, Savarino et al 2007). As no clear pattern was observed in our study sites, all these
340 sources may be contributing to nitrate in precipitation in Ardley Island. Dry deposition of
341 nitrogen was not measured in the present study; however, model simulations indicate that it
342 may play a minor role in total deposition of reactive nitrogen in maritime Antarctica,
343 reaching ca. 20-30% in the South Shetlands region (Li et al. 2010).

344 In relation to P, a gaseous reduced form of P called phosphine is emitted at
345 extraordinary high rates at the Penguin Colony on Ardley Island (Zhu et al. 2014), which
346 can be oxidized to water soluble phosphate in precipitation and, therefore, be transported
347 long distances. A similar pattern of phosphate concentration in the rainwater was identified
348 by this study and which were much higher directly in the *guanera* and declining towards
349 the margins of the Penguin Colony and Patterned Ground sites. Similarly to ammonium, the
350 effect of phosphate was at a small scale and did not reach the paleo-beaches located upwind
351 from the Penguin Colony. It has been documented that the main source of N to plants in
352 Sub-Antarctic islands is N originating from guano (Erskine et al. 1998; Crittenden et al.
353 2015) and may even be sourced from abandoned-ancient penguin rookeries (Wasley et al.
354 2006). Because the sites are located in the upwind direction of the Penguin Colony, its
355 effect on BNF performed by BSCs is on a small scale on Ardley Island.

356

357 The effect of guano, moisture and temperature on BNF.

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

360 Artic regions, where moisture and temperature are found the main factors controlling BNF
361 in soil and vegetation (Zielke et al. 2005, Sorensen et al. 2006). However, BNF is
362 completely inhibited even during favourable years at the Penguin Colony with high N
363 availability. Moreover, we report that enhanced inputs of N via guano have a significant
364 negative effect on BNF performed by BSCs which cover soils with lower N availability
365 than in the Penguin Colony and distant from the effect of guano aerosols in rainwater. A
366 similar inhibition of BNF was reported for soils under the influence of breeding snow
367 petrels (*Pagodroma nivea*), in Dronning Maud Land, continental Antarctica (Cocks et al.
368 1998). These findings suggests that the ultimate control on BNF in northern maritime
369 Antarctica under the effect of penguin colonies and in regions of continental Antarctica
370 under bird influence is N availability.

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

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

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

388 In the present study, we report that during wetter and warmer years, BNF can reach
389 higher levels of up to $3 \text{ kg N ha}^{-1} \text{ y}^{-1}$ on the oldest paleo-beach PB 7, which is fifteen times
390 higher than N inputs via wet deposition. Similar estimations of BNF on Signy Island
391 (maritime Antarctica) have reported levels of 0.46 and $1.92 \text{ kg N ha}^{-1} \text{ y}^{-1}$ for dry turf and
392 wet carpets respectively (Vincent 2000). Ranges of symbiotic N fixation reported from
393 other types of cold biomes such as the moist and alpine tundra are $1 - 4.9 \text{ kg N ha}^{-1} \text{ y}^{-1}$
394 (Reed et al. 2011) and up to $1.3 \text{ kg N ha}^{-1} \text{ y}^{-1}$ in the Arctic tundra of northern Alaska
395 (Hobara et al. 2006). However, even higher rates have been reported for soils crusts in a
396 low Arctic tundra landscape of Canada and in the Sub-Arctic region of Sweden of up to 11
397 $\text{kg N ha}^{-1} \text{ y}^{-1}$ (Stewart et al. 2011; Sorensen et al. 2006). Together all these data suggest that
398 even in the colder high latitude biomes such as maritime Antarctica and Arctic, “hot spots”
399 of BNF can be present where free of the influence of sea bird guano.

400

401 Potential denitrification rates.

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

408 2014. Conversely, we found no trend along the chronosequence, as the young and oldest
409 palaeo-beach did not present significant differences in denitrification rates. In other studies
410 it has been found that there was a small increase in potential denitrification rates with time
411 since glacier retreat in amended (with potassium nitrate and dextrose) soils on Anvers
412 Island, linked to an increase in nitrate content in the soils, although at very low rates < 1
413 $\mu\text{mol N m}^{-2} \text{ h}^{-1}$ (Strauss et al. 2009, 2012). Our mean values for the palaeo-beaches of 0.21
414 $\mu\text{mol N m}^{-2} \text{ h}^{-1}$ are in the lower ranges in comparison to the Anvers Island chronosequence.
415 The highest denitrification rates within the Penguin Colony ($12 \mu\text{mol N m}^{-2} \text{ h}^{-1}$) during
416 2014 is one order of magnitude higher than potential denitrification in the amended and
417 older soils on Anvers Island. However, even higher emission rates of $\text{N}_2\text{O-N}$ in field assays
418 that varied from 53-194 $\mu\text{mol N m}^{-2} \text{ h}^{-1}$ have been estimated from the Penguin Colony on
419 Ardley Island (Zhu et al. 2008).

420

421 Conclusions

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

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

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

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593 Figure Legends

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

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

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

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

608 Figure 5. The effect of the addition of water on acetylene reduction activity in laboratory
609 incubation of biological soil crusts in Ardley Island during February 2012 (a), 2013 (b) and
610 2014 (c). The asterisks indicate significant differences among treatments (Tukey tests
611 $p < 0.05$). Bars indicate *SE* of mean values ($n=6$). Different letters among sites indicate
612 statistically significant differences according to a-posteriori Tukey tests ($p < 0.05$).

613 Figure 6. Potential denitrification rates in surface soils of Ardley Island during February
614 2012, 2013 and 2014. Bars indicate *SE* of mean values ($n=6$). Different letters among sites
615 indicate statistically significant differences according to a-posteriori Tukey tests ($p < 0.05$).

616

Figure 1

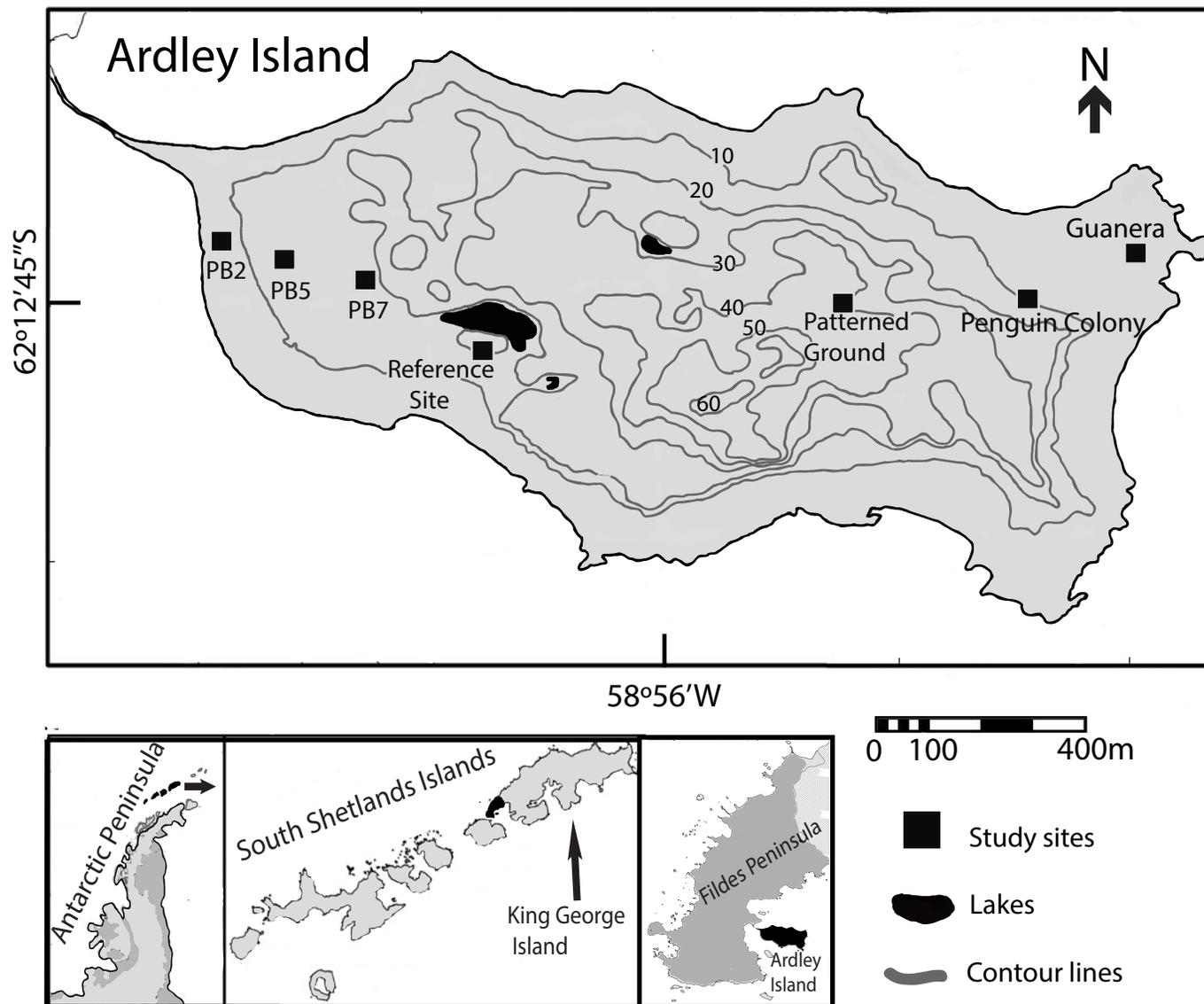


Figure 2

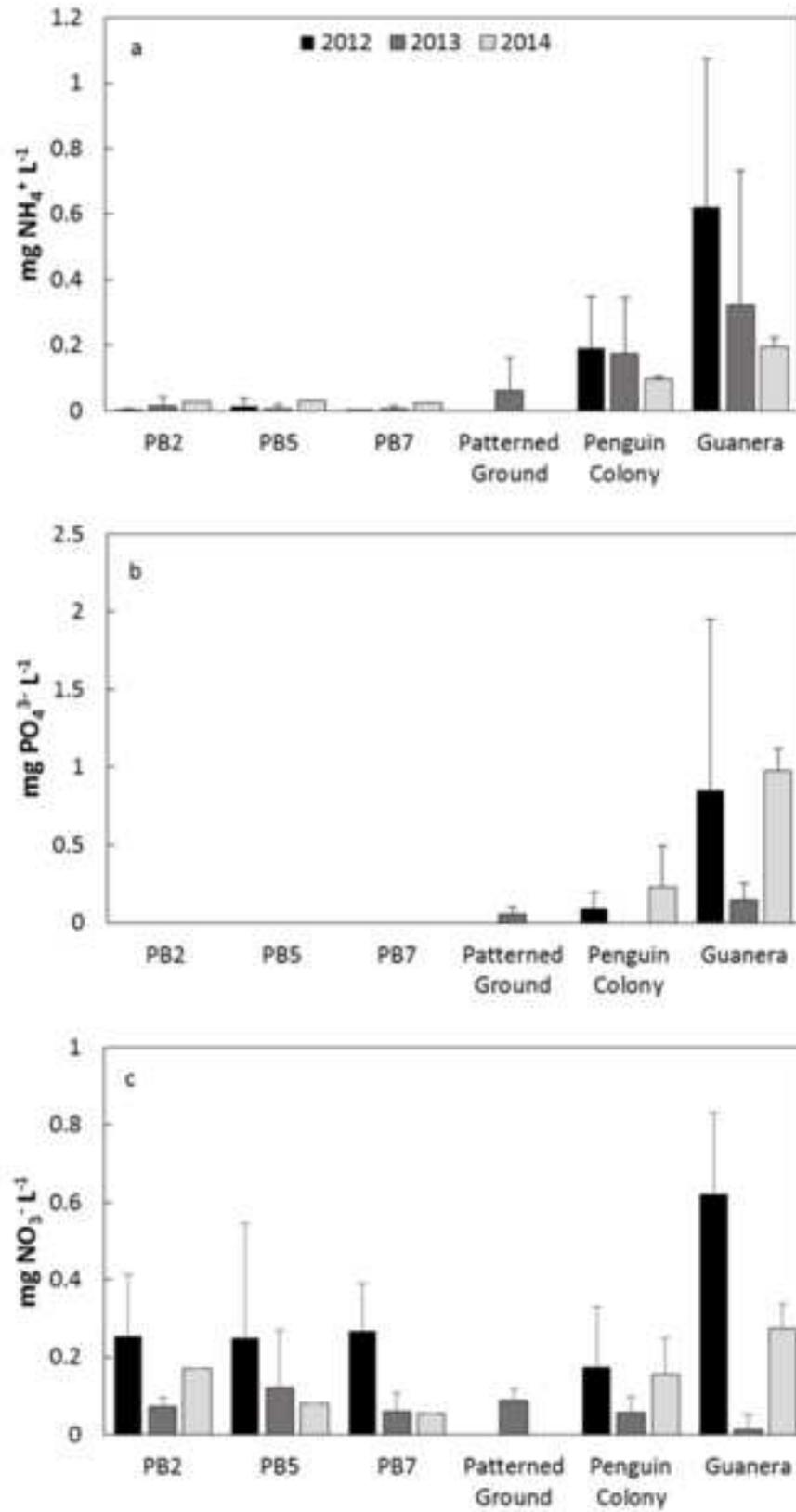
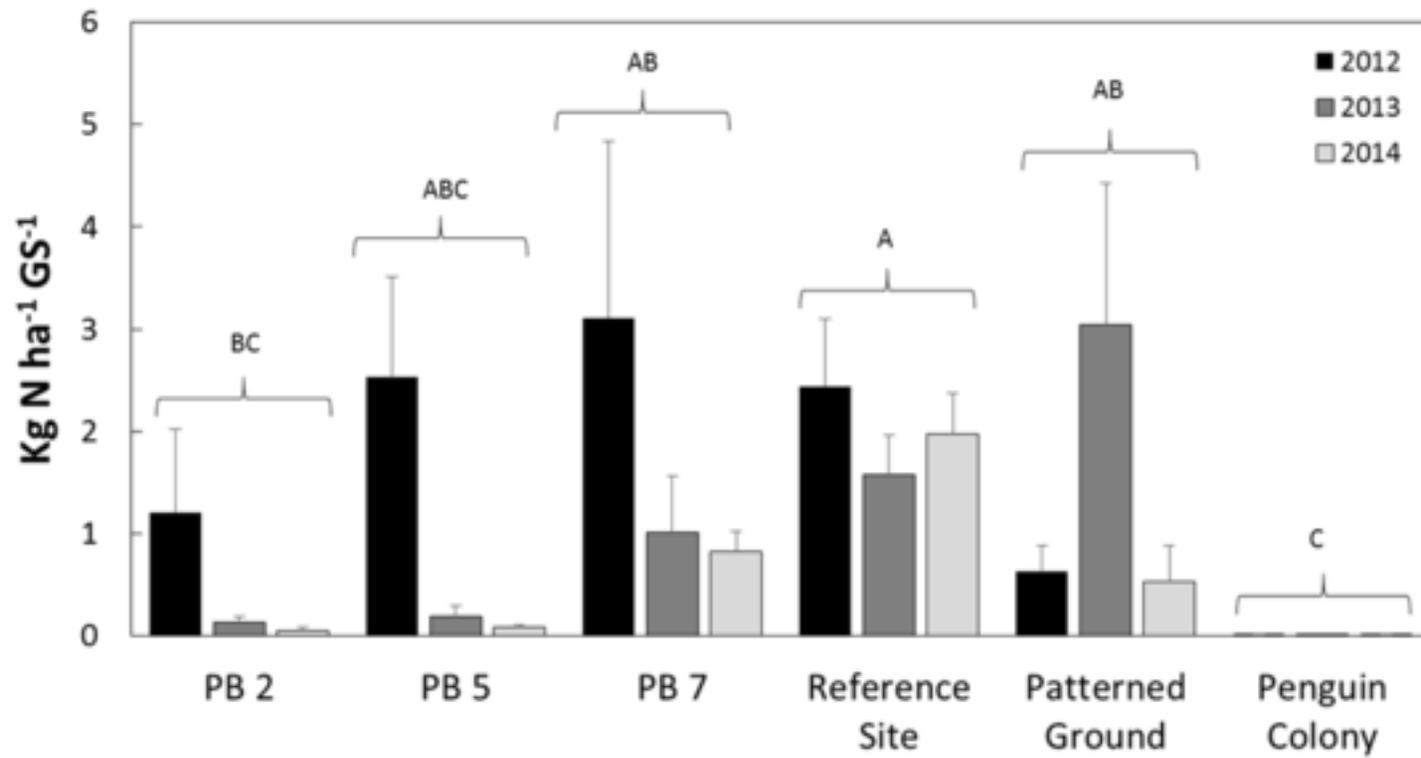
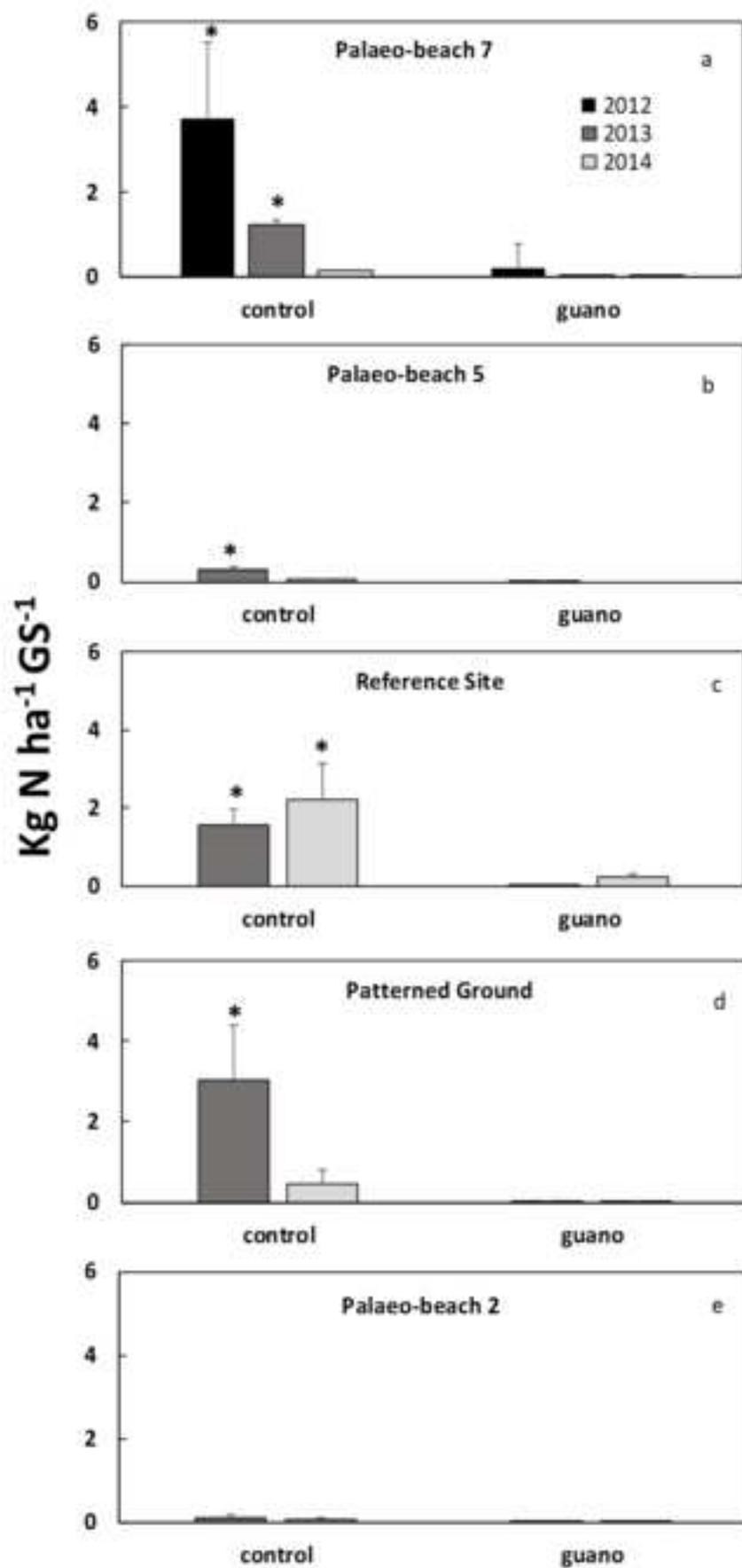


Figure 3





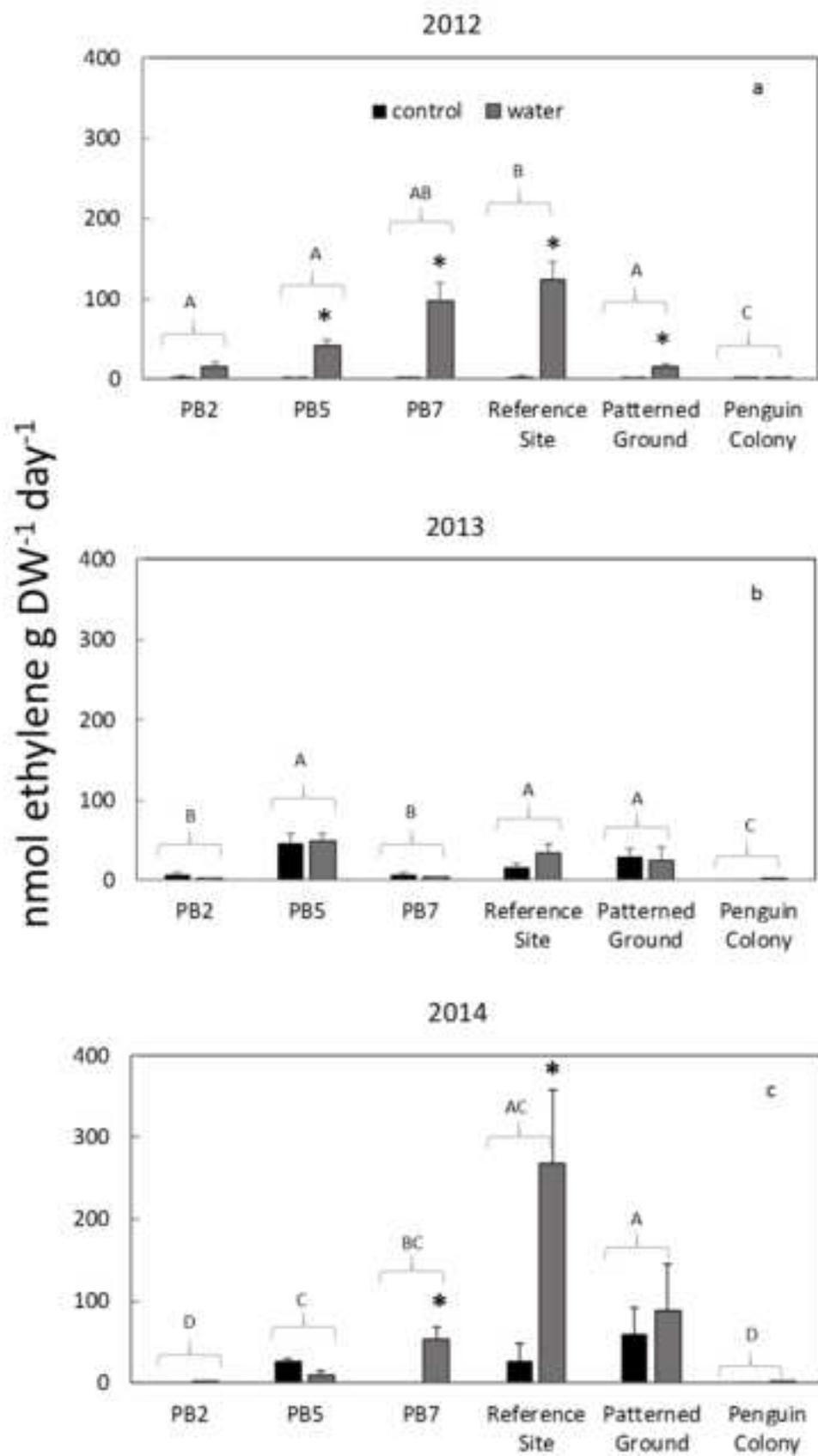
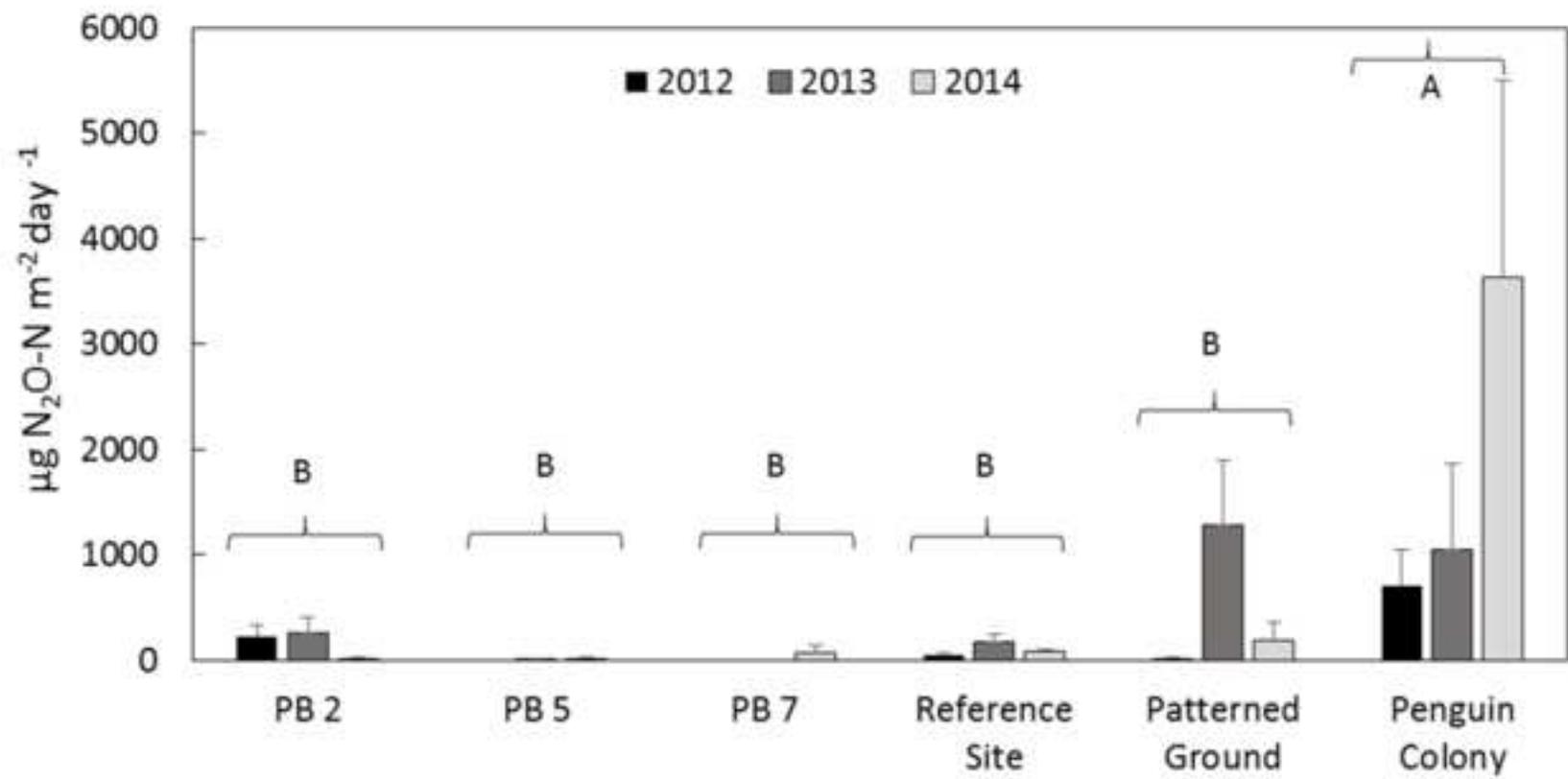


Figure 6



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

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	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

4

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

4

	N_{in} (mg kg⁻¹)	P_a (mg kg⁻¹)	pH (H₂O)	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)

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

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 4

	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)

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