

1 **Soil bacterial functional diversity mirrors the loss of plant diversity by the expansion of a native tall-**
2 **grass in high mountain grasslands**

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8

9 **Abstract**

10 *Background and Aims* In highland ecosystems, global change processes are intense and foster vegetation
11 shifts that may have an impact on soil functioning. Soil bacterial communities may be particularly
12 sensitive to these changing scenarios. The aim of this research is to determine whether the loss of
13 floristic diversity caused by the unusual dominance of a native component -the perennial grass
14 *Brachypodium rupestre* (L.) Beauv., which is expanding aggressively in natural grasslands of the Western
15 Pyrenees-, parallels a decrease of the soil bacterial functional diversity and their potential for nutrient
16 transformations.

17 *Methods.* We conducted the study in eight grasslands exposed to different degrees of *B. rupestre*
18 spreading. Soil community physiological profiles of the heterotrophic bacteria, enzymatic activities
19 related to C, P and N cycles, C and N microbial biomasses, N components and soil physical and chemical
20 properties were determined.

21 *Results* Soils below low-diversity grasslands had lower bacterial functional richness and diversity but
22 greater urease activity, pH and nitrate than soils in diverse grasslands. Ammonium pools, C and N
23 microbial biomasses and enzymatic activities related to C and P did not differ between grasslands.

24 *Conclusions* The expansion of *B. rupestre* and the decrease of plant diversity coincided with a significant
25 decline of bacterial functional diversity and an alteration of the N cycle. Not only plant composition but
26 the prevailing disturbance regime may account for the results. Results also suggest that *B. rupestre* may
27 rely on its capability to use N efficiently rather than on a soil bacteria-mediated N availability.

28

29 **Keywords**

30 Grassland diversity · Native plant spread · *Brachypodium rupestre* · Disturbance regime · Enzymatic
31 activities · N cycle

32

33 **Introduction**

34 Global change is a driving force causing dramatic alterations in natural mountain ecosystems, affecting
35 the composition and diversity of plant communities at regional and local scales (Pauchard et al. 2009).
36 The conditions of change, i.e. climatic variation and disrupted regime of disturbances, enhance in some
37 ecosystems the success of particular plant species. The spreading of the species is boosted at the
38 expense of the rest of taxa, and the equilibrium attained among species constituting a community is
39 modified (Buckland et al. 2001; Valery et al. 2009a; 2009b). *Brachypodium rupestre* (L.) Beauv., a
40 perennial tall-grass native to Europe, is experiencing a comparable process. The species, common in
41 chalk grasslands of Western and Central Europe, has rapidly expanded in the last decades due to the

42 atmospheric N deposition (Bobbink et al. 1988) and to the disruption of fire and herbivory regimes (Baba
43 2003; Catorci et al. 2011; Kohler et al. 2005). In the Western Pyrenees, the decrease of grazing pressure
44 and the high frequency of controlled burnings to reduce necromass build-ups have enhanced the
45 expansion of *B. rupestre* in many grassland communities, to the detriment of many sympatric species
46 (Canals et al. 2014).

47 The current configuration of soil microbial communities largely depends on climate and land-use
48 legacies (Delgado-Baquerizo et al. 2017; Jangid et al. 2011) and, at the community level, on the plant
49 species composition resulting from these legacies (Lambers et al. 2009). Changes occurring aboveground
50 may exert a strong influence on the microbial functional diversity and the biological mechanisms of
51 nutrient cycling (Bever et al. 1997; Knops et al. 2002; Milcu et al. 2010; Westover et al. 1997), ultimately
52 affecting the resistance and resilience of plant communities to change (De Deyn and Van der Putten
53 2005; Wardle et al. 2004).

54 The modification of the functional properties of the soils by alien invasive species has devoted much
55 research in the last decades. Exotic species disrupt soils through a variety of processes, which in the mid-
56 term tend to cause a significant simplification of functions (Vitousek et al. 1996). Native soil microbiota
57 may be affected by changes in the quality and quantity of the litter and the root exudates, and by
58 different patterns of plant nutrients acquisition (Canals et al. 2005; Jo et al. 2015; Mack and D'Antonio
59 2003). Microbial processes related to the nitrogen (N) cycle, such as N-enzymatic activities and N-
60 mineralization and nitrification processes, may be particularly affected by exotic plant invasions
61 (DeCappreo et al. 2017; Ehrenfeld 2003; Evans et al. 2001; Kourtev et al. 2002). In comparison, the
62 circumstance of a native species, common component of the ecosystem, changing the expansion
63 patterns in its own, native habitat is a particular situation fostered by the global change (Valery, 2009a;
64 Valery, 2009b). Although previous plant-soil interactions may remain, functions and processes may be
65 altered by the increased dominance of a specific taxa and the loss of plant diversity. The question that
66 arises is, to what extent microbial communities and mediated nutrient processes in soils are resistant to
67 the loss of diversity caused by the aggressive expansion of a particular native species?.

68 The microbiome developing in highlands has to cope with severe abiotic constraints (Lipson et al. 2002).
69 Besides the extreme climate, soils in these environments are generally limited by acidity and
70 oligotrophy, and a low transformation and availability of nutrients to plants and to microbes occurs
71 (Jaeger et al. 1999; van der Heijden et al. 2003). Bacteria and fungi are both important mediators of the
72 biochemical processes in highlands, but the bacterial community is expected to be more sensitive than
73 the fungal community to the prevailing constraints (Lipson 2007; Nemergut et al. 2005). The aim of this
74 research is to determine whether the loss of floristic diversity in a community caused by the unusual
75 dominance of a native component parallels a decrease of the soil bacterial functional diversity and their
76 potential for nutrient transformations. We hypothesize that plant communities degraded by the
77 expansion of *B. rupestre*, which carry a legacy of altered regimes of fire and herbivory disturbance,
78 harbour lower microbial biomass, bacterial functional diversity, and associated nutrient, particularly N,
79 availability compared to high diverse communities. These losses, if occurring, may hinder the recovery
80 of degraded communities, even though the historical regime of disturbances is eventually restored.

81

82 **Materials and Methods**

83 **Study site**

84 The study was carried out at eight experimental sites in Aezkoa Valley, in the Western Pyrenees (43°3'
85 N 1°13' W), in two mountain ranges between 800-1,450 m a.s.l. encompassing a surface of 1,929 ha.
86 Consequence of the altitude and the influence of the Atlantic Ocean (55 km far in a straight line), climate
87 is cold and snowy in winter, and mild and foggy in summer. Mean annual temperature is 9.3 °C and mean
88 annual precipitation is 1,856 mm (last 20 years records in the Irabia climatic station). The dominant

89 substrates are sandstones and calcareous clays, which develop deep, acidic and organic soils with loamy
90 and clay-loamy textures. Vegetation is a mosaic of beech forests, shrublands dominated by *Ulex gallii*
91 Planch. and *Erica vagans* L. and grasslands constituted by perennial grasses (such as *Festuca rubra* gr.,
92 *Agrostis capillaris* L., *Brachypodium rupestre* (L.) Beauv., *Danthonia decumbens* (L.) DC. and *Avenula*
93 *sulcata* (J. Gay ex Boiss.) Dumort.), forbs (such as *Galium saxatile* L., *Potentilla erecta* (L.) Ræusch,
94 *Potentilla montana* Brot. and *Hipochaeris radicata* L.), and legumes (such as *Trifolium repens* L.).
95

96 Grasslands in the study site support an extensive free mixed grazing regime by sheep, cows and horses
97 during the mild season (May to October). Domestic herbivory has been intense for centuries but, in the
98 last decades, it has largely reduced. The relaxation of the herbivory has promoted the expansion of the
99 native, low-palatable tall-grass *B. rupestre* in many areas. The species builds up a dense layer of
100 necromass each year, which is reduced by frequent surface burnings. As a consequence, despite pastoral
101 fires have been traditionally practiced in the area to prevent shrub encroachment (every 6-7 years on
102 uneven clumps of ungrazed vegetation) a different regime of recurrent widespread burnings (every 2-4
103 winters) has developed in the last decades in many areas. This new regime, although reducing rapidly
104 *B. rupestre* biomass, promotes the expansion of the species in the mid term (Canals et al. 2014).

105

106 Survey areas characterization and soil samplings

107 After the consultation of a detailed grassland cartography (Ferrer and Canals 2008) and the completion
108 of an exploratory field survey, eight homogeneous sites encompassing a minimum surface of 4 ha were
109 chosen for the study. The sites selected included a range of abundance of *B. rupestre*, from species-poor
110 communities visually dominated by the grass to species-rich swards where *B. rupestre* coexisted with
111 many other species. In spring 2013, we undertook a general characterization of the sites, by describing
112 the main environmental variables (altitude, topography, slope, aspect, substrate and soil type) and the
113 intensity of the current management (grazing pressure and burning recurrence) (Table 1). This depiction
114 was completed in summer by conducting systematic floristic inventories using the point-intercept
115 methodology (2 transect lines of 20 m per site, with 50 intercepted points per line) for plant community
116 typification (see Canals et al. 2017 for more details).
117

118 The soil samplings were done at the peak of the summer 2013. Highland soils meet optimal conditions
119 for the development of fast-growing bacterial populations in this period of the year, where mild
120 temperatures, soil moisture and labile root exudates are expected to rise the bacterial-to-fungal ratios
121 and improve bacterial community characterization (Andersen et al. 2013). At each site, five sampling
122 points -separated each one 100-150 m in distance- were randomly selected and, at each point, three
123 topsoil samples were collected (10 cm depth, cores of 9 cm diameter). As a result, 120 soil samples, 15
124 samples per site, were gathered and kept cold in portable fridges until the laboratory.

125

126 Soil analyses

127 In the laboratory, 40 composite and homogenised soil samples were obtained after mixing the three
128 samples collected at each point. A half portion of each sample was sieved to 2mm, stored in
129 polyethylene bags and kept refrigerated at +4 °C for further analyses. A fraction of the non-sieved soils
130 were sent to an official laboratory (Nasertic, Pamplona, Spain) where the main physical and chemical
131 parameters (texture, organic matter, total C and N, available P and K, cation exchangeable capacity and
132 exchangeable cations) were determined by standardised methods. The remnant fractions of the non-
133 sieved soils were analysed in our laboratory for gravimetric moisture (SWC), pH, ammonium (NH₄⁺) and
134 nitrate (NO₃⁻) (in 2M KCL extracts by continuous flow colorimetry, Braun+Luebbe segmented flow
135 analyser AA3 Norderstedt, Germany). Also, they were analysed for contents of C and N in the microbial

136 biomasses (MBC and MBN) using the chloroform fumigation-extraction method (in 0.05M K₂SO₄
137 extracts, (Brookes et al. 1985), assuming a fumigation efficiency of 0.45 (K_N and K_C) (Joergensen et al.
138 2011), for dissolved organic nitrogen (DON, subtracting the mineral-N pool from N contents in non-
139 fumigated extracts) and for dissolved organic carbon (DOC, from non-fumigated extracts).

140 Enzymatic activities of β-glucosidase, phosphatase alkaline and urease, related to the C, P, and N cycles
141 respectively, were determined in the sieved soils. For the β-glucosidase and alkaline phosphatase
142 enzyme activities we used the 96-well microplate approach (German et al. 2011). The method is based
143 on the release of p-nitrophenol after a reaction of a centrifuged and filtered extract of soil water (1:3
144 soil-to-H₂O, 1h) with the synthetic substrates, p-nitrophenyl-b-d-glucopyranoside (PNG) for β-
145 glucosidase, and p-nitrophenyl phosphate hexahydrate (PNP) for alkaline phosphatase, in a modified
146 universal buffer (60 mM, pH 6.0). After incubation at 37°C for 1 hour the reaction was stopped with the
147 addition of 0.5M NaOH, and the absorbance was measured at 410 nm with a microplate
148 spectrophotometer (Multiskan™ GO, Thermo Scientific). Urease activity was determined by the
149 colorimetric method of Kandeler and Gerber (1988), modified by Rodriguez-Loinaz et al. (2008). One
150 gram of soil was wetted with borate and 100 mM buffer pH 10.0, then 820 mM urea was added and the
151 solution was extracted with 2 M KCl. The extract was incubated at 37° for 1 hour, centrifuged, and the
152 suspension diluted with water. Then, the reagents A (salicylate-nitroprusside solution in 3M NaOH) and
153 dicholorisocyanurate were added to the suspension and, after an incubation of 30 min at room
154 temperature, the absorbance was measured at 670 nm (Multiskan™ GO, Thermo Scientific).

155 The physiological profiles of the heterotrophic bacterial soil communities (CLPP) were determined by
156 means of Biolog-EcoPlates™ (BIOLOG, Inc., Hayward, CA) (Garland 1997). The method estimates the
157 potential metabolic diversity of the soil bacterial community by using microplates with 31 different
158 carbon sources and a negative control (water), replicated three times. The determination of community
159 level physiological profiles using the BIOLOG technique has been widely used in ecological and
160 agronomic studies to assess, i.e., the effects of toxic metals and pollutants (Borymski et al. 2018; Galazka
161 et al. 2018; Ratcliff et al. 2006; Thompson et al. 1999) and the impacts on soils of different managements
162 (Buyer and Drinkwater 1997; Cesarano et al. 2017). Given that CLPP measurements rely on the culturing
163 of the microflora in a collection of selected substrates, the results may represent a subset of the whole
164 bacterial community present in the soil (Garland and Mills 1991; Preston-Mafham et al. 2002). However,
165 CLPP data have demonstrated to be sensitive indicators of changes in the soil microbial function in
166 comparison to the measurements of microbial biomasses and enzymatic activities, which may respond
167 to more general community-level processes (Rogers and Tate 2001). Microorganisms were extracted
168 from the soil samples in water (125 rpm, 1 h) and a 150 µl soil suspension (1/1000 m/v) was dispensed
169 into each of the 96 wells. Then, microplates were incubated at 30°C in the dark for 7 days. Absorbance
170 measurements at 595 nm were done every 12 hours during a week. From the absorbance values we
171 determined the average well colour development (AWCD), as an estimate of the bacterial community
172 catabolic activity, and the richness (S), diversity (Shannon-Wiener and Simpson indexes) and evenness
173 (Pielou's index) of bacterial substrate utilization as estimates of the bacterial functional composition.

174

175 Statistical analyses

176 We assessed the soil-related drivers of plant community composition using redundancy analysis (RDA).
177 We used non-transformed relative frequency data of our plant community data as response variable,
178 and the main soil variables (pH, SWC, ammonium, nitrate, DON, DOC, BMN, BMC, AWCD, urease,
179 phosphatase, glucosidase and urease) as explanatory variables. Soil variables were standardised to
180 mean zero and standard deviation one to account for different measurement scales of soil parameters.
181 We used a forward selection procedure (ordistep function, vegan package; Oksanen et al. 2015) to
182 determine the subset of soil variables explaining most variation in plant species composition. The
183 statistical power of the analysis was assessed by Monte Carlo permutation tests (n= 999). Linear mixed

184 models (*nlme* package; Pinheiro et al. 2015) were performed to assess the effects of grassland
185 community type (low-diversity and high-diversity) on the soil physical and chemical variables, and on
186 the soil functional and microbiological variables. The model included the type of grassland community
187 as the fixed factor and the individual sites as the random factor. The sites were included as random
188 factors to account for the inherent variation of each particular site. Data were log-transformed when
189 necessary to improve normality and homoscedasticity of errors, and the best variance structure for the
190 residuals was chosen using the likelihood ratio test (restricted maximum likelihood estimation
191 procedures). The significance of the fixed effects was analysed in a similar way by maximum likelihood
192 estimation procedures (Zuur et al. 2009). Multivariate approaches by PCA were used to study the
193 relationships between grassland types and bacterial metabolic groups (*vegan* package; Oksanen et al.
194 2015), using as response variables the AWCD of each group (amines, amino acids, carboxylic acids,
195 polymers, carbohydrates and miscellaneous). In order to determine the relationships among soil
196 microbial variables in low and high-diversity grasslands, Pearson's correlation coefficients were
197 determined (*rcorr* function, *Hmisc* package; Harrell 2017). Means and SE presented in the text and
198 figures were calculated using non-transformed data.

199

200 **Results**

201 Grasslands ordination

202 RDA analysis revealed that soil-related explanatory variables explained a significant amount of variation
203 of plant composition (55.66%). Forward selection of soil parameters resulted in the parameters nitrate
204 and bacterial catabolic activity (AWCD) as most relevant soil variables (Figure 1A, red arrows). In the
205 ordination plot, we overlaid the species scores of the most abundant grasses (Figure 1A, blue arrows)
206 showing that the first axis separated high diverse and low diverse grasslands according to a gradient of
207 soil nitrate and abundance of *B. rupestre* (Figure 1A). In low diversity grasslands, estimates of plant
208 richness and diversity were more variable but consistent with the changing cover of *B. rupestre*.

209

210 Soil physical and chemical parameters

211 No significant differences between soils under low and high-diversity grasslands were found for most of
212 the abiotic variables analysed. All soils had a silty clay loam texture, high organic matter (OM) and total
213 N content (on average 9.42 % and 0.49 % respectively), low pools of phosphorus (P_2O_5 averaged 17.08
214 $mg \cdot kg^{-1}$), and a high cation exchange capacity (CEC averaged $18.89 \text{ cmol}^{(+)} \cdot kg^{-1}$), occupied by protons
215 (H^+) and Al^{+3} and, to a lesser extent, by the major cations ($Ca^{+2} + Mg^{+2} + Na^{+1} + K^{+1} < 7.5 \text{ cmol}^{(+)} \cdot kg^{-1}$) (Table
216 2).

217 Soil pH, exchangeable magnesium and nitrate pools were the only variables that differed significantly
218 between low- and high-diversity grasslands. Despite all soils being very acidic (< 5.84), pH and
219 exchangeable magnesium were higher in soils under low-diversity grasslands (Table 2). The inorganic
220 pools of N were very small at all sites ($NO_3^- < 0.6 \text{ mg N} \cdot kg^{-1}$ at average, Figure 2), but nitrate was
221 significantly higher (4-fold increase) in low- than in high-diversity grasslands (Table 2). Nitrate pools were
222 positively correlated with soil pH. The site displaying the highest abundance of *B. rupestre* (LD3, Table
223 1), exhibited a high peak of nitrate (around $1 \text{ mg N} \cdot kg^{-1}$) compared to the rest of sites (Figure 2B).

224

225 Soil functional and microbiological parameters

226 Although no significant differences were reported for C and N microbial biomasses (Table 3), the
227 physiological profiles of the heterotrophic bacterial communities differed significantly between soils

228 developing under low- and high-diversity grasslands. Soils in high-diversity grasslands had a significantly
229 higher bacterial activity and functional richness, diversity and evenness than soils in low-diversity
230 grasslands (Table 3). In addition, high-diversity grasslands exhibited a more consistent pattern among
231 sites (regarding bacterial catabolic activity and bacterial functional composition, Figure 2f & 3), in
232 comparison to low-diversity grasslands where a high variability of results existed among sampled
233 locations.

234 The multivariate analysis on the functional microbial groups responding to substrates in the microplates
235 indicated that the first two axes of the PCA explained a high percentage of the variance, 87.32 %. The
236 first axis accounted for 66.48 % of the variance and the second axis for 20.84 % (Figure 4). The
237 segregation of samples from soils below high-diversity grasslands in the left side of the PCA suggested a
238 high level of functional microbial groups related to amino acids, amines, carboxylic acids and polymers.
239 Figure 5 details the substrate activities observed between grasslands. Overall, the group of
240 miscellaneous substrates (pyruvic acid methyl ester in particular) were the most utilised by bacteria,
241 followed by the carbohydrates (N-acetyl-D-glucosamine, L-methyl-D-glucoside and D-mannitol) and the
242 amino acids (L-asparagine, L-phenylalanine and L-serine). Soils below high-diversity grasslands displayed
243 a higher bacterial use of N-rich organic substrates, such as amino acids ($LR = 5.849$, $p = 0.016$, mainly
244 glycyl-L-glutamic acid and L-asparagine) and amines ($LR = 5.140$, $p = 0.023$, mainly putrescine),
245 suggesting a relevance of N bacterial transformations. Carboxylic acids ($LR = 3.391$; $p = 0.065$, mainly
246 itaconic acid) and polymers ($LR = 3.576$, $p = 0.086$, mainly Tween 80) also tended to be comparatively
247 higher in high-diversity grasslands. Despite these results, the activity of the enzyme urease, which
248 degrades urea into ammonia, the substrate for nitrification, was lower in soils of high- compared to low-
249 diversity grasslands ($LR = 5.689$, $p = 0.017$; Figure 2d). Enzyme activities of phosphatase-alkaline and β -
250 glucosidase did not differ between grasslands. Eventually, two carbohydrates of the five tested, tended
251 to be more utilised in low- than in high-diversity grasslands, L-methyl-D-glucoside and D-cellobiose
252 (Figure 5).

253 Figure 6 displays the matrix of correlations among soil functional bacterial diversity parameters and
254 microbial biomasses in the two grassland types. In low-diversity grasslands, BMN correlated significantly
255 and positively with bacterial functional richness, and a similar tendency was found for BMC. This pattern
256 was not observed in high-diversity communities. In the latter, a high similarity among sites, which
257 hinders the detection of trends, and/or a higher functional redundancy compared to low-diversity sites
258 may explain the results.

259

260

261 **Discussion**

262 Differences in soil bacterial functional diversity between grasslands

263 In this research, soils in low-diversity grasslands had a significant lower bacterial functional richness,
264 diversity, evenness and catabolic activity (AWCP) than soils in high-diversity grasslands, suggesting that
265 soil bacterial functional diversity reflected the loss of plant diversity. Experimental studies in the last
266 decade describe similar results, indicating a positive link between above- and belowground diversity and
267 proposing plant diversity as a decisive determinant of soil biodiversity (Eisenhauer et al. 2010; He et al.
268 2008; Milcu et al. 2010). One of the possible mechanisms underlying this relationship is the increased
269 heterogeneity of soil organic inputs promoted by diverse plant communities (Eisenhauer 2016). In this
270 research, soils in diverse grasslands had a higher activity of bacterial groups involved in organic N
271 transformations (amines and amino acids) compared to soils in low-diversity grasslands. Bacterial-guild
272 specific differences among sites are expected to respond to the aptitude of the microbial communities
273 to degrade the main specific categories of carbon compounds in each particular soil (Zak et al. 1994).
274 During the growing season in high altitude regions, root exudates are the main source of labile nutrients

275 to soil microbes (Nemergut et al. 2005) and summer microbial communities are mostly composed by
276 fast-growing organisms that feed on these labile nutrients (Lipson et al. 2002). During this period, root
277 exudates are expected to be mostly composed by sugars, organic acids and, to a lesser extent, amino
278 acids and phenols (Marschner and 1985). The observed higher diversity of bacterial functional groups
279 and the higher level of catabolic activity related to N in diverse compared to poor grasslands, may reflect
280 an enhanced availability of N organic compounds from root exudates (due to a high number of species
281 and functional groups, including N-rich legumes), or/and a higher input of plant labile carbon (which
282 stimulates N microbial immobilisation, Knops et al. 2002) in diverse grasslands. In low-diversity
283 grasslands, *B. rupestre* generates a high amount of plant litter with high C:N ratios (Canals et al. 2017)
284 and the soil microbiome may be presumably responding to the recalcitrant nature of these tissues (for
285 instance, by an expecting increase of the fungal community, Paterson et al. 2008).

286

287 Contrary to the results of some studies identifying a significant relationship between plant diversity and
288 soil microbial biomass (Eisenhauer et al. 2010; Lange et al. 2015), we did not find such a clear
289 relationship. No significant differences in soil C and N microbial biomasses were reported between
290 grasslands. However, a tendency for MBC to increase in high-diversity grasslands and a positive,
291 significant correlation between the functional bacterial richness and BMN (a tendency in BMC) in soils
292 below low-diversity grasslands was detected. Altogether, this information may suggest a comparatively
293 higher bacterial functional redundancy in soils of high-diversity grasslands. In the current scenario of
294 change, microbial functional redundancy is a positive asset, since it may result in a high ecosystem
295 stability, i.e., an alteration in the microbial composition may not involve a change in key ecosystem
296 processes (Allison and Martiny 2008). Besides, when alternative niches are available, a high bacterial
297 richness is prone to stimulate a high evolutionary diversification (Jousset et al. 2016), which is also
298 positive for adaptation.

299

300 Differences in N dynamics between grasslands

301 Soil urease activity, nitrate pools and pH were significantly different below high- and low-diversity
302 grasslands. In natural soils, the enzyme-driven depolymerisation stage is the most limiting step for the
303 generation of available N (Chapin et al. 2011; Schimel and Bennet 2004). In this research, low-diversity
304 grasslands exhibited a higher urease-enzyme activity, nitrate concentration and pH than high-diversity
305 grasslands. Since ammonia is the final product of the urease activity and the substrate of the nitrification
306 the increased soil pH and nitrate content in *B. rupestre* dominated grasslands may be associated to the
307 enhancement of this enzymatic activity.

308 The ability to alter the N cycling for its own benefit has been demonstrated in many exotic invaders, i.e.,
309 increasing soil N availability and nitrification rates (Adair and Burke 2010; Booth et al. 2003; Ehrenfeld
310 2003; Kourtev et al. 2003). In N-limited ecosystems, the capability to change the internal N cycling may
311 allow expansive species to gain a clear competitive advantage over established species (Laungani and
312 Knops 2009). In the case of *B. rupestre*, we do not have evidence for an active role of the grass in the
313 urease enhancement. The enzyme may originate from plant and microbial sources and the mechanisms
314 underlying the increase of urease activity in *B. rupestre* dominated soils need a specific research to
315 determine whether the grass is actively promoting the enzymatic activity or just keeping track of the
316 pulses of N availability occurring in the soil. Previous experimental research in *B. rupestre* has reported
317 a good responsiveness of the species to particular inorganic N pulses, as those caused by atmospheric N
318 deposition (Hanstein et al. 1999) and by prescribed fires (Canals et al. 2014; Hanstein et al. 1999).
319 Alternatively, the species has demonstrated to adopt efficient N-foraging and conservative strategies in
320 N-limited soils (Canals et al. 2017; Hurst and John 1999; Ryser 1996; Ryser and Lambers 1995; Tardella
321 et al. 2017). Altogether, the results suggest a very competent use of the N by *B. rupestre*, both in
322 enriched and in poor N environments.

323

324 Concluding remarks

325 The hypothesis that grasslands degraded by the expansion of *B. rupestre*, subjected to a disrupted regime
326 of fire and herbivory, have a loss of soil microbial biomass, bacterial functional diversity, and N
327 availability is partially supported by the results of this study. Despite the soil microbial functional
328 diversity mirrors the floristic degradation occurring aboveground, microbial biomasses, enzyme
329 activities and the concentration of the main available nutrients remain little affected or even increase in
330 low-diversity grasslands.

331 Low-diversity grasslands dominated by *B. rupestre* experience a recurrent regime of burnings that, even
332 having low intensity, affect the chemical, functional and microbiological properties of the soils. Detailed
333 monitoring of close prescribed burnings reports transitory pulses of N-inorganic, slight pH increases and
334 declines in urease activity, microbial biomass and bacterial C-substrate utilization diversity (Fonturbel et
335 al. 2016; San Emeterio et al. 2016). According to this, the higher nitrate availability, increased pH, and
336 lower functional bacterial diversity in low-diversity grasslands compared to high-diversity, may result
337 from a combined, synergic effect of the fire regime and the current plant composition, which is not
338 discernible in this research.

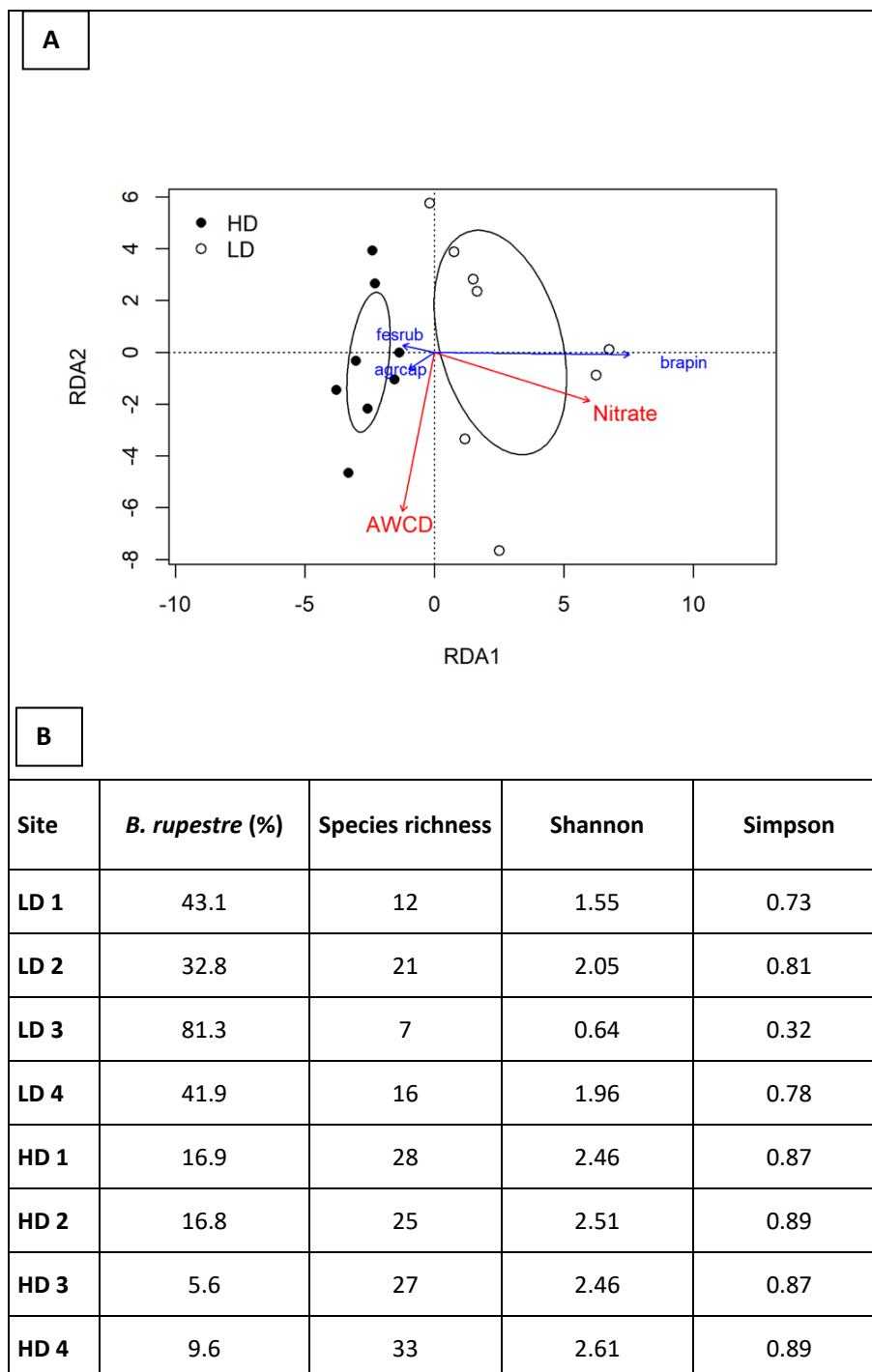
339 Leaving aside the key role that fungal communities may play in these environments, we should expect
340 that the decrease of soil bacterial functional diversity in grasslands experiencing the expansion of *B.*
341 *rupestre* might conduct to a lower adaptability and capacity to cope with new constraints compared to
342 high-diversity grasslands. However, the stability over time of *B. rupestre* grasslands observed in the field
343 and the state of current research on the species, lead us to suggest the hypothesis described in figure 7.
344 According to this, diverse grasslands and *B. rupestre*-dominated grasslands would rely on two different
345 strategies to meet their demands on N, the most limiting and mobile nutrient in soils. While high-
346 diversity grasslands would establish an intimate mediation with the bacterial microbiome to access
347 different forms of N (N-soil reliant strategy), low-diversity grasslands would develop a strategy more
348 based on the successful capability of *B. rupestre* to access and preserve N sources (N-plant reliant
349 strategy). To what extent this potential self-sufficiency in *B. rupestre* entails a high resistance and
350 resilience to change for the whole community is a matter of future discussion.

351

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359



360

361 **Fig. 1.** Differences in plant composition among high and low diversity grasslands and related drivers of compositional
 362 differences. A) Ordination plot showing soil related drivers of plant composition (red arrows) and species scores of the most
 363 abundant grasses (blue arrows). Results are based on RDA and only significant factors are displayed. B) Summary table of
 364 differences in *B. rupestre* cover, plant richness and diversity estimates between study sites.

365 LD: Low-diversity grasslands. HD: High-diversity grasslands. Brapin, *Brachypodium rupestre*; fesrub, *Festuca g. rubra*; agrcap,
 366 *Agrostis capillaris*; agrcur, *Agrostis curtisii*.

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370 **Table 1.** General description of the study sites. LD: Low-diversity grasslands. HD: High-diversity grasslands. See Canals *et al*
 371 (2017) for visual location of the sites.

Site	Location	Altitude (m.a.s.l.)	Slope (°)	Aspect	Soil classification	Fire recurrence	Stocking rate
LD1	Erroitzate	1091	33	SW-W-NW	Lithic Udorthents	High	Medium
LD2	Armorieta	861	28	E	Lithic Udorthents	Medium	Low
LD3	Arpea	943	26	NE	Dystric Eutrudepts/Typic Dystrudepts	High	Low
LD4	Abodi	1306	21	SW-W	Lithic Udorthents/Lithic Hapludolls	Medium	Medium
HD1	Ezkanda	1062	7	SW	Dystric Eutrudepts/Typic Dystrudepts	Low	Medium
HD2	Zalbeta	1015	4	SW-W-NW	Dystric Eutrudepts/Typic Dystrudepts	Low	Medium
HD3	Urkulu	1290	25	SW-W	Dystric Eutrudepts/Typic Dystrudepts	Low	Medium
HD4	Azalegi	1074	14	NW	Dystric Eutrudepts/Typic Dystrudepts	Low	High

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374 **Table 2.** Soil physical and chemical parameters measured under low-diversity and high-diversity grasslands and statistical
 375 significance using GLM models. These data are partially published in Canals *et al* (2017).

	Low-diversity	High-diversity	±SE	L. ratio	Significance
	Average	Average			
Physical parameters					
Sand (0.05-2 mm) (%)	11.62	11.55	5.93	0.0396	0.8422
Silt (0.002-0.05 mm) (%)	46.46	49.01	2.95	0.9403	0.3322
Clay (<0.002 mm) (%)	41.92	39.44	5.50	0.2629	0.6081
Water Soil Content (%)	27.30	27.10	0.05	0.0022	0.9627
Chemical parameters					
pH in water (1:2.5)	5.38	5.17	0.10	4.3613	0.0368
Organic Matter (%)	9.86	8.98	1.11	0.7991	0.3714
C/N ratio	9.91	9.86	0.54	0.0087	0.9255
Total N (%)	0.47	0.51	0.04	1.0180	0.3130
Dissolved organic nitrogen (mg N · kg ⁻¹)	6.18	4.75	2.30	0.2419	0.6228
Dissolved organic carbon (mg C · kg ⁻¹)	61.09	65.77	7.18	0.4423	0.5060
Total CEC (cmol ⁽⁺⁾ · kg ⁻¹)	20.01	17.78	2.30	1.1682	0.2798
Exchangeable Ca (cmol ⁽⁺⁾ · kg ⁻¹)	4.53	4.35	0.98	0.0457	0.8308
Exchangeable Mg (cmol ⁽⁺⁾ · kg ⁻¹)	1.41	1.11	0.14	4.5341	0.0332
Exchangeable Na (cmol ⁽⁺⁾ · kg ⁻¹)	0.71	0.68	0.06	0.3073	0.5793
Exchangeable K (cmol ⁽⁺⁾ · kg ⁻¹)	0.53	0.44	0.10	0.8652	0.3523
Exchangeable Al (cmol ⁽⁺⁾ · kg ⁻¹)	3.77	3.65	1.07	0.0160	0.8993
Mineral nutrient pools					
Ammonium (N-NH ₄ ⁺) (mg N · kg ⁻¹)	8.11	7.84	1.81	0.3527	0.5526
Nitrate (N-NO ₃ ⁻) (mg N · kg ⁻¹)	0.44	0.14	0.13	5.0827	0.0242
Phosphorus (P ₂ O ₅) (mg · kg ⁻¹)	17.70	16.47	2.61	0.2393	0.6247
Potassium (K ₂ O) (mg · kg ⁻¹)	246.95	206.20	49.08	0.8700	0.3510

376 *Significances with p < 0.05 are indicated in bold type.

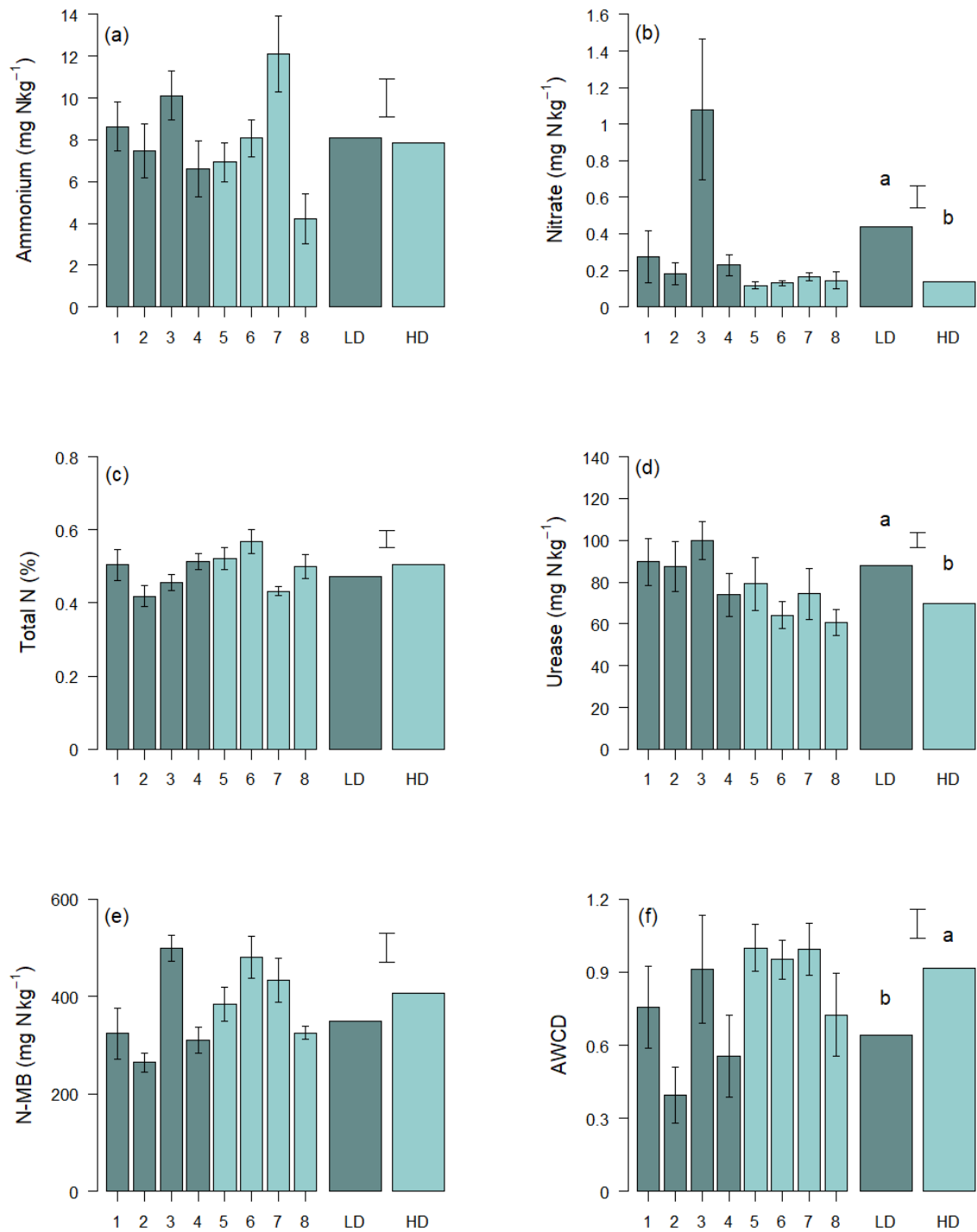
377

378 **Table 3.** Soil bacterial community parameters, microbial biomasses and enzymatic activities under low-diversity and high-
 379 diversity grasslands and statistical significance using GLM models.

	Low- diversity	High- diversity			
	Average	Average	±SE	L. ratio	Significance
Community level physiological profiles					
Average Well Colour Development	0.64	0.92	0.12	5.0115	0.0252
Richness	21.37	25.00	1.51	5.4683	0.0194
Shannon-Wiener diversity index	2.50	2.93	0.17	5.8529	0.0156
Pielou's evenness index	1.89	2.10	0.09	4.6407	0.0312
Microbial biomasses					
Carbon (mg C·kg ⁻¹ soil)	1291.90	1431.97	95.28	2.5907	0.1075
Nitrogen (mg N·kg ⁻¹ soil)	349.64	406.32	61.18	1.4303	0.2317
Enzymatic activities					
Phosphatase (mmol PN·g ⁻¹ soil)	160.75	164.45	26.65	1.7672	0.1837
β-Glucosidase (mmol PN·g ⁻¹ soil)	66.39	60.41	4.93	1.5177	0.2180
Urease (mg N·kg ⁻¹ soil)	87.76	69.60	7.23	5.6887	0.0171

380 *Significances with p < 0.05 are indicated in bold type.

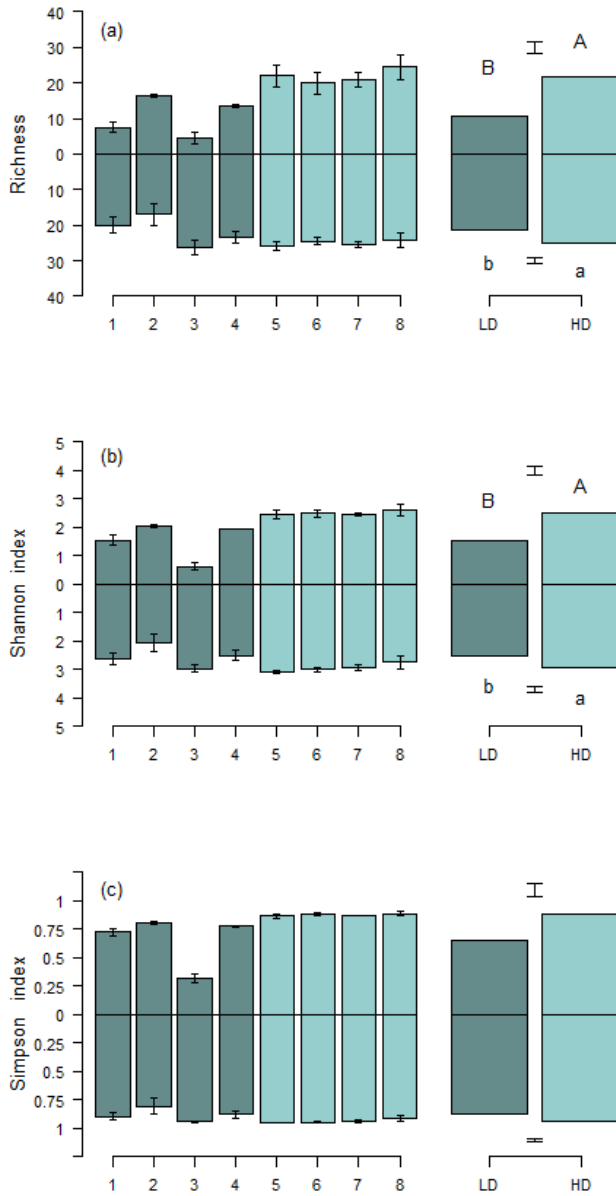
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384 **Fig. 2.** Soil inorganic N contents -(a) ammonium (mg N·kg⁻¹) and (b) nitrate (mg N·kg⁻¹), (c) total N (%), (d) urease enzyme activity
 385 (mg N·kg⁻¹), (e) N in microbial biomass (mg N·kg⁻¹) and (f) average well colour development (physiological profile of the carbon
 386 sources used by the soil bacterial communities). Different numbers in X-axis are the eight study sites: 1-4 are low-diversity
 387 grasslands (LD) and 5-8 are high-diversity grasslands (HD). LD and HD represent means values. Dark pale turquoise are LD
 388 grasslands and light pale turquoise HD grasslands. Different letters stand for significant differences between covers.

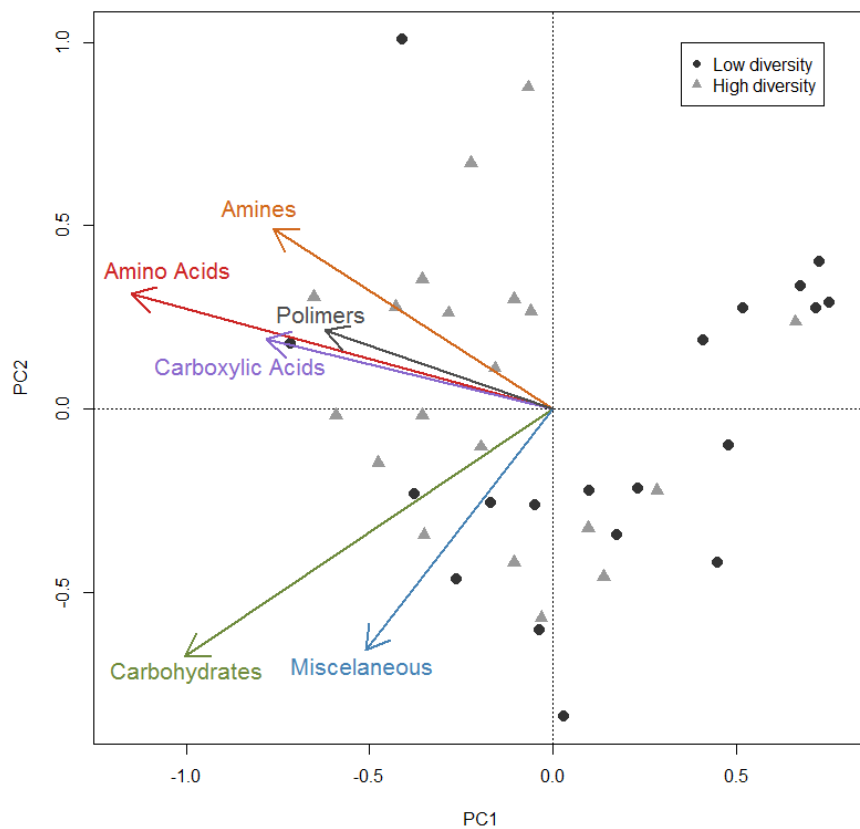
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392 **Fig. 3.** The plant community is represented in the positive Y-axis and the soil bacterial community in the negative Y-axis. (a)
 393 Richness –number of species for the plant community and number of functional groups for the soil bacterial community-, (b)
 394 Shannon-Wiener diversity index and (c) Simpson diversity index in low-diverse and high-diverse grasslands. Different numbers
 395 in X-axis are the eight study sites: 1-4 are low-diversity grasslands (LD) and 5-8 are high-diversity grasslands (HD). LD and HD
 396 represent means values. Dark pale turquoise are LD grasslands and light pale turquoise HD grasslands. Different capital letters
 397 stand for significant differences between plant communities and different lowercase letters stand for significant differences
 398 between bacterial communities.

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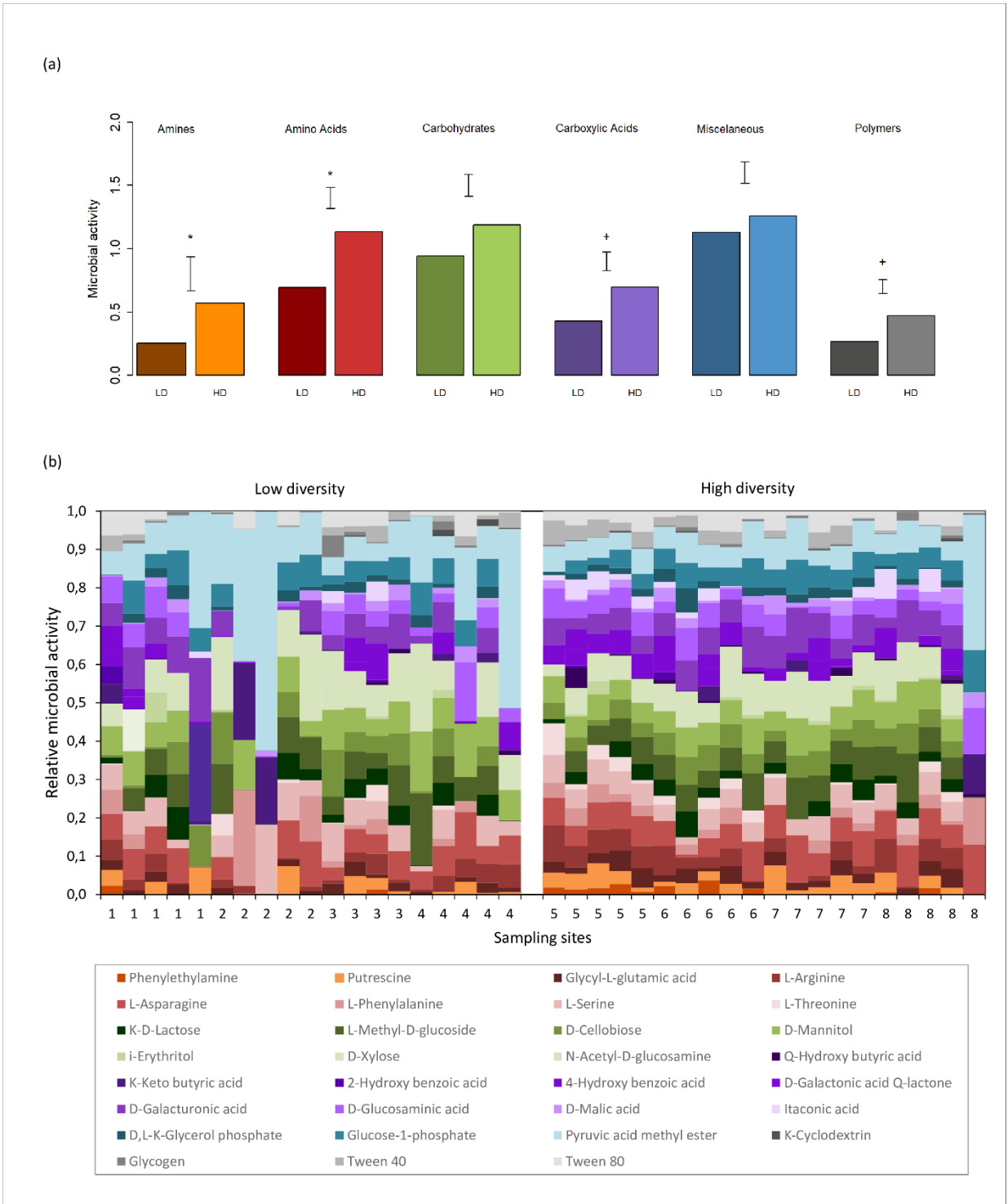
401 **Fig.4.** Principal Component Analysis on AWCD (Average Well Colour Development) of the bacterial metabolic groups tested.
 402 The first axis accounts for 66.48 % of the variance and the second axis 20.84 %.

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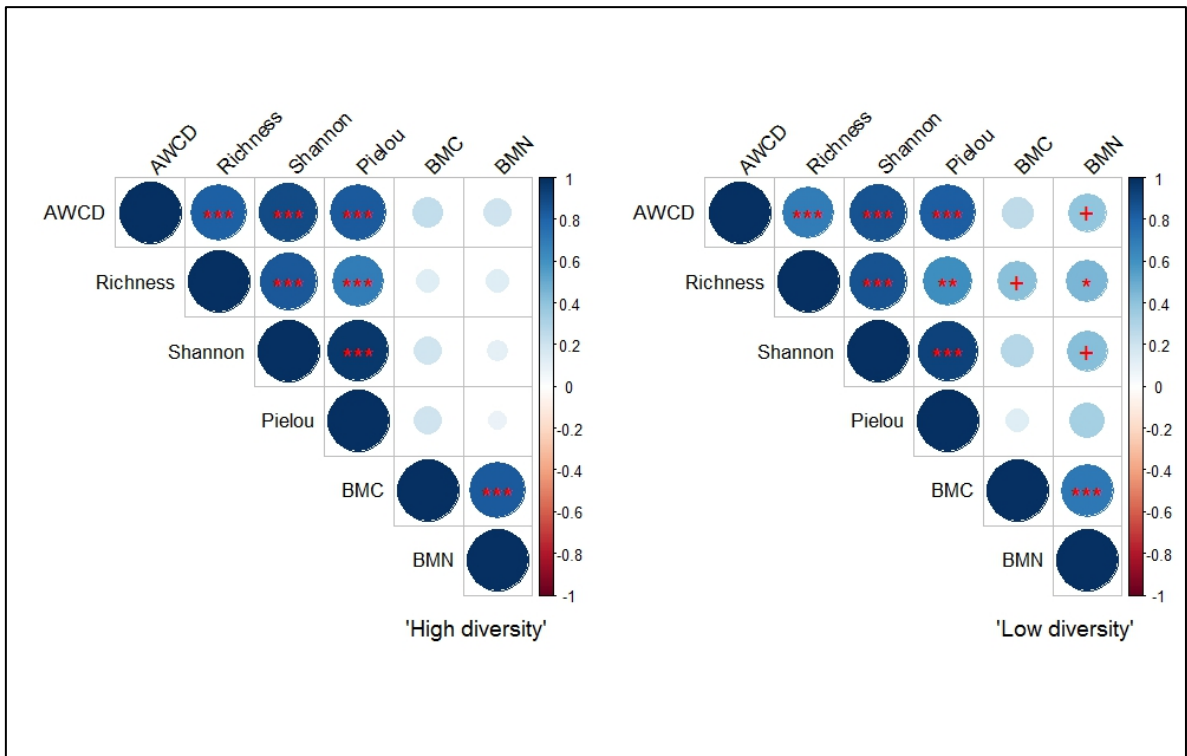
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Fig. 5. Microbial activity in the different substrates tested in low-diversity (LD) and high-diversity (HD) grasslands. Chart (a) summarises the information detailed in chart (b). Statistical significances using GLM models * $p < 0.05$; + $p < 0.07$.



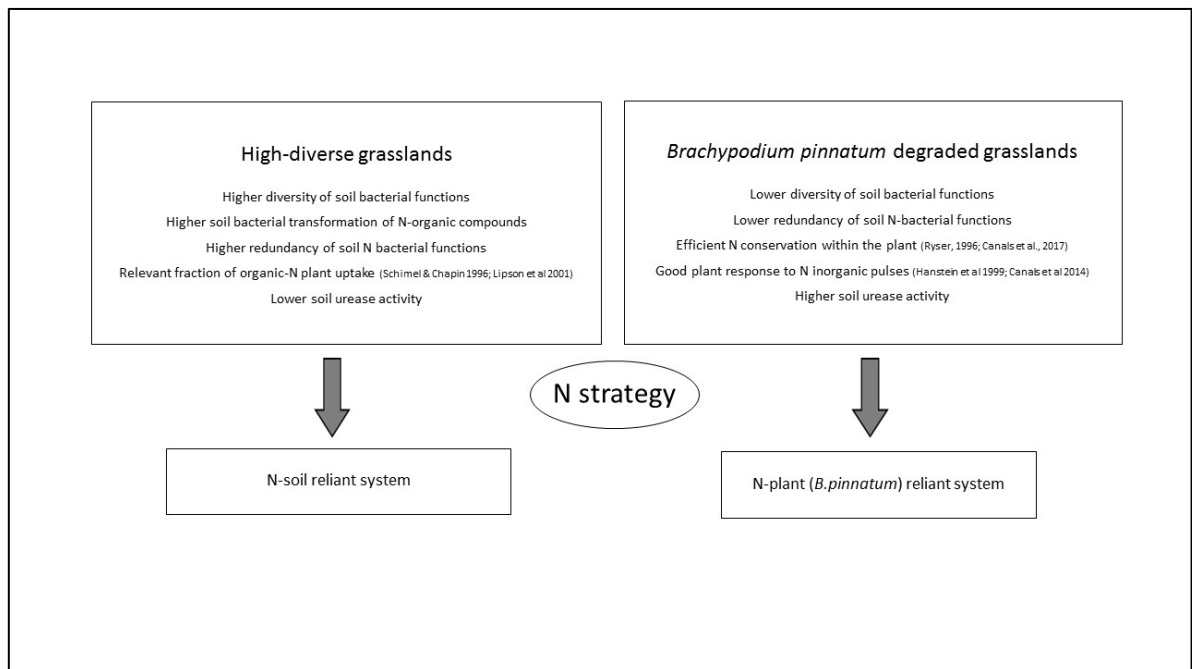
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411 Fig. 6. Pearson correlation coefficients between soil microbial variables in high-diversity and low-diversity grasslands. Dark
 412 colours indicate strong correlations among variables. AWCD, average well colour development, MBC, microbial biomass carbon;
 413 MBN, microbial biomass nitrogen. *** p<0.001, ** p<0.01, * p<0.05, + p<0.10.

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418 Fig 7. New hypothesis about the type of N strategy occurring in diverse and in degraded covers of *B. rupestre*. Authors cited:
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