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

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Abstract

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

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

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

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

Keywords

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

Introduction

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

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

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

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

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

Materials and Methods

Study site

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

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

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

Survey areas characterization and soil samplings

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

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

Soil analyses

In the laboratory, 40 composite and homogenised soil samples were obtained after mixing the three samples collected at each point. A half portion of each sample was sieved to 2mm, stored in polyethylene bags and kept refrigerated at +4 ºC for further analyses. A fraction of the non-sieved soils were sent to an official laboratory (Nasertic, Pamplona, Spain) where the main physical and chemical parameters (texture, organic matter, total C and N, available P and K, cation exchangeable capacity and exchangeable cations) were determined by standardised methods. The remnant fractions of the non-sieved soils were analysed in our laboratory for gravimetric moisture (SWC), pH, ammonium (NH_4^+) and nitrate (NO_3^-) (in 2M KCL extracts by continuous flow colorimetry, Braun+Luebbe segmented flow analyser AA3 Norderstedt, Germany). Also, they were analysed for contents of C and N in the microbial

biomasses (MBC and MBN) using the chloroform fumigation-extraction method (in 0.05M K_2SO_4 extracts, (Brookes et al. 1985), assuming a fumigation efficiency of 0.45 (K_N and K_C) (Joergensen et al. 2011), for dissolved organic nitrogen (DON, subtracting the mineral-N pool from N contents in non-fumigated extracts) and for dissolved organic carbon (DOC, from non-fumigated extracts).

Enzymatic activities of β-glucosidase, phosphatase alkaline and urease, related to the C, P, and N cycles respectively, were determined in the sieved soils. For the β-glucosidase and alkaline phosphatase enzyme activities we used the 96-well microplate approach (German et al. 2011). The method is based on the release of ρ-nitrophenol after a reaction of a centrifuged and filtered extract of soil water (1:3 soil-to-H_2O, 1h) with the synthetic substrates, p-nitrophenyl-b-d-glucopyranoside (PNG) for β-glucosidase, and ρ-nitrophenyl phosphate hexahydrate (PNP) for alkaline phosphatase, in a modified universal buffer (60 mM, pH 6.0). After incubation at 37°C for 1 hour the reaction was stopped with the addition of 0.5M NaOH, and the absorbance was measured at 410 nm with a microplate spectrophotometer (Multiskan™ GO, Thermo Scientific). Urease activity was determined by the colorimetric method of Kandeler and Gerber (1988), modified by Rodriguez-Loinaz et al. (2008). One gram of soil was wetted with borate and 100 mM buffer pH 10.0, then 820 mM urea was added and the solution was extracted with 2 M KCl. The extract was incubated at 37° for 1 hour, centrifuged, and the suspension diluted with water. Then, the reagents A (salicytate-nitroprusside solution in 3M NaOH) and dicholorisocyanurate were added to the suspension and, after an incubation of 30 min at room temperature, the absorbance was measured at 670 nm (Multiskan™ GO, Thermo Scientific).

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

Statistical analyses

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

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

Results

Grasslands ordination

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

Soil physical and chemical parameters

No significant differences between soils under low and high-diversity grasslands were found for most of the abiotic variables analysed. All soils had a silty clay loam texture, high organic matter (OM) and total N content (on average 9.42 % and 0.49 % respectively), low pools of phosphorus ($P_2 0_5$ averaged 17.08 $mg.kg^{-1}$), and a high cation exchange capacity (CEC averaged 18.89 $cmol^{(+)} \cdot kg^{-1}$), occupied by protons (H^+) and Al^{+3} and, to a lesser extent, by the major cations ($Ca^{+2}+Mg^{+2}+Na^{+1}+K^{+1} < 7.5$ $cmol^{(+)}.kg^{-1}$) (Table 2).

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

Soil functional and microbiological parameters

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

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

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

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

Discussion

Differences in soil bacterial functional diversity between grasslands

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

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

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

Differences in N dynamics between grasslands

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

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

Concluding remarks

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

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

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

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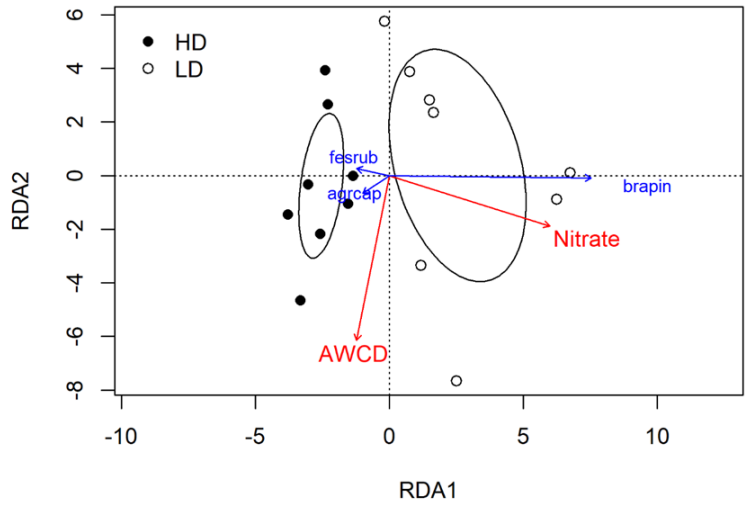
A



B

Site	*B. rupestre* (%)	Species richness	Shannon	Simpson
LD 1	43.1	12	1.55	0.73
LD 2	32.8	21	2.05	0.81
LD 3	81.3	7	0.64	0.32
LD 4	41.9	16	1.96	0.78
HD 1	16.9	28	2.46	0.87
HD 2	16.8	25	2.51	0.89
HD 3	5.6	27	2.46	0.87
HD 4	9.6	33	2.61	0.89

Fig. 1. Differences in plant composition among high and low diversity grasslands and related drivers of compositional differences. A) Ordination plot showing soil related drivers of plant composition (red arrows) and species scores of the most abundant grasses (blue arrows). Results are based on RDA and only significant factors are displayed. B) Summary table of differences in *B. rupestre* cover, plant richness and diversity estimates between study sites.
LD: Low-diversity grasslands. HD: High-diversity grasslands. Brapin, *Brachypodium rupestre*; fesrub, *Festuca* g. *rubra*; agrcap, *Agrostis capillaris*; agrcur, *Agrostis curtisii*.

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	Zalbetea	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			
	Average	**Average**	**±SE**	**L. ratio**	**Significance**
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 $\cdot kg^{-1}$)	6.18	4.75	2.30	0.2419	0.6228
Dissolved organic carbon (mg C $\cdot kg^{-1}$)	61.09	65.77	7.18	0.4423	0.5060
Total CEC ($cmol^{(+)} \cdot kg^{-1}$)	20.01	17.78	2.30	1.1682	0.2798
Exchangeable Ca ($cmol^{(+)} \cdot kg^{-1}$)	4.53	4.35	0.98	0.0457	0.8308
Exchangeable Mg ($cmol^{(+)} \cdot kg^{-1}$)	1.41	1.11	0.14	**4.5341**	**0.0332**
Exchangeable Na ($cmol^{(+)} \cdot kg^{-1}$)	0.71	0.68	0.06	0.3073	0.5793
Exchangeable K ($cmol^{(+)} \cdot kg^{-1}$)	0.53	0.44	0.10	0.8652	0.3523
Exchangeable Al ($cmol^{(+)} \cdot kg^{-1}$)	3.77	3.65	1.07	0.0160	0.8993
Mineral nutrient pools					
Ammonium ($N\text{-}NH_4^+$) (mg N $\cdot kg^{-1}$)	8.11	7.84	1.81	0.3527	0.5526
Nitrate ($N\text{-}NO_3^-$) (mg N $\cdot kg^{-1}$)	0.44	0.14	0.13	**5.0827**	**0.0242**
Phosphorus (P_2O_5) (mg$\cdot kg^{-1}$)	17.70	16.47	2.61	0.2393	0.6247
Potassium (K_2O) (mg$\cdot kg^{-1}$)	246.95	206.20	49.08	0.8700	0.3510

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

377

Table 3. Soil bacterial community parameters, microbial biomasses and enzymatic activities under low-diversity and high-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**

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

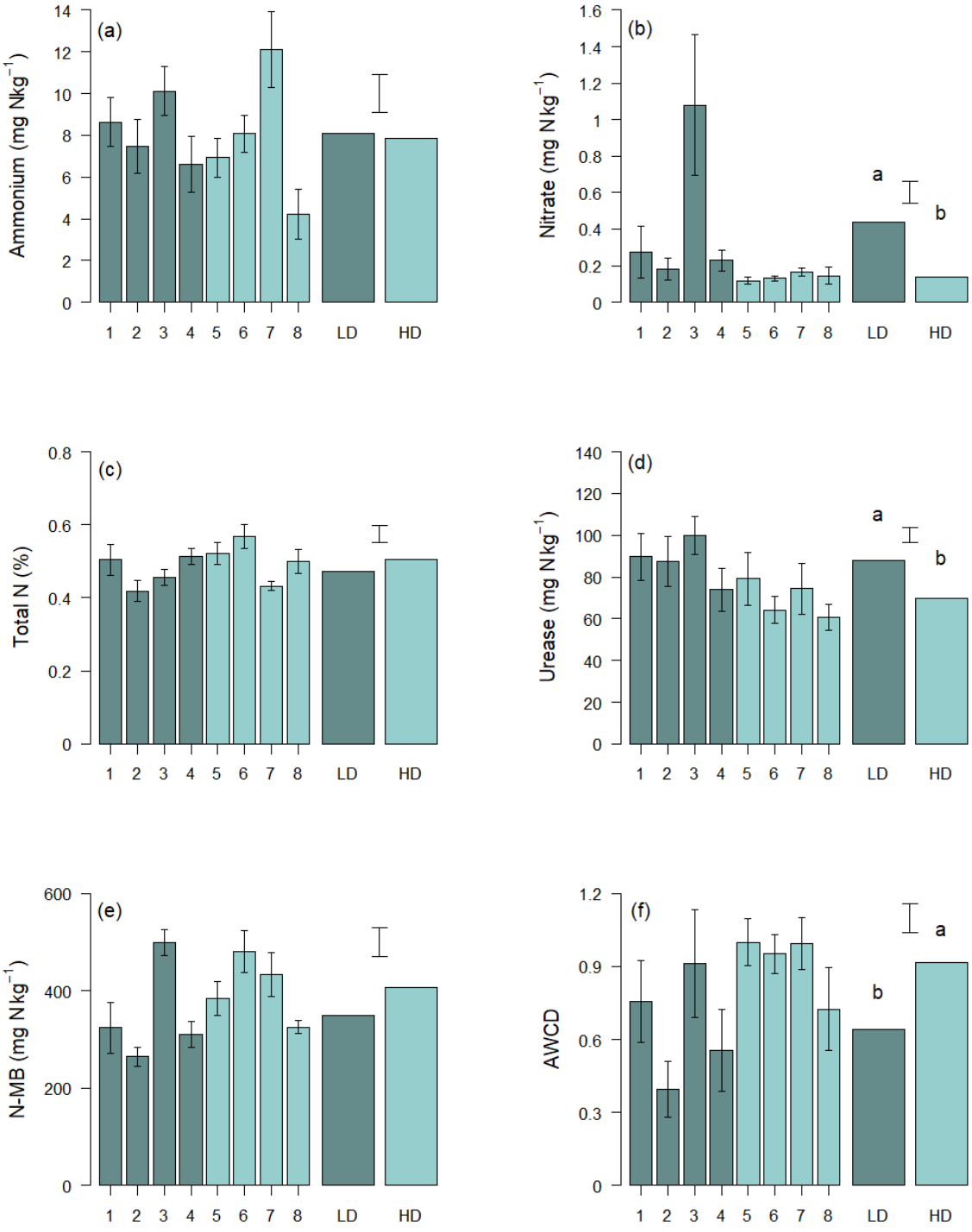


Fig. 2. Soil inorganic N contents -(a) ammonium (mg N·kg^{-1}) and (b) nitrate (mg N·kg^{-1})-, (c) total N (%), (d) urease enzyme activity (mg N·kg^{-1}), (e) N in microbial biomass (mg N·kg^{-1}) and (f) average well colour development (physiological profile of the carbon sources used by the soil bacterial communities). Different numbers 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 represent means values. Dark pale turquoise are LD grasslands and light pale turquoise HD grasslands. Different letters stand for significant differences between covers.

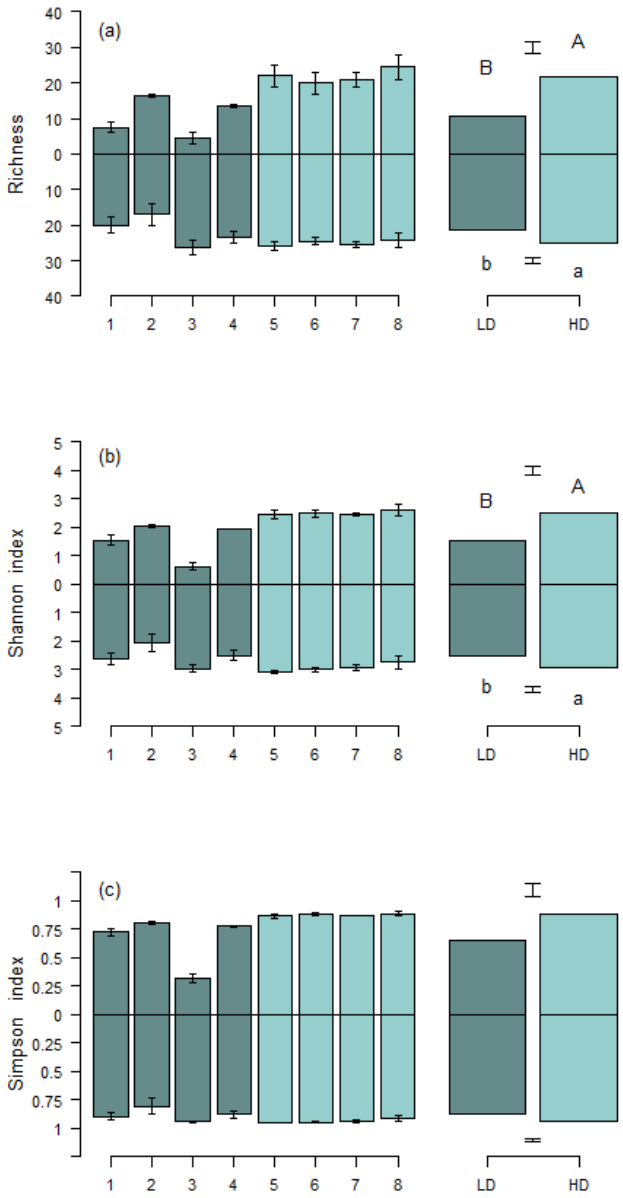
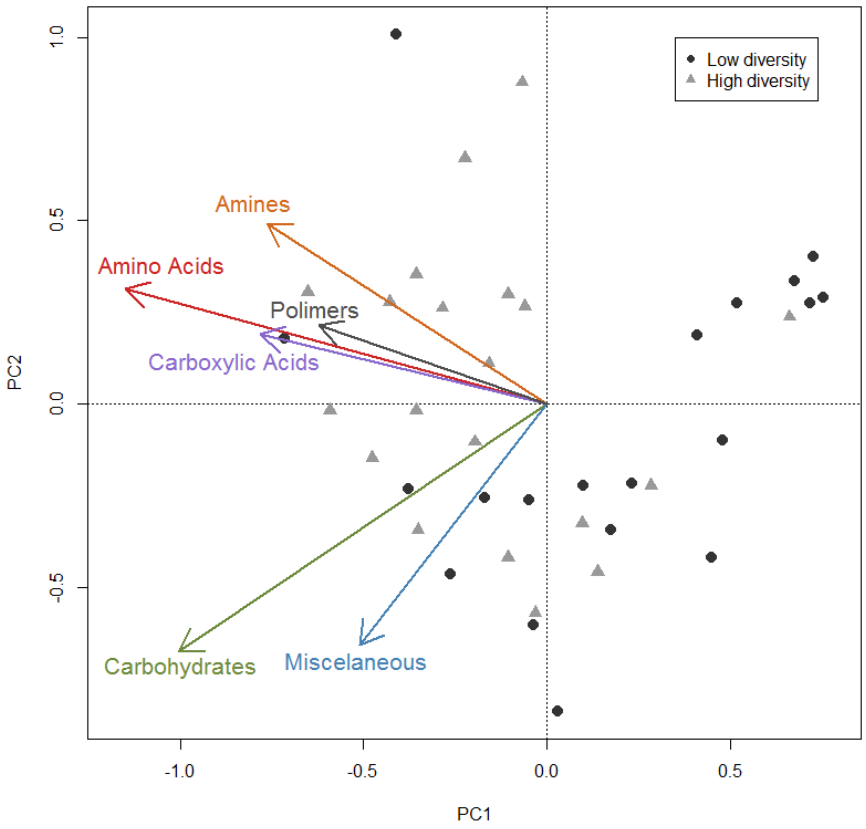


Fig. 3. The plant community is represented in the positive Y-axis and the soil bacterial community in the negative Y-axis. (a) Richness –number of species for the plant community and number of functional groups for the soil bacterial community-, (b) Shannon-Wiener diversity index and (c) Simpson diversity index in low-diverse and high-diverse grasslands. Different numbers 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 represent means values. Dark pale turquoise are LD grasslands and light pale turquoise HD grasslands. Different capital letters stand for significant differences between plant communities and different lowercase letters stand for significant differences between bacterial communities.



400

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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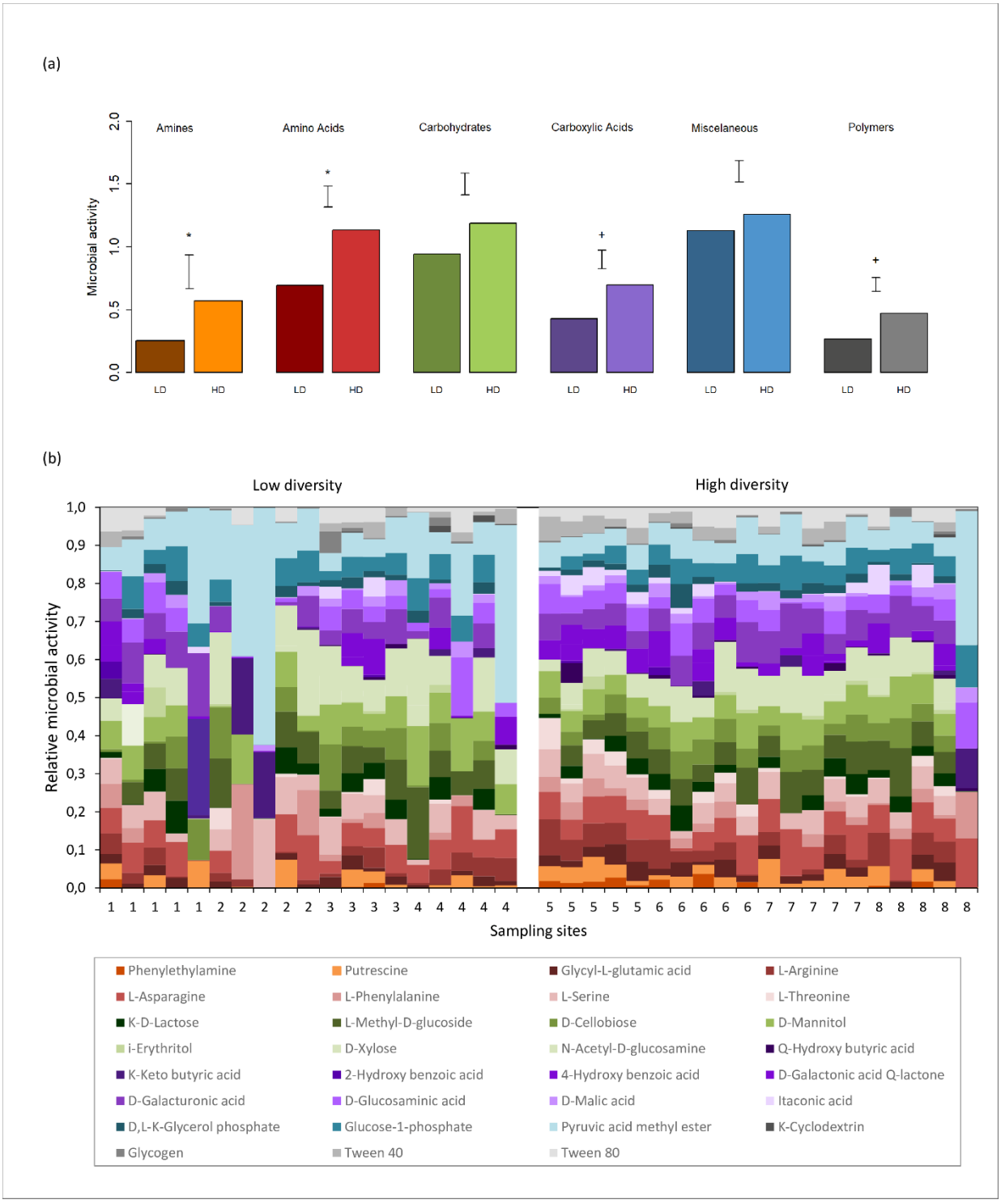
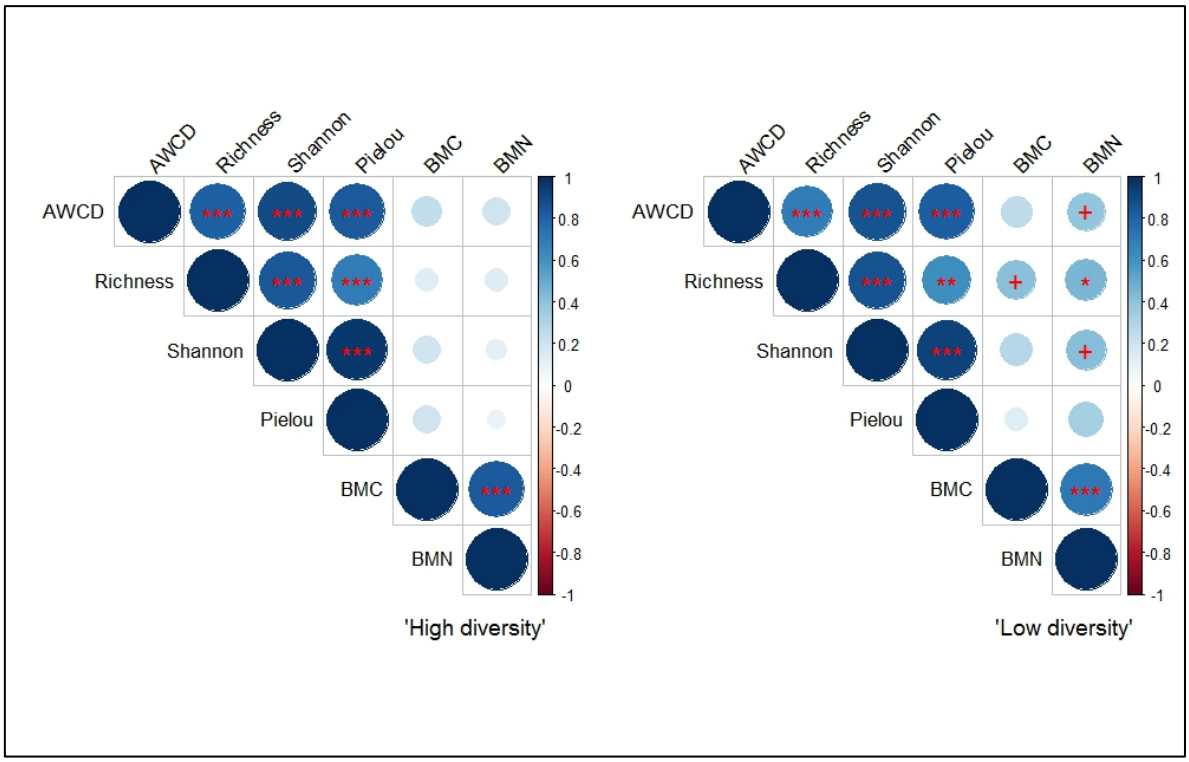


Fig. 5. Microbial activity in the different substrates tested in low-diversity (LD) and high-diversity (HD) grasslands. Chart (a) summarises the information detailed in chart (b). Statistical significances using GLM models *p < 0.05; + p < 0.07.



410

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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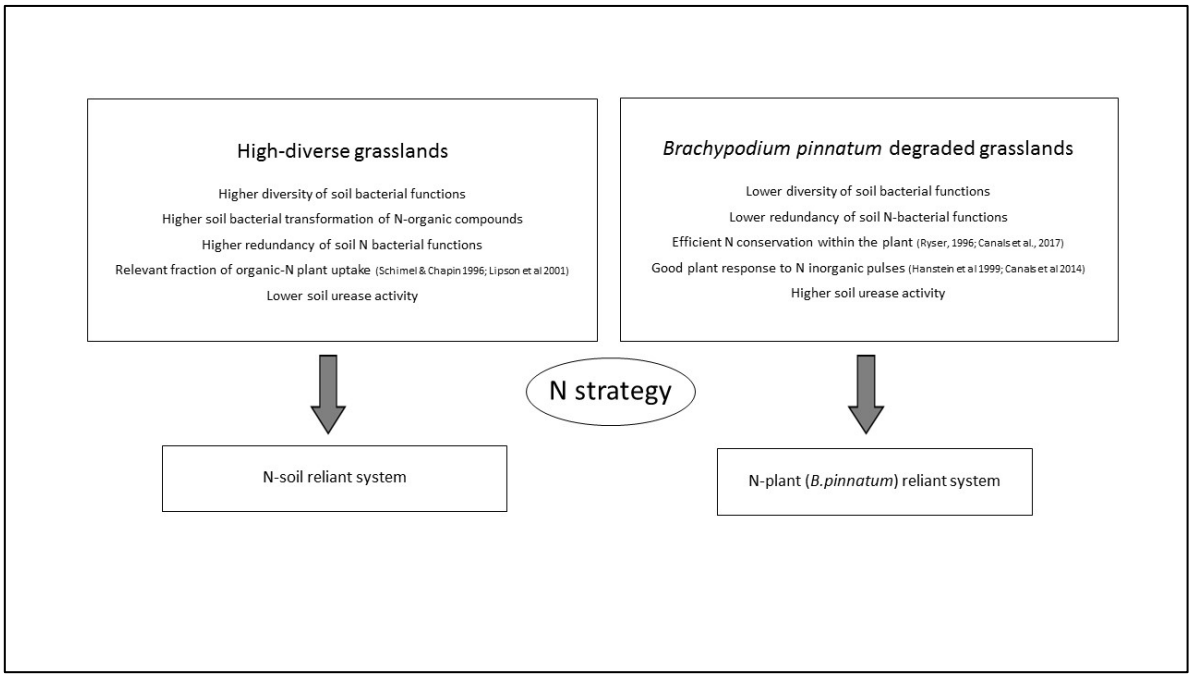

417

418 **Fig 7**. New hypothesis about the type of N strategy occurring in diverse and in degraded covers of *B.rupestre*. Authors cited:
419 Ryser 1996; Schimel 1996; Hanstein 1999; Lipson 2001; Canals 2014; Canals 2017).

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