Plants reverse the positive effect of nutrient addition on the drought resistance of soil multifunctionality

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ABSTRACT

Global change affects soil microbial communities and the multiple functions they control in soil. However, our understanding of the combined effects of multiple global change factors on soil multifunctionality (SMF), and how plant-soil interactions shape these effects remain limited. In this study, we used a mountain grassland soil to test the interactive effect of mineral nutrient (Nitrogen and Phosphorous) addition and drought on SMF with and without plant in a mesocosm experiment. We calculated SMF based on 8 microbial properties associated with the capacity of soil microbes to store carbon (C), nitrogen (N) and phosphorous (P) in their biomass, and to process these elements through organic matter depolymerization, mineralization, nitrification and denitrification processes. To investigate mechanisms underlying the SMF response we characterized the associated changes in soil nutrients stoichiometry and microbial community composition using 16S and 18S rRNA amplicon sequencing. Our results showed that nutrient addition decreased the SMF drought resistance when plants were present, but the opposite was observed in unplanted soil. We suggest this was due to the interaction of plant, fertilization and drought in influencing four coupled properties related to high SMF: high soil moisture, low microbial C limitation, high bacterial diversity and low bacteria gram positive:gram negative ratio. Our study revealed that plant presence can reverse the response of SMF to interacting global change factors, and further showed that combining stoichiometric and biodiversity assessment represents a powerful approach to disentangle the underlying mechanisms.

1 INTRODUCTION

Soil microbial communities drive many key functions in terrestrial biogeochemical cycles underlying ecosystem services like maintenance of soil fertility and climate mitigation through carbon sequestration (Crowther et al. 2019). The concept of ecosystem multifunctionality has been developed to aggregate such high number of ecosystem functions in integrative indexes (Zavaleta et al. 2010, Maestre et al. 2012). Soil multifunctionality (SMF) represent the capacity of the soil to maintain simultaneously multiple functions including storing and recycling C and nutrients (Garland et al. 2021), with most of them being controlled by

soil microbial communities (Delgado-Baquerizo et al. 2016b). Global changes are impacting SMF but we still have a limited understanding of the impact of the interaction between multiple global change factors on SMF (Rillig et al. 2019). To predict these complex responses it is essential to understand the mechanisms that control SMF. Plants likely play a central role through their effect on soil C, nutrients and microbial communities (Bardgett et al. 2008). Microbial processes are highly influenced by the dynamics of abiotic factors of their soil environment such as pH, humidity, redox potential and temperature (Fierer 2017). Their functioning also depend on their nutritional resource that are often investigated through stoichiometric, which relies on the balance between chemical elements to predict their storage and circulation among different biotic and abiotic pools (Zechmeister-Boltenstern et al. 2015). Stoichiometric theory predicts that soil microorganisms immobilize and store the most limiting element for their growth whereas mineralization is accelerated for elements available in excess. However, for some specific microbial functions, including nitrification, denitrification or depolymerization of specific substrates depend the presence of specific microbes or microbial consortium (Crowther et al. 2019). Therefore high diversity of microbes should sustain multiple soil functions, while functional redundancy between microbes, especially for generalist functions, was suspected to potentially weaken this relationship (Allison and Martiny 2008, Delgado-Baquerizo et al. 2016a). But functional redundancy seems of limited importance for soil functions (Trivedi et al. 2019, Yang et al. 2024). Thus, recent studies confirmed a positive biodiversity-SMF relationship, with high magnitude and stability of SMF associated with higher microbial diversity and specific community composition (Delgado-Baquerizo et al. 2016b, 2017, Maron et al. 2018). This suggest that some specific abiotic and stoichiometric conditions along with a diverse microbial community able to realize multiple functions are necessary to maximize SMF. Hence, it sounds appealing that microbial-MF response to multiple global change factors could be predicted through their impact on soil conditions, stoichiometry and microbial diversity.

In the last decades nutrient inputs to terrestrial ecosystems have drastically increased worldwide, especially in agricultural land with increasing fertilizer use combined with atmospheric deposition (Galloway et al. 2008). On the other hand, drought intensity and frequency have increased in many regions, with dramatic consequences on ecosystem functioning (Shukla et al. 2019). Nevertheless, how increased nutrient inputs influence the ecosystem response to drought, remains overlooked. Experiments showed that plant and microbial responses to drought are tightly coupled, with N competition playing an important role (Bloor et al. 2018, Piton et al. 2020a, 2020c). Indeed, nutrient can be vital for microbes to cope with water stress, for instance to produce osmolytes which may represent an important part of their bacterial-N budget during drought (Schimel et al. 2007). An increase of the microbial biomass C:N has often been observed with drought, a pattern attributed to the selection of drought-resistant fungi that are less N demanding than bacteria (Strickland and Rousk 2010, Sun et al. 2020). Yet, how this response varies under high nutrient level remains to be tested. Life history strategy theory predict that r-strategy microbes (copiotrophes) thrive under conditions of high nutrient availability and exhibit a low resistance to climatic stress (De Vries and Shade 2013). Some recent experiments support this theory (Karlowsky et al. 2018, Piton et al. 2020a, 2020c) but it remains unclear if this selection of drought sensitive

microbes is driven by nutrient level or by the associated response of the plants. Recent studies also showed that nutrient addition to soil can decrease bacterial and fungal communities richness and alter their compositions (Zhou et al. 2020). Such biodiversity changes can lead to a decrease resistance of ecosystem function to stress, while it remains debated if this biodiversity effect is rather driven by microbial alpha diversity (eg. richness) or by associated changes in community composition (Yachi and Loreau 1999, Tardy et al. 2014, Yang et al. 2022, Osburn et al. 2023). One consistent shift in the bacterial community composition reported following drought is an increase of the gram positive : gram negative ratio, explained by the thicker membrane of gram positive bacteria (Naylor and Coleman-Derr 2018). This consistent pattern led to the proposition that factor changing the gram positive : gram negative ratio, such as C limitation (Fanin et al. 2018), could have important consequences for microbial community stability (De Vries and Shade 2013). Overall, more experimental studies are needed to fully understand how increasing soil nutrient levels affect the drought response of the plant-soil system, and the consequences for soil microbial diversity and SMF.

This study aimed at investigating the role of plant in the response of SMF to the interaction between nutrient addition and drought, two important global change factors. We hypothesized that fertilization, drought and plant presence together affect SMF through their joint effect on soil nutrient, soil moisture and microbial diversity. More precisely, factors increasing soil water deficit and microbial limitation and/or decreasing soil microbial diversity and/or selecting for drought sensitive taxa are expected to decrease SMF (Table 1).

Table 1 : Hypotheses on how water stress, soil stoichiometry, and soil microbial diversity will respond to plant presence and nutrient inputs, individually or combined.

	Without plant		With plant		
	No nutrient addition	With nutrient addition	No nutrient addition	With nutrient addition	
Water deficit under drought	-	-	-- Plant water uptake increase water deficit for microbe	--- High growth of fertilized plant accentuates water deficit	
Stoichiometric imbalance under drought	- Imbalance +. Increase of microbial biomass C:N.	-- Imbalance ++. Microbial biomass C:N increases while C:N of available resource in soil is decreased by nutrient addition.	--- Imbalance +++. Increase of microbial biomass C:N while plant reduce N uptake during drought	---- Imbalance ++++. Increase of microbial biomass C:N while plant reduce N uptake and nutrient addition decreased C:N of soil resources.	(Sun et al. 2020)
Microbial diversity response to drought	0	- Nutrient addition decreases microbial diversity	0 Plant presence increases microbial diversity	- Nutrient addition decreases microbial diversity	(Zhou et al. 2020)

2 MATERIALS AND METHODS

2.1 SOIL SAMPLING

Soil was collected in a grassland localized in the Chartreuse mountain range of French Alps (45°36’ N, 5°90’E), at 930m a.s.l. with a mean annual temperature of 8.1°C and 1049mm of mean annual precipitation. Management was extensive since decades with one mowing per year and no fertilization. The 15 first cm of the topsoil (3.6% of C and 0.4% of N) were extracted in spring (5th and 6th of April 2018), and sieved at 7mm to remove plant residues. After homogenization, soil was used to fill 32 mesocosms (25cm diameter and 40cm depth). All mesocosms were transported in the alpine green-house of the Jardin du Lautaret (Grenoble), with an

air temperature maintained at 20°C during the experiment, approximating the study site's daily mean temperature during the growing season. Mesocosms were weighted prior the start of the experiment (mean fresh soil weight: 11.8 kg, 95% confidence interval ± 0.1kg). All along the experiment, control mesocosms (unplanted soil, control climate) were weighted twice a week and compared with their initial weight to assess water loss. All mesocosms received the same amount of water equal to this water loss in control mesocosms (in average 300mL per week), to maintain a stable soil moisture in mesocosms equal to the initial field value (0.24 g water per g of fresh soil, 66% of maximum water holding capacity). During the first 30 days, soil water content was kept constant for all mesocosms. In parallel, seeds of *Lolium perenne* from Arbiotech® (Saint Gilles, France) were germinated in individual pots filled with the same soil used in the mesocosms until seedling establishment. *L. perenne* was used as a phytometer because it is usually sown in managed grasslands in the French Alps (Loucougaray et al. 2015, Legay et al. 2017). After 30 days of growth, 25 individuals of *L. perenne* were planted in 16 mesocosms, with a 4 cm distance between each individual and with mesocosm edge. At day 45, half of the mesocosms (planted and unplanted soil) were fertilized with mineral N (50%-N as NO_3^- and 50%-N as NH_4^+) and P (P_2O_5) equivalent to 150 kg N/ha and 80kg P_2O_5/ha representing the regional recommendation for nutrient addition in intensive sown grasslands (Guide régional de fertilisation 2016, Chambre d'Agriculture). Twenty days after fertilization (day 65), watering of 16 mesocosms (4 unplanted soil/unfertilized, 4 unplanted soil/fertilized, 4 planted soil/unfertilized, 4 planted soil/fertilized) was stopped during 32 days (until day 97). At day 97, three soil cores (5cm diameter, 15cm depth) were sampled on the location of three *L. perenne* individuals in the center of each mesocosm, and pooled in a composite sample to assess plant biomass and soil stoichiometry, microbial community biomass stoichiometry, activity and diversity.

2.2 PLANT ANALYSES

Plants shoots and roots in planted mesocosms were manually sorted from the three soil cores. Plants shoots and roots were separated and dried at 70°C for a week and weighed to measure shoots and roots biomass. All plant material was ground and analyzed for C and N contents using an elemental analyzer (FlashEA 1112: Fisher 302 Scientific, Waltham, Massachusetts, USA).

2.3 SOIL ANALYSES

Composite soil samples were split in subsamples, some of which were immediately frozen in liquid nitrogen and stored at -80°C until molecular analyses. Subsamples for enzymatic analyses were frozen at -20°C until analysis. Subsamples for C, N and P pools were stored at 4°C and analyzed within 24h.

Subsamples of 5g of fresh soil were oven-dried at 70°C for 1 week and weighed to determine soil water content (SWC) (Robertson et al. 1999). Soil subsamples were air dried and ground to powder to measure total C and N contents using a FlashEA 1112 elemental analyzer (FlashEA 1112: Fisher 302 Scientific, Waltham, Massachusetts, USA), and to determine soil pH in a 1:2.5 (soil:distilled water) solution. Dissolved organic carbon (DOC), nitrate (NO_3^-), ammonium (NH_4^+), total dissolved nitrogen (TDN) were extracted on 10 g of

fresh soil using 0.5M K_2SO_4 solution following Jones and Willett (2006). DOC concentration was measured on a Shimadzu TOC-L analyzer (Shimadzu Inc.). N concentrations were measured on an automated photometric analyzer using standard colorimetric methods (Gallery Plus: Thermo Fisher Scientific, Waltham, Massachusetts, USA). Dissolved organic nitrogen (DON) was calculated as the difference between TDN and total inorganic N ($N\text{-}NO_3^-$+ $N\text{-}NH_4^+$).PO_4 was extracted on 10g of fresh soil using 0.5M $NaHCO_3$ solution as described by (Brookes et al. 1982).

2.4 MICROBIAL BIOMASS AND ACTIVITIES

Microbial biomass carbon (MBC), nitrogen (MBN) and phosphorus (MBP) contents were measured on 10-g of fresh soil, using chloroform fumigation extraction-methods as described by (Vance et al. 1987) for MBC and MBN, and by Brooks et al. (1981) for MBP. A spiking procedure (25µgP-PO_4/g soil were added on two samples) was used to estimate and correct for P recovery during extraction (Brookes et al. 1982). Microbial biomass element contents were calculated as the difference between fumigated and non-fumigated samples, and adjusted using conversion factors of 0.45, 0.45 and 0.40 for MBC, MBN and MBP respectively (Jenkinson et al. 2004). Indicators of the stoichiometric imbalances between microbial biomass and their resources were calculated as the ratios of resource A:B in the soil solution over microbial biomass A:B (Mooshammer et al. 2014), with A:B being C:N, C:P or N:P ratios. Assuming that the microbial biomass composition represent its nutritional need to grow, an increase of such indicator above 1 indicate that the element B become more limiting over A, in other words its availability relative to A become too low to build new biomass. Values below 1 indicate the opposite situation.

Seven potential extracellular enzymes activities (EEA) involved in the decomposition of C-rich substrates (α-Glucosidase, β-1,4-Glucosidase, β-D-Cellobiosidase, and β-Xylosidase), N-rich substrates (β-1,4-N-acetylglucosaminidase and leucine aminopeptidase) and P-rich substrates (phosphatase) were estimated using standardized fluorimetric techniques (Bell et al. 2013). Briefly, 2.75-g of soil were homogenized (1-min in a Waring blender) in 200-ml of sodium acetate buffer solution adjusted at soil pH (5.8). The soil slurries were added in duplicate to 96-deep-well microplates followed by the addition of a substrate solution for each enzyme at enzyme saturation concentration. Duplicated standard curves (0-100-µM concentration) were prepared by mixing 800-ml of soil slurry with 200-ml of 4-methylumbellfferone (MUB) or 7-amino-4-methylcoumarin (MUC) in 96-deep-well microplates for each soil sample. Microplates were incubated during 3-h (dark, 175-rpm, 20°C), and centrifuged at 2900-g for 3-min. Then soil slurries (250-µL) were transferred into black Greiner flat-bottomed microplate and scanned on a Varioskan Flash reader (Thermo Scientific) using excitation at 365-nm and emission at 450-nm (Bell et al. 2013). After correcting for negative controls, potential enzyme activities were expressed as nmol g $soil^{-1}$ h^{-1}. The activities of the seven enzymes were summed to provide a measure of total extracellular enzyme activity (EEA).

Potential N mineralization (PNM) was estimated after incubation of 10-g of fresh soil under anaerobic conditions for 7 days at 40°C in the dark (Wienhold 2007), inducing accumulation of mineralized NH_4^+. PNM

rate was calculated as the difference between NH_4^+ content before and after incubation (µgN/g dry soil/day). Potential nitrification enzyme activity (NEA) was estimated according to (Koper et al. 2010) following (Dassonville et al. 2011). Briefly, 3 g of each soil were incubated under aerobic conditions (180 rpm, 28°C, 10 h) in a solution of 50µg N-(NH4)2SO4/g dry soil and rates of NO_2 and NO_3 production were measured after 2, 4, 8 and 10 hr by ionic chromatography (DX120; Dionex, Salt Lake City, UT, USA). Maximal nitrification rate (NEA) was assessed by plotting nitrification rates along the gradient of NH_4–N concentrations (Lineweaver and Burk 1934). Potential denitrification activity (DEA) was estimated following Attard et al. (2011). Briefly, 10 g dry weight (dw) soil were placed at 28°C under anaerobic conditions using 90:10 He:C_2H_2 mixture inhibiting N_2O-reductase activity. Each sample was supplemented with 3 ml KNO_3 (50 µg N–NO_3 g^{-1}dw), glucose (0.5 g C/g dw) and sodium glutamate (0.5 g C/g dw) (Attard et al. 2011), completed with distilled water to reach water holding capacity. N_2O was measured at 2, 3, 4, 5 and 6 hr using a gas chromatograph (microGC R3000; SRA Instruments, Marcy l'Etoile, France). Substrate-induced respiration (SIR) was measured following (Anderson and Domsch 1978). Briefly, 5 g of soil samples were placed in airtight flask, with 1.2 mg of C-glucose/g dry soil, completed with distilled water to reach water holding capacity. Then, samples were incubated at 28°C during 5 hours with CO_2 concentrations measured each hour. The slope of the linear regression between time and with CO_2 concentrations was used to estimate aerobic respiration (g C-CO_2^{-1} h^{-1}).

2.5 SOIL MULTIFUNCTIONALITY

A soil multifunctionality (SMF) index was calculated using the widely used method presented by Maestre et al. (2012). Eight microbial properties and functions (MBC, MBN, MBP, SIR, EEA, NMP, NEA, DEA) were first standardized using Z-score transformation and then averaged. These variables were selected because they capture the capacity of soil microbes to store C, N and P in their biomass and to process these elements through organic matter depolymerisation, mineralization and downward transformations (nitrification and denitrification).

2.6 MOLECULAR ANALYSES

Soil biodiversity was estimated using environmental DNA metabarcoding targeting two universal DNA markers (Table S1), one amplifying all Bacteria (v4 region of the 16S rRNA gene) and one amplifying all Eukaryota (v7 region of the 18S rRNA gene). Extracellular DNA was extracted from 10g of soil using the phosphate buffer procedure described in (Taberlet et al. 2012). PCR amplifications were performed in a 20-µL volume containing 10 µL of AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 µM of each primer, 0.16 µL of 20 mg.ml-1 bovine serum albumin (BSA; Roche Diagnostics, Basel, Switzerland), and 2 µL of 1/10 diluted DNA extract. Thermocycling conditions (Table S1) followed recommendation from Taberlet et al. (2018) and Guardiola et al. (2015). The forward and reverse primers were tagged with unique combinations of eight-nucleotide labels. PCR products were purified using the MinElute™ PCR purification kit (Qiagen, Hilden, Germany) and mixed in equal volumes. Library preparation and

sequencing were performed at Fasteris (Fasteris SA, Geneva, Switzerland) using the MetaFast protocol (www.fasteris.com/metafast). The bacterial and eukaryote libraries were sequenced on an Illumina MiSeq (2 x 250 bp paired-end reads) and HiSeq 2500 ((2x150 bp paired-end reads) platforms, respectively. Potential contaminations were tracked by including negative controls at the extraction and PCR steps. Tag jumps caused by chimeras were accounted for at the bioinformatic level by monitoring unused tag combinations (Schnell et al. 2015).

Sequencing data were then analysed using the OBITools software package (Boyer et al. 2016) and R scripts following (Zinger et al. 2019). Firstly, paired-end reads were assembled and then assigned to the corresponding PCR replicate on the basis of primer sequences (two mismatches allowed) and tag combinations (no mismatches allowed). Secondly, reads were dereplicated. Low quality sequences (i.e. sequences containing "Ns"), sequences observed less than 100 times, and sequences whose length fell outside the expected length range (<45bp and <36bp for the bacterial and eukaryote markers, respectively) were discarded (Taberlet et al. 2018). Thirdly, unique sequences were clustered at 97% similarity into operational taxonomic units (OTUs) using the Sumaclust package (Mercier et al. 2013). Only the cluster centres were kept for taxonomic annotation with the ecotag program from the OBITools package (Boyer et al. 2016). OTUs whose similarity score of the best match (best identity score from ecotage program) in the reference database was below 0.95% were excluded. Then, potential contaminations were removed by excluding all OTUs whose absolute number of reads was highest in negatives control. PCR replicates were summed for each sample and rarefied to the same sequencing depth equal to the lowest number of sequences observed in a sample. Rarefaction curves were also obtained for each sample using the *vegan* R package (Oksanen et al. 2011) to assess to which extent the sequencing depth captured the diversity present in our samples (SI Figure 1). All fungal sequences were extracted from the Eukaryota dataset to narrow down the subsequent analyses to bacterial and fungal communities. The gram positive to gram negative ratio of soil bacteria was calculated using relative abundance of 16S rDNA OTUs as in Orwin et al. (2018), which relied on the Gupta (2011) classification of bacteria phylum based on the presence of the Hsp60 insert in their genomes (Gram positive : Actinobacteria, Firmicutes, Deinococcus-Thermus and Chloroflexi ; Gram negative : Proteobacteria, Acidobacteria, Bacteroidetes, Chlamydiae, Gemmatimonadetes, Nitrospirae, Planctomycetes, Spirochaetes, Verrucomicrobia, Armatimonadetes, Elusimicrobia and Fusobacteria).

2.7 STATISTICAL ANALYSES

Three-way analysis of variance (ANOVA) were used to test the effect of plant presence, fertilization, drought, and their interactions on microbial and soil properties. Two-way ANOVA was used to test the effect of fertilization, drought and their interaction on total plant biomass. Model residuals were tested for normality and homoscedasticity and transformed when necessary. Three-way permutational multivariate analysis of variance (PERMANOVA) using Bray-Curtis distance matrices were used to test the treatment effects on

bacterial and fungal community composition. Microbial community composition data were transformed using Hellinger transformation before PERMANOVA analyses. Significance was set to $P < 0.05$.

3 RESULTS

SMF response to drought significantly changed with both the presence of plant and the fertilization (Figure 1). Drought decreased SMF in unplanted soil only in the absence of fertilization, indicating a lower resistance of SMF. Conversely, when plants were present, SMF did not decrease any more with drought in unfertilized treatment but did decrease in the fertilized one. Across all treatments, the lowest SMF was observed in the presence of plants, when drought and fertilization are combined. Looking at each function independently (Table S2, and Figure S2), the lower resistance observed in unplanted soil without fertilization was triggered by a decrease of MBC, SIR and MBP, whereas unplanted fertilized soil did not show this negative drought effect, instead positive responses to drought were observed for PNM and MBN. However, this higher resistance associated with fertilization in unplanted soil was not associated with significantly higher value of microbial functions for fertilized soils compared to unfertilized soils under drought (excepted for MBN) because in most cases fertilization has already altered microbial functions in the same direction as drought did (Figure S2). In the presence of plant, drought significantly impacted microbial functions in the unfertilized treatment, whereas it induced a decrease of MBC, SIR, MBN and PNM when plant had been fertilized. Fertilization and drought also affected plant biomass with an average increase of 220% with fertilization, and a decrease by 30% with drought (Figure S3).

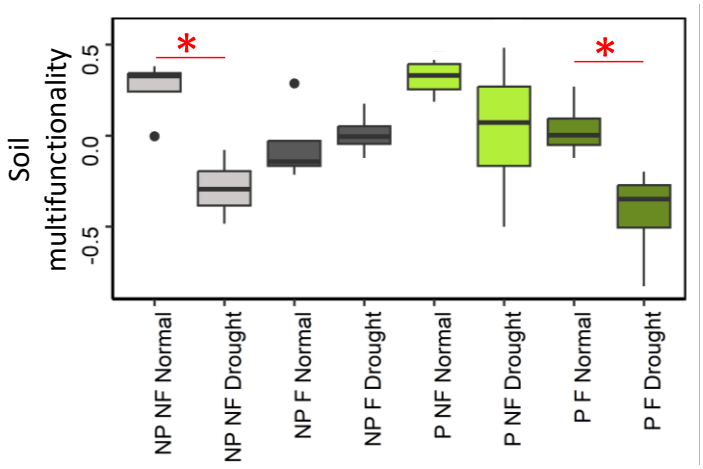

Figure 1. SMF response to drought and fertilisation, with or without plant. Star indicate significant difference between drought and normal climate treatment. NP=No plant, P=Plant presence, F=Fertilized, NF=No fertilization

Metabarcoding analyses identified 3,181 bacterial OTUs and 624 fungal OTUs across all treatments. Drought significantly decreased bacterial Shannon index but increased fungal richness (Table S2, Figure S4). Bacterial alpha-diversity (Shannon index and OTU richness) also decreased with fertilisation but only in the presence of plants, and fungi showed the lowest richness values when fertilization, plant presence and normal climate were combined (Table S2, Figure S4). Plant presence, drought and fertilisation significantly influenced the

composition of bacterial and fungal communities. According to the PERMANOVA models (Table 1), these experimental treatments explained a total of 43% and 39% of the variation in bacterial and fungal community composition, respectively. No significant interaction was observed for fungal community composition, whereas interaction between fertilisation and plant presence was significant for bacteria. Indeed, posthoc tests showed significant effect of fertilization on bacterial community composition in planted soil ($p<0.001$) but not in unplanted soil ($p=0.12$).

Table 1. P-values and R^2 associated with the effects of Drought, Fertilization, Plant presence and their interactions on bacterial and fungal community composition. Effects were assessed by PERMANOVA on a Bray-Curtis distance matrix based on bacterial (16S rRNA) and fungal (18S rRNA) operational taxonomic units (OTUs).

	Drought (D)	Fertilization (F)	Plant presence (P)	D × F	D × P	F × P	D × F × P	Total R^2
Bacteria								
p-value	**p<001**	**p<001**	**p<001**	0.07	0.10	**0.03**	0.15	
R^2	**0.10**	**0.12**	**0.07**	0.03	0.03	**0.04**	0.03	0.43
Fungi								
p-value	**p<001**	**0.02**	**p<001**	0.60	0.33	0.38	0.30	
R^2	**0.10**	**0.05**	**0.13**	0.02	0.03	0.03	0.03	0.39

While SMF showed contrasting response to drought and fertilization depending on plants presence, SMF showed consistent relationships with some soil and microbial properties across planting treatments (Figure S5 and S6). In both planted and unplanted soils, SMF was positively correlated with soil moisture, C:N imbalance and bacterial diversity, and negatively correlated with the bacterial gram positive : gram negative ratio (Figure 3). These 4 variables were selected for their consistent relationships with SMF, and variance partitioning showed that 25% was commonly explained by them (SI Figure 5). Microbial properties explained 24 additional %, with 8 % explained by both microbial properties and 16% explained only by the GP:GN ratio.

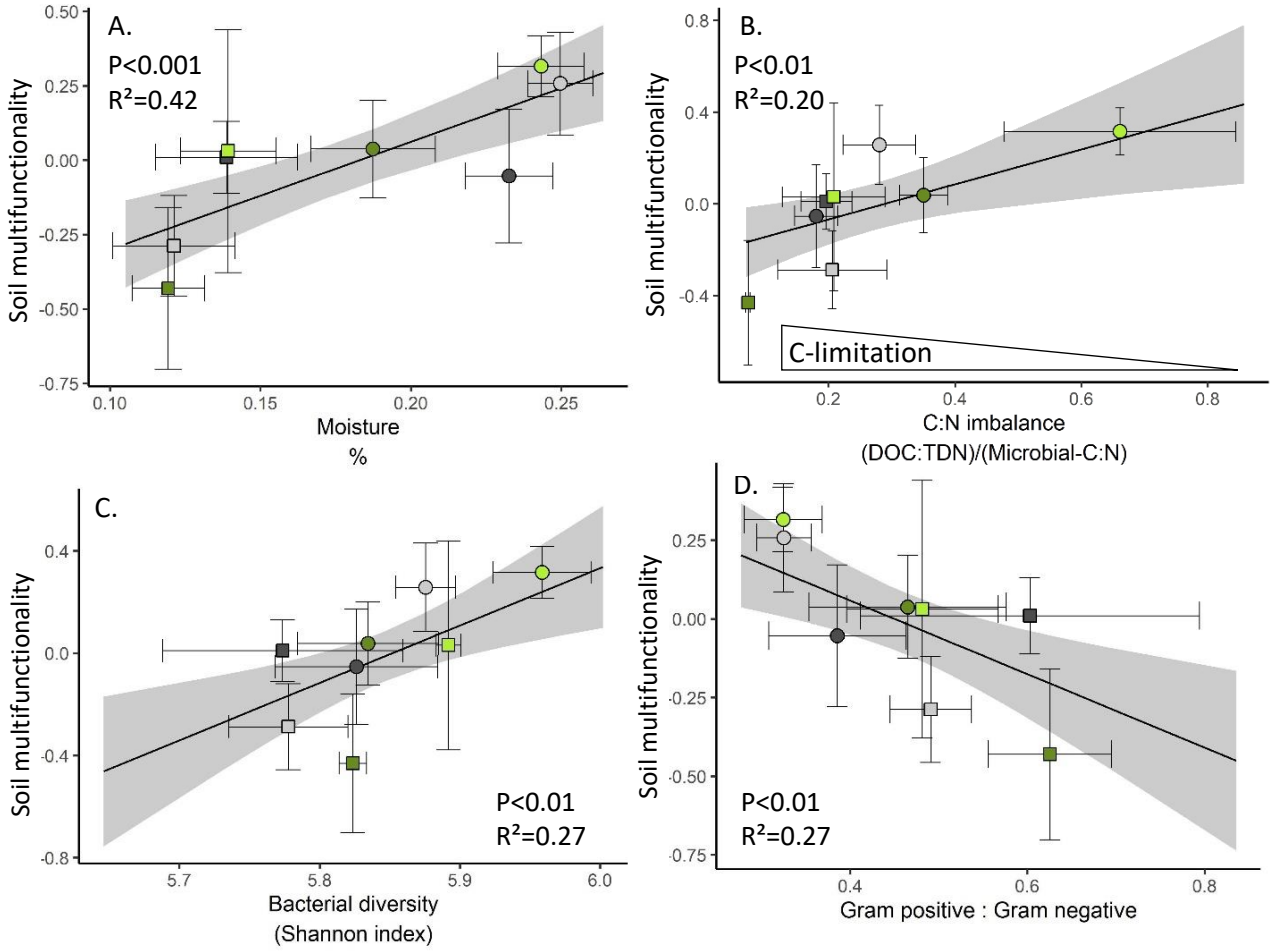

Figure 2. Correlations between SMF and soil moisture (A), C:N imbalance (B.), bacterial alpha diversity (C) and Gram positive : Gram negative ratio (D). Lines represent predicted value and shades represent 95% confidence interval from regression model using all observations (n=32). Points represent mean of each treatments. Error bar around points are 95% confidence interval (n=4). Grey points = unplanted soil, green points = planted soil, light colour points = unfertilized soil, dark colour points =fertilized soil, circle=control climate, square=drought.

4 DISCUSSION

We hypothesized a negative effect of fertilization on the soil multifunctionality (SMF) resistance to drought. Our study showed that drought decreased SMF in unplanted soil without fertilization but not when soil was fertilized, contrary to our expectations. Although fertilization decreased SMF under normal climate. Adding plants to the unfertilized soil, this also improve the resistance of SMF to drought. However, when both plant presence and fertilization were combined, we did observe a large negative effect of drought on SMF. Our results thus support our hypothesis in planted soil but showed opposite in unplanted soil. This demonstrate how plants

can play a central role in controlling the interaction between global change effects on SMF, here reversing from positive to negative the effect of nutrient addition on the drought resistance of SMF.

Starting with the unfertilized treatments, our study showed that plant can improve the drought resistance of SMF, contrasting with other studies reporting negative effect of plant presence for soil microbial community composition and respiration resistance to drought (Orwin and Wardle 2005, Koyama et al. 2017). Our study also tested the role of plant presence on the interacting effects of drought and nutrient addition on SMF, an interaction that remained unexplored in a unique experiment. Using soil incubation (no plants), Luo et al. (2019) also found a positive effect of mineral fertilization on the resistance of SMF (indices based on 7 enzyme activities) to dry/rewetting cycles, consistent with our results in unplanted soils. Alternatively, another soil incubation study tested the effect of an induced N-deficiency and such difference for drought resistance was not observed (Dong et al. 2022). On the other hand, Eskelinen et al. (2020) studied the interaction between fertilization and watering on the plants and the soil of a Mediteranean grassland, and observed that fertilization increased the amount of soil nutrients under water stress (no watering), but with no effect on microbial biomass and processes. This suggests that the negative effect of fertilization of SMF resistance to drought that we observed in planted soil might not be a generic pattern and likely depends on specific conditions. Supporting this idea, Preece et al. (2020) showed that interaction between nutrient input and drought on microbial respiration and metabolic profile was significant or not depending on which plant species was present. More studies, using different plant community composition and diversity are necessary to fully appreciate this role of plant on the SMF response to drought and nutrient addition. To understand and predict this interaction, it is also essential to address the underlying mechanisms.

Coupled changes of soil moisture, stoichiometry and bacterial community explain soil SMF responses

We hypothesized that changes of soil stoichiometry and microbial diversity could represent the mechanism underlying the response of SMF to interacting factors (i.e. drought, nutrient addition and plant presence). Indeed, whether plants were present or not, we observed a consistent link between high SMF and four coupled properties: high soil moisture, C:N imbalance closer to 1 (indicating lower microbial C limitation), high bacterial diversity and low bacteria gram positive : gram negative ratio. These links with soil moisture and stoichiometry confirmed the importance of microbial growth favorable conditions to maximize their multifunctionality, as predicted by stoichiometric and ecohydrological models of microbial biomass and processes (Manzoni et al. 2019). The positive link with bacterial diversity, and the higher diversity observed in the planted soil without fertilization depicting high SMF resistance, supports increasing evidences that a diverse microbial community might also be key to maintain high SMF in planted ecosystems exposed to global changes (Delgado-Baquerizo et al. 2016b). Our results are also consistent with the studies that indicated stronger relationship between SMF and bacterial diversity rather than fungal one (Jia et al. 2022, 2024). SMF also increased with gram positive : gram negative ratio, a trend highly associated with an increase of *Actinobacteria*, a gram positive phylum, and with a decrease of *Proteobacteria*, a gram negative phylum. Abundances of gram

negative bacteria, such as *Proteobacteria*, are known to decrease with C limitation (Fierer et al. 2007, Fanin et al. 2018), and water stress (Naylor and Coleman-Derr 2018), as observed in our mesocosm study. Although such agregation of bacteria at high phylogentic level is robust only for deeply conserved traits and could thus miss some difference between taxa that exist at lower level (Martiny et al. 2015, Ho et al. 2017), high level classification show some ecological coherence and have been key in the developpemnt of generic theory in microbial ecology (Philippot et al. 2010). With this consideration in mind, Gram negative bacteria was associated with copiotrophic (r strategy) bacteria that use labile C to sustain fast growth (De Vries and Shade 2013, Fanin et al. 2018). Such growth strategy can promote microbial biomass and enzymatic processes (Piton et al. 2020b), and eventually the high SMF that we observed. Altogether, a large proportion (25%) of SMF was explained by these four properties (soil moisture, C:N imbalance, bacterial diversity and GPGN) stressing their coupling in the soil system. However, about same proportion (24%) was additionally explained only by the microbial properties (bacterial diversity and GPGN), suggesting that high SMF not only emerged from favorable stoichiometric and moisture conditions for microbial growth, but also relied on microbial diversity.

Looking at the effect of plant, fertilization and drought on soil stoichiometry and microbial diversity, we disentangled the complex responses of SMF to our experimental treatments. When plant were present, fertilization logically promoted plant growth, and associated water uptake. Consequently, this response of the vegetation increased drought related water stress and C limitation for microbes, and had a negative impact on bacterial diversity and a positive effect on GPGN leading to reduced SMF under drought. In unplanted soil, drought induced a weaker effect on soil moisture, soil nutrient stoichiometry and bacteria community, which eventually limited the consequences for SMF. Moreover, we observed that microbial biomass in unplanted soil adapted their stoichiometry thanks to the large quantity of N provided by the fertilization, and was likely to accumulate osmolytes in response to drying stress (Schimel et al. 2007). This response of the microbial biomass composition tracked the change of soil resource stoichiometry and limited stoichiometric imbalance and consequences for SMF. Altogether, our study showed that combining stoichiometric and biodiversity-MF theories represents a powerful approach to understand the mechanisms underlying the complex responses of SMF to global change factors.

1 CONCLUSION

Our study demonstrated the central importance of integrating plants when investigating global change effect on soil multifunctionality. Indeed, we showed that plant presence can reverse the interaction between nutrient addition and drought. Nutrient addition decreased the drought resistance of soil multifunctionality when plant were present whereas the opposite was observed in unplanted soil. This soil multifunctionality response could be explained by how plant responded to fertilisation and drought, and changed soil resources and microbial diversity that promoted SMF. In agricultural field, nutrient are usually applied as fertilizers on soil covered by crops. Our results thus suggest that management strategy aiming at reducing nutrient addition to these soils could promote the maintenance of their multifunctionality under the increasing droughts.

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COMPETING INTERESTS

The authors declare no competing interests.

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SUPPLEMENTARY MATERIALS

SUPPLEMENTARY FIGURES

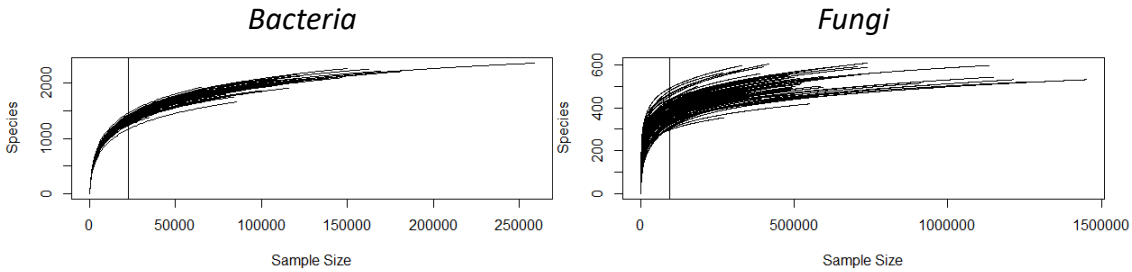


Figure S1. Rarefaction curves for Bacteria and Fungi. Vertical lines represent resampling size used for data rarefaction of each sample to have similar sequencing depth equal to the number of reads of the sample with the lowest reads numbers (Bacteria: 22 571 sequences/sample, Eukaryota : 93 742 sequences/sample).

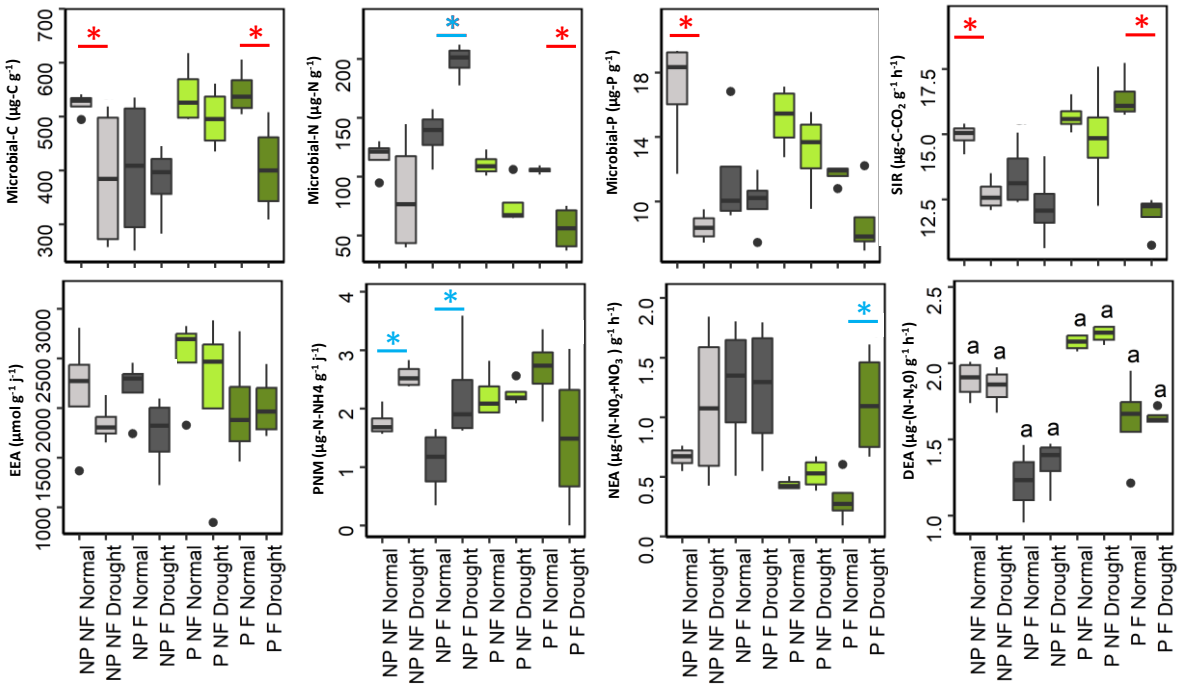


Figure S2. Effect of drought, fertilization and plant presence on soil microbial functions. Star indicate significant difference between drought and normal climate treatment. SIR=Substrate-induced-respiration. PNM=Potential N mineralization. NEA=Potential nitrification activity. DEA=Potential denitrification activity. NP=No plant, P=Plant presence, F=Fertilized, NF=No fertilization

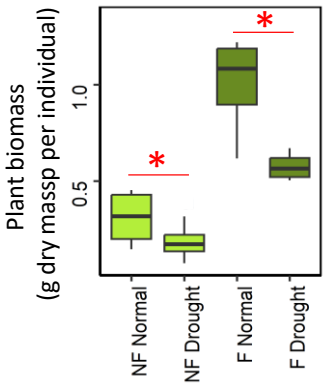


Figure S3. Plant biomass response to drought with or without fertilisation. Star indicate significant difference between drought and normal climate treatment.

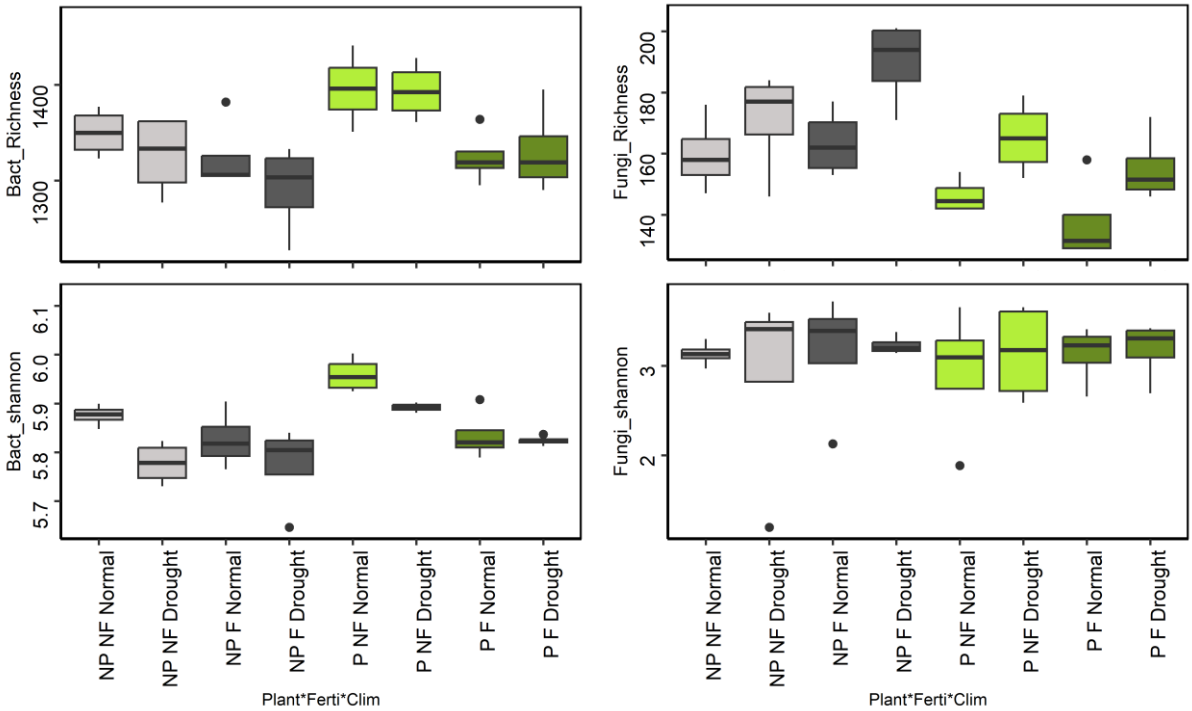


Figure S4. Soil microbial diversity response to drought, fertilization and plant presence. Model statistics are provided in Table S2. NP=No plant, P=Plant presence, F=Fertilized, NF=No fertilization

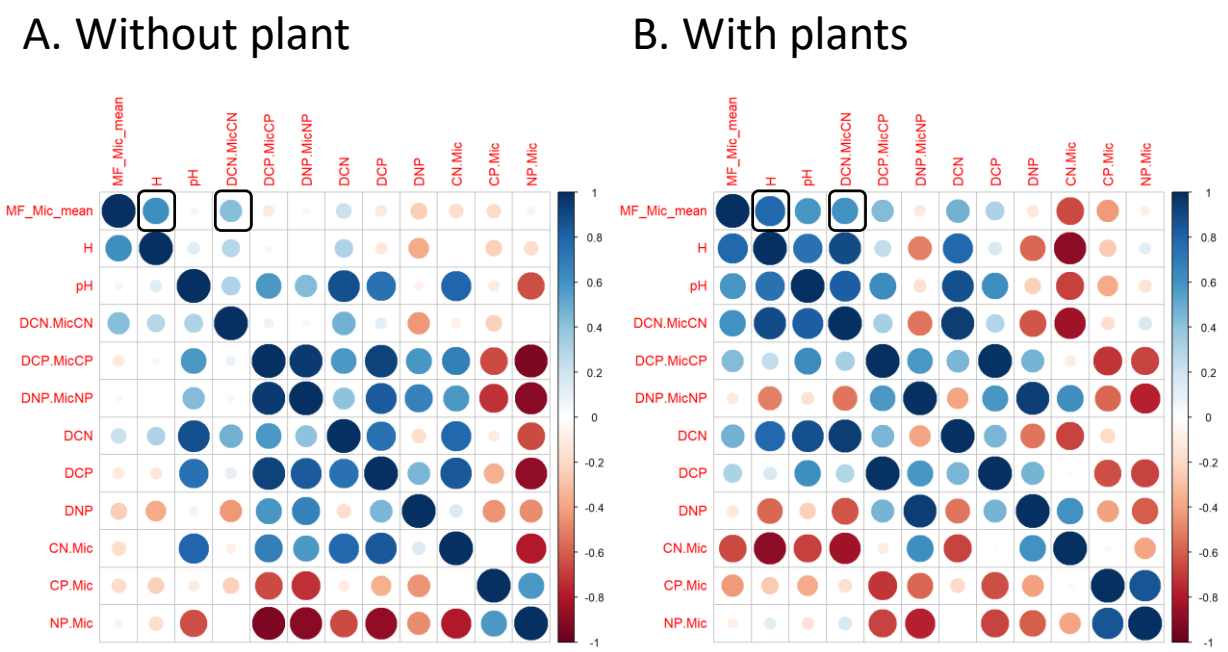


Figure S5. Spearman correlation matrix between soil multifunctionality (MF_Mic_mean), soil moisture, pH and stoichiometry (DCN=DOC:TDN, DCP=DOC:PO4, DNP=TDN:PO4) in soil without plants (A) or with plants (B). Circle color represent correlation direction and circle size and color intensity represent correlation coefficient.

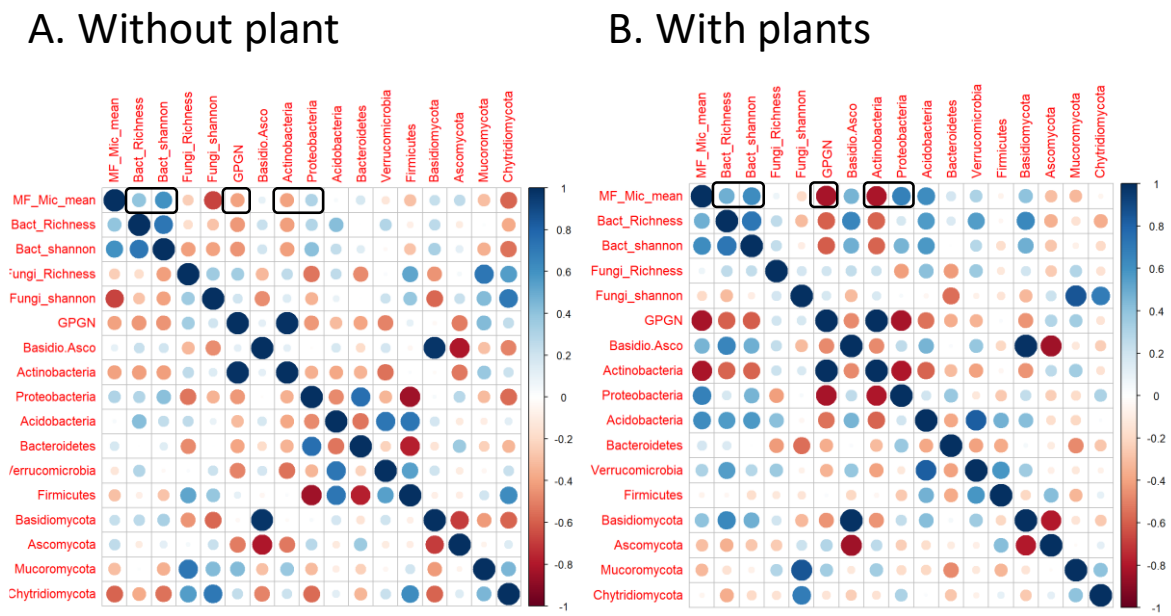
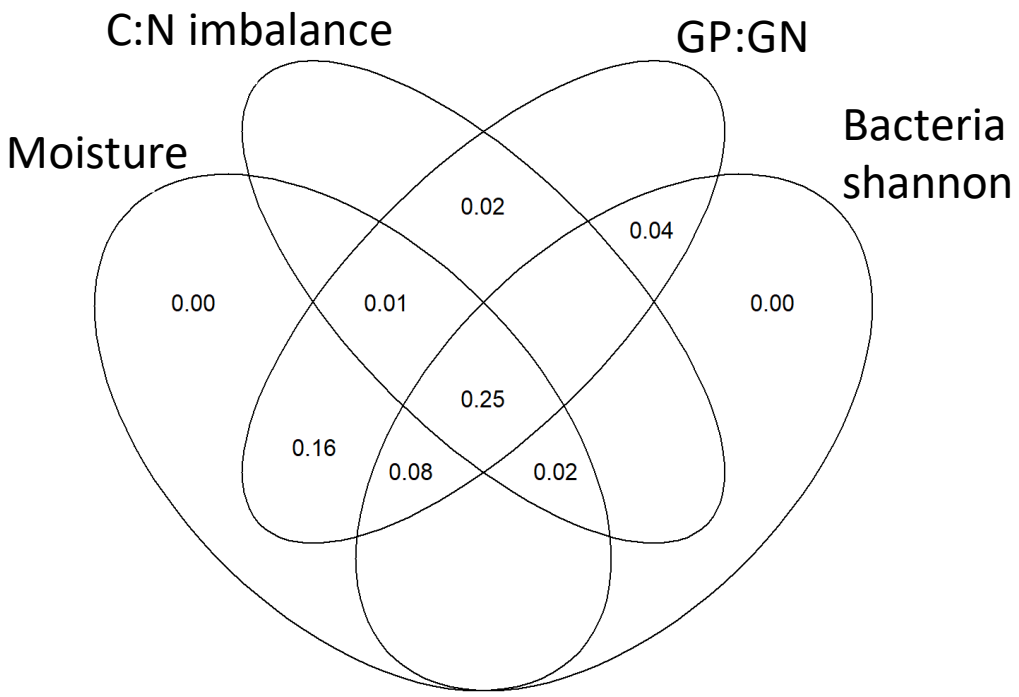


Figure S6. Spearman correlation matrix between soil multifunctionality (MF_Mic_mean), soil microbial community diversity, phylum relative abundances and gram positive : gram negative ratio (GPGN) in soil without plant (A) or with plants (B). Circle color represent correlation direction and circle size and color intensity represent correlation coefficient. Cells in black frames are consistent relationship across soil with and without plants.



SI Figure 5. Venn diagram of variance partitioning analysis illustrating the effect of moisture, C:N imbalance, GP:GN ratio, and Bacterial Shannon index on SMF. Values show the percentage of explained variance by each variable, and the percentage commonly explained in the intersections.

Supplementary Tables

Table S1. Overview of primers characteristics and cycling conditions used for polymerase chain reaction (PCR) and amplicon sequencing

	Bacteria	**Eukaryota**
Primers set		
Targeted region	V5-V6 regions, 16S rRNA gene	V7 region, 18S rRNA gene
Forward primer (5'–3')	GGATTAGATACCCTGGTAGT†	TTTGTCTGSTTAATTSCG‡
Reverse primer (5'–3')	CACGACACGAGCTGACG†	TCACAGACCTGTTATTGC‡
PCR conditions		
Number of cycles	35	45
PCR cycles characteristics (Denaturation, Annealing, Elongation – Final elongation)	95°C(30s), 57°C(30s), 72°C(90s) – 72°C(7min)	95°C(30s), 45°C(30s), 72°C(60s) – 72°C(7min)
Illumina platform	MiSeq 2x250	HiSeq 2500 2x150

† (Taberlet et al. 2018), ‡ (Guardiola et al. 2015)

Table S2. P-values of ANOVA 3 testing the effect of Drought, Fertilization, Plant presence and their interactions in SMF, microbial functions, soil chemistry, stoechiometry and microbial diversity.

	Drought (D)	Fertilisation (F)	Plant presence (P)	D x F	D x P	F x P	D x F x P
SMF	**0,001**	**0,029**	0,934	0,201	0,410	**0,034**	**0,022**
MBC	**0,010**	0,094	**0,024**	0,874	0,820	0,745	0,096
MBN	0,119	**0,003**	**0,000**	**0,025**	**0,002**	**0,000**	**0,003**
MBP	**0,000**	**0,002**	0,593	0,090	0,156	0,249	**0,031**
SIR	**0,000**	**0,018**	**0,002**	0,126	0,274	0,869	**0,008**
EEA	0,140	0,314	0,324	0,743	0,523	0,443	0,501
EEC	0,070	0,208	0,364	0,953	0,550	0,495	0,830
EEN	0,186	0,145	0,430	0,757	0,563	0,142	0,328
EEP	0,208	0,619	0,279	0,579	0,512	0,740	0,470
NMP	0,389	0,184	0,325	0,406	**0,004**	0,506	0,120
NEA	**0,036**	0,059	**0,004**	0,669	0,433	0,666	0,059
DEA	0,525	**0,000**	**0,000**	0,552	0,987	0,620	0,395
H	**0,000**	**0,005**	**0,042**	**0,009**	0,052	**0,005**	0,953
DOC	**0,012**	0,126	**0,000**	0,979	0,084	0,297	0,109
NO3	**0,000**	**0,000**	**0,000**	0,651	0,290	0,989	0,665
NH4	**0,000**	**0,000**	**0,000**	0,061	0,418	0,098	**0,010**
DON	0,247	0,398	0,934	0,282	0,188	0,945	0,832
PO4	0,195	**0,000**	0,069	0,142	0,189	0,722	0,751
Microbial C:N	0,062	**0,000**	**0,000**	0,112	**0,001**	**0,000**	**0,036**
Microbial C:P	0,258	0,202	0,387	0,549	0,591	0,254	0,538
Microbial N:P	0,178	**0,000**	**0,000**	0,326	**0,009**	**0,009**	0,221
DOC:TDN	**0,000**	**0,000**	**0,000**	0,208	**0,000**	0,122	0,434
DOC:PO4	0,341	**0,000**	0,924	0,198	0,859	0,829	0,759
TDN:PO4	**0,002**	0,107	**0,003**	0,123	0,374	0,854	0,525
C:N imbalance	**0,000**	**0,000**	**0,001**	**0,036**	**0,000**	**0,010**	0,484
C:P imbalance	0,937	**0,000**	0,580	0,519	0,541	0,616	0,766
N:P imbalance	**0,027**	**0,035**	0,442	0,171	0,176	0,368	0,444
GP:GN bacteria	**0,000**	**0,003**	0,510	0,691	0,638	0,420	0,729
Bacteria richness	0,339	**0,001**	**0,009**	0,996	0,272	0,178	0,736
Bacteria Shannon	**0,002**	**0,001**	**0,001**	0,139	0,282	**0,046**	0,871
Fungi Richness	**0,000**	0,825	**0,000**	0,440	0,956	**0,029**	0,364
Fungi Shannon	0,901	0,500	0,974	0,874	0,622	0,889	0,588