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# 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

33 soil microbial communities (Delgado-Baquerizo et al. 2016b). Global changes are impacting SMF but we still  
34 have a limited understanding of the impact of the interaction between multiple global change factors on SMF  
35 (Rillig et al. 2019). To predict these complex responses it is essential to understand the mechanisms that control  
36 SMF. Plants likely play a central role through their effect on soil C, nutrients and microbial communities  
37 (Bardgett et al. 2008). Microbial processes are highly influenced by the dynamics of abiotic factors of their soil  
38 environment such as pH, humidity, redox potential and temperature (Fierer 2017). Their functioning also depend  
39 on their nutritional resource that are often investigated through stoichiometric, which relies on the balance  
40 between chemical elements to predict their storage and circulation among different biotic and abiotic pools  
41 (Zechmeister-Boltenstern et al. 2015). Stoichiometric theory predicts that soil microorganisms immobilize and  
42 store the most limiting element for their growth whereas mineralization is accelerated for elements available in  
43 excess. However, for some specific microbial functions, including nitrification, denitrification or  
44 depolymerization of specific substrates depend the presence of specific microbes or microbial consortium  
45 (Crowther et al. 2019). Therefore high diversity of microbes should sustain multiple soil functions, while  
46 functional redundancy between microbes, especially for generalist functions, was suspected to potentially  
47 weaken this relationship (Allison and Martiny 2008, Delgado-Baquerizo et al. 2016a). But functional  
48 redundancy seems of limited importance for soil functions (Trivedi et al. 2019, Yang et al. 2024). Thus, recent  
49 studies confirmed a positive biodiversity-SMF relationship, with high magnitude and stability of SMF  
50 associated with higher microbial diversity and specific community composition (Delgado-Baquerizo et al.  
51 2016b, 2017, Maron et al. 2018). This suggest that some specific abiotic and stoichiometric conditions along  
52 with a diverse microbial community able to realize multiple functions are necessary to maximize SMF. Hence,  
53 it sounds appealing that microbial-MF response to multiple global change factors could be predicted through  
54 their impact on soil conditions, stoichiometry and microbial diversity.

55 In the last decades nutrient inputs to terrestrial ecosystems have drastically increased worldwide, especially  
56 in agricultural land with increasing fertilizer use combined with atmospheric deposition (Galloway et al. 2008).  
57 On the other hand, drought intensity and frequency have increased in many regions, with dramatic consequences  
58 on ecosystem functioning (Shukla et al. 2019). Nevertheless, how increased nutrient inputs influence the  
59 ecosystem response to drought, remains overlooked. Experiments showed that plant and microbial responses to  
60 drought are tightly coupled, with N competition playing an important role (Bloor et al. 2018, Piton et al. 2020a,  
61 2020c). Indeed, nutrient can be vital for microbes to cope with water stress, for instance to produce osmolytes  
62 which may represent an important part of their bacterial-N budget during drought (Schimel et al. 2007). An  
63 increase of the microbial biomass C:N has often been observed with drought, a pattern attributed to the selection  
64 of drought-resistant fungi that are less N demanding than bacteria (Strickland and Rousk 2010, Sun et al. 2020).  
65 Yet, how this response varies under high nutrient level remains to be tested. Life history strategy theory predict  
66 that r-strategy microbes (copiotrophes) thrive under conditions of high nutrient availability and exhibit a low  
67 resistance to climatic stress (De Vries and Shade 2013). Some recent experiments support this theory  
68 (Karlowsky et al. 2018, Piton et al. 2020a, 2020c) but it remains unclear if this selection of drought sensitive

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

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

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99 Table 1 : Hypotheses on how water stress, soil stoichiometry, and soil microbial diversity will respond to  
 100 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             | -  | -   | --<br>Plant water uptake increase water deficit for microbe   | ---<br>High growth of fertilized plant accentuates water deficit  |                    |
| Stoichiometric imbalance under drought  | -<br>Imbalance +. Increase of microbial biomass C:N. | --<br>Imbalance ++.<br>Microbial biomass C:N increases while C:N of available resource in soil is decreased by nutrient addition. | ---<br>Imbalance +++.<br>Increase of microbial biomass C:N while plant reduce N uptake during drought | ----<br>Imbalance ++++.<br>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  | -<br>Nutrient addition decreases microbial diversity  | 0<br>Plant presence increases microbial diversity   | -<br>Nutrient addition decreases microbial diversity  | (Zhou et al. 2020) |

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## 102 2 MATERIALS AND METHODS

### 103 2.1 SOIL SAMPLING

104 Soil was collected in a grassland localized in the Chartreuse mountain range of French Alps (45°36' N,  
 105 5°90'E), at 930m a.s.l. with a mean annual temperature of 8.1°C and 1049mm of mean annual precipitation.  
 106 Management was extensive since decades with one mowing per year and no fertilization. The 15 first cm of the  
 107 topsoil (3.6% of C and 0.4% of N) were extracted in spring (5<sup>th</sup> and 6<sup>th</sup> of April 2018), and sieved at 7mm to  
 108 remove plant residues. After homogenization, soil was used to fill 32 mesocosms (25cm diameter and 40cm  
 109 depth). All mesocosms were transported in the alpine green-house of the Jardin du Lautaret (Grenoble), with an

110 air temperature maintained at 20°C during the experiment, approximating the study site's daily mean  
111 temperature during the growing season. Mesocosms were weighted prior the start of the experiment (mean fresh  
112 soil weight: 11.8 kg, 95% confidence interval  $\pm$  0.1kg). All along the experiment, control mesocosms (unplanted  
113 soil, control climate) were weighted twice a week and compared with their initial weight to assess water loss.  
114 All mesocosms received the same amount of water equal to this water loss in control mesocosms (in average  
115 300mL per week), to maintain a stable soil moisture in mesocosms equal to the initial field value (0.24 g water  
116 per g of fresh soil, 66% of maximum water holding capacity). During the first 30 days, soil water content was  
117 kept constant for all mesocosms. In parallel, seeds of *Lolium perenne* from Arbiotech® (Saint Gilles, France)  
118 were germinated in individual pots filled with the same soil used in the mesocosms until seedling establishment.  
119 *L. perenne* was used as a phytometer because it is usually sown in managed grasslands in the French Alps  
120 (Loucougaray et al. 2015, Legay et al. 2017). After 30 days of growth, 25 individuals of *L. perenne* were planted  
121 in 16 mesocosms, with a 4 cm distance between each individual and with mesocosm edge. At day 45, half of  
122 the mesocosms (planted and unplanted soil) were fertilized with mineral N (50%-N as NO<sub>3</sub><sup>-</sup> and 50%-N as  
123 NH<sub>4</sub><sup>+</sup>) and P (P<sub>2</sub>O<sub>5</sub>) equivalent to 150 kg N/ha and 80kg P<sub>2</sub>O<sub>5</sub>/ha representing the regional recommendation for  
124 nutrient addition in intensive sown grasslands (Guide régional de fertilisation 2016, Chambre d'Agriculture).  
125 Twenty days after fertilization (day 65), watering of 16 mesocosms (4 unplanted soil/unfertilized, 4 unplanted  
126 soil/fertilized, 4 planted soil/unfertilized, 4 planted soil/fertilized) was stopped during 32 days (until day 97).  
127 At day 97, three soil cores (5cm diameter, 15cm depth) were sampled on the location of three *L. perenne*  
128 individuals in the center of each mesocosm, and pooled in a composite sample to assess plant biomass and soil  
129 stoichiometry, microbial community biomass stoichiometry, activity and diversity.

## 130 **2.2 PLANT ANALYSES**

131 Plants shoots and roots in planted mesocosms were manually sorted from the three soil cores. Plants shoots  
132 and roots were separated and dried at 70°C for a week and weighed to measure shoots and roots biomass. All  
133 plant material was ground and analyzed for C and N contents using an elemental analyzer (FlashEA 1112: Fisher  
134 302 Scientific, Waltham, Massachusetts, USA).

## 135 **2.3 SOIL ANALYSES**

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

139 Subsamples of 5g of fresh soil were oven-dried at 70°C for 1 week and weighed to determine soil water  
140 content (SWC) (Robertson et al. 1999). Soil subsamples were air dried and ground to powder to measure total  
141 C and N contents using a FlashEA 1112 elemental analyzer (FlashEA 1112: Fisher 302 Scientific, Waltham,  
142 Massachusetts, USA), and to determine soil pH in a 1:2.5 (soil:distilled water) solution. Dissolved organic  
143 carbon (DOC), nitrate (NO<sub>3</sub><sup>-</sup>), ammonium (NH<sub>4</sub><sup>+</sup>), total dissolved nitrogen (TDN) were extracted on 10 g of

144 fresh soil using 0.5M K<sub>2</sub>SO<sub>4</sub> solution following Jones and Willett (2006). DOC concentration was measured on  
145 a Shimadzu TOC-L analyzer (Shimadzu Inc.). N concentrations were measured on an automated photometric  
146 analyzer using standard colorimetric methods (Gallery Plus: Thermo Fisher Scientific, Waltham,  
147 Massachusetts, USA). Dissolved organic nitrogen (DON) was calculated as the difference between TDN and  
148 total inorganic N (N-NO<sub>3</sub><sup>-</sup>+ N-NH<sub>4</sub><sup>+</sup>).PO<sub>4</sub> was extracted on 10g of fresh soil using 0.5M NaHCO<sub>3</sub> solution as  
149 described by (Brookes et al. 1982).

## 150 **2.4 MICROBIAL BIOMASS AND ACTIVITIES**

151 Microbial biomass carbon (MBC), nitrogen (MBN) and phosphorus (MBP) contents were measured on 10-  
152 g of fresh soil, using chloroform fumigation extraction-methods as described by (Vance et al. 1987) for MBC  
153 and MBN, and by Brooks et al. (1981) for MBP. A spiking procedure (25µgP-PO<sub>4</sub>/g soil were added on two  
154 samples) was used to estimate and correct for P recovery during extraction (Brookes et al. 1982). Microbial  
155 biomass element contents were calculated as the difference between fumigated and non-fumigated samples, and  
156 adjusted using conversion factors of 0.45, 0.45 and 0.40 for MBC, MBN and MBP respectively (Jenkinson et  
157 al. 2004). Indicators of the stoichiometric imbalances between microbial biomass and their resources were  
158 calculated as the ratios of resource A:B in the soil solution over microbial biomass A:B (Mooshammer et al.  
159 2014), with A:B being C:N, C:P or N:P ratios. Assuming that the microbial biomass composition represent its  
160 nutritional need to grow, an increase of such indicator above 1 indicate that the element B become more limiting  
161 over A, in other words its availability relative to A become too low to build new biomass. Values below 1  
162 indicate the opposite situation.

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

177 Potential N mineralization (PNM) was estimated after incubation of 10-g of fresh soil under anaerobic  
178 conditions for 7 days at 40°C in the dark (Wienhold 2007), inducing accumulation of mineralized NH<sub>4</sub><sup>+</sup>. PNM

179 rate was calculated as the difference between  $\text{NH}_4^+$  content before and after incubation ( $\mu\text{gN/g}$  dry soil/day).  
180 Potential nitrification enzyme activity (NEA) was estimated according to (Koper et al. 2010) following  
181 (Dassonville et al. 2011). Briefly, 3 g of each soil were incubated under aerobic conditions (180 rpm,  $28^\circ\text{C}$ , 10  
182 h) in a solution of  $50\mu\text{g N}-(\text{NH}_4)_2\text{SO}_4/\text{g}$  dry soil and rates of  $\text{NO}_2$  and  $\text{NO}_3$  production were measured after 2,  
183 4, 8 and 10 hr by ionic chromatography (DX120; Dionex, Salt Lake City, UT, USA). Maximal nitrification rate  
184 (NEA) was assessed by plotting nitrification rates along the gradient of  $\text{NH}_4\text{-N}$  concentrations (Lineweaver and  
185 Burk 1934). Potential denitrification activity (DEA) was estimated following Attard et al. (2011). Briefly, 10 g  
186 dry weight (dw) soil were placed at  $28^\circ\text{C}$  under anaerobic conditions using 90:10  $\text{He}:\text{C}_2\text{H}_2$  mixture inhibiting  
187  $\text{N}_2\text{O}$ -reductase activity. Each sample was supplemented with 3 ml  $\text{KNO}_3$  ( $50\mu\text{g N-NO}_3\text{ g}^{-1}\text{dw}$ ), glucose ( $0.5\text{ g}$   
188  $\text{C/g dw}$ ) and sodium glutamate ( $0.5\text{ g C/g dw}$ ) (Attard et al. 2011), completed with distilled water to reach water  
189 holding capacity.  $\text{N}_2\text{O}$  was measured at 2, 3, 4, 5 and 6 hr using a gas chromatograph (microGC R3000; SRA  
190 Instruments, Marcy l'Etoile, France). Substrate-induced respiration (SIR) was measured following (Anderson  
191 and Domsch 1978). Briefly, 5 g of soil samples were placed in airtight flask, with 1.2 mg of C-glucose/g dry  
192 soil, completed with distilled water to reach water holding capacity. Then, samples were incubated at  $28^\circ\text{C}$   
193 during 5 hours with  $\text{CO}_2$  concentrations measured each hour. The slope of the linear regression between time  
194 and with  $\text{CO}_2$  concentrations was used to estimate aerobic respiration ( $\text{g C-CO}_2^{-1}\text{ h}^{-1}$ ).

## 195 **2.5 SOIL MULTIFUNCTIONALITY**

196 A soil multifunctionality (SMF) index was calculated using the widely used method presented by Maestre et al.  
197 (2012). Eight microbial properties and functions (MBC, MBN, MBP, SIR, EEA, NMP, NEA, DEA) were first  
198 standardized using Z-score transformation and then averaged. These variables were selected because they  
199 capture the capacity of soil microbes to store C, N and P in their biomass and to process these elements through  
200 organic matter depolymerisation, mineralization and downward transformations (nitrification and  
201 denitrification).

## 202 **2.6 MOLECULAR ANALYSES**

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



213 sequencing were performed at FASTERIS (FASTERIS SA, Geneva, Switzerland) using the MetaFast protocol  
214 ([www.fasteris.com/metafast](http://www.fasteris.com/metafast)). The bacterial and eukaryote libraries were sequenced on an Illumina MiSeq (2 x  
215 250 bp paired-end reads) and HiSeq 2500 ((2x150 bp paired-end reads) platforms, respectively. Potential  
216 contaminations were tracked by including negative controls at the extraction and PCR steps. Tag jumps caused  
217 by chimeras were accounted for at the bioinformatic level by monitoring unused tag combinations (Schnell et  
218 al. 2015).

219 Sequencing data were then analysed using the OBITools software package (Boyer et al. 2016) and R scripts  
220 following (Zinger et al. 2019). Firstly, paired-end reads were assembled and then assigned to the corresponding  
221 PCR replicate on the basis of primer sequences (two mismatches allowed) and tag combinations (no mismatches  
222 allowed). Secondly, reads were dereplicated. Low quality sequences (i.e. sequences containing “Ns”), sequences  
223 observed less than 100 times, and sequences whose length fell outside the expected length range (<45bp and  
224 <36bp for the bacterial and eukaryote markers, respectively) were discarded (Taberlet et al. 2018). Thirdly,  
225 unique sequences were clustered at 97% similarity into operational taxonomic units (OTUs) using the Sumacust  
226 package (Mercier et al. 2013). Only the cluster centres were kept for taxonomic annotation with the ecotag  
227 program from the OBITools package (Boyer et al. 2016). OTUs whose similarity score of the best match (best  
228 identity score from ecotag program) in the reference database was below 0.95% were excluded. Then, potential  
229 contaminations were removed by excluding all OTUs whose absolute number of reads was highest in negatives  
230 control. PCR replicates were summed for each sample and rarefied to the same sequencing depth equal to the  
231 lowest number of sequences observed in a sample. Rarefaction curves were also obtained for each sample using  
232 the *vegan* R package (Oksanen et al. 2011) to assess to which extent the sequencing depth captured the diversity  
233 present in our samples (SI Figure 1). All fungal sequences were extracted from the Eukaryota dataset to narrow  
234 down the subsequent analyses to bacterial and fungal communities. The gram positive to gram negative ratio of  
235 soil bacteria was calculated using relative abundance of 16S rDNA OTUs as in Orwin et al. (2018), which relied  
236 on the Gupta (2011) classification of bacteria phylum based on the presence of the Hsp60 insert in their genomes  
237 (Gram positive : Actinobacteria, Firmicutes, Deinococcus-Thermus and Chloroflexi ; Gram negative :  
238 Proteobacteria, Acidobacteria, Bacteroidetes, Chlamydiae, Gemmatimonadetes, Nitrospirae, Planctomycetes,  
239 Spirochaetes, Verrucomicrobia, Armatimonadetes, Elusimicrobia and Fusobacteria).

240

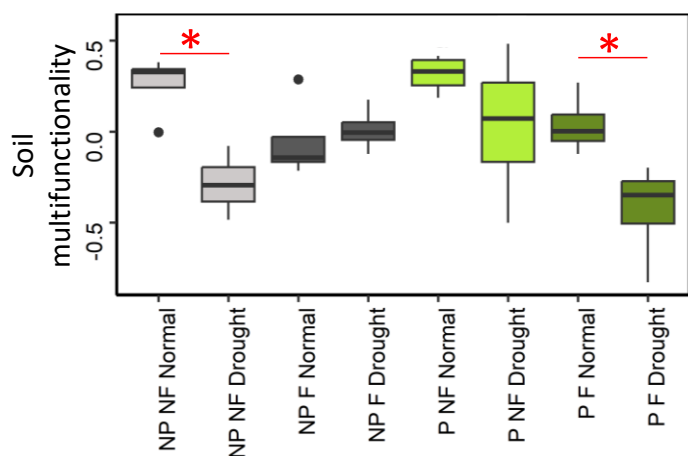
## 241 **2.7 STATISTICAL ANALYSES**

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

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

### 249 3 RESULTS

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



265

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

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

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

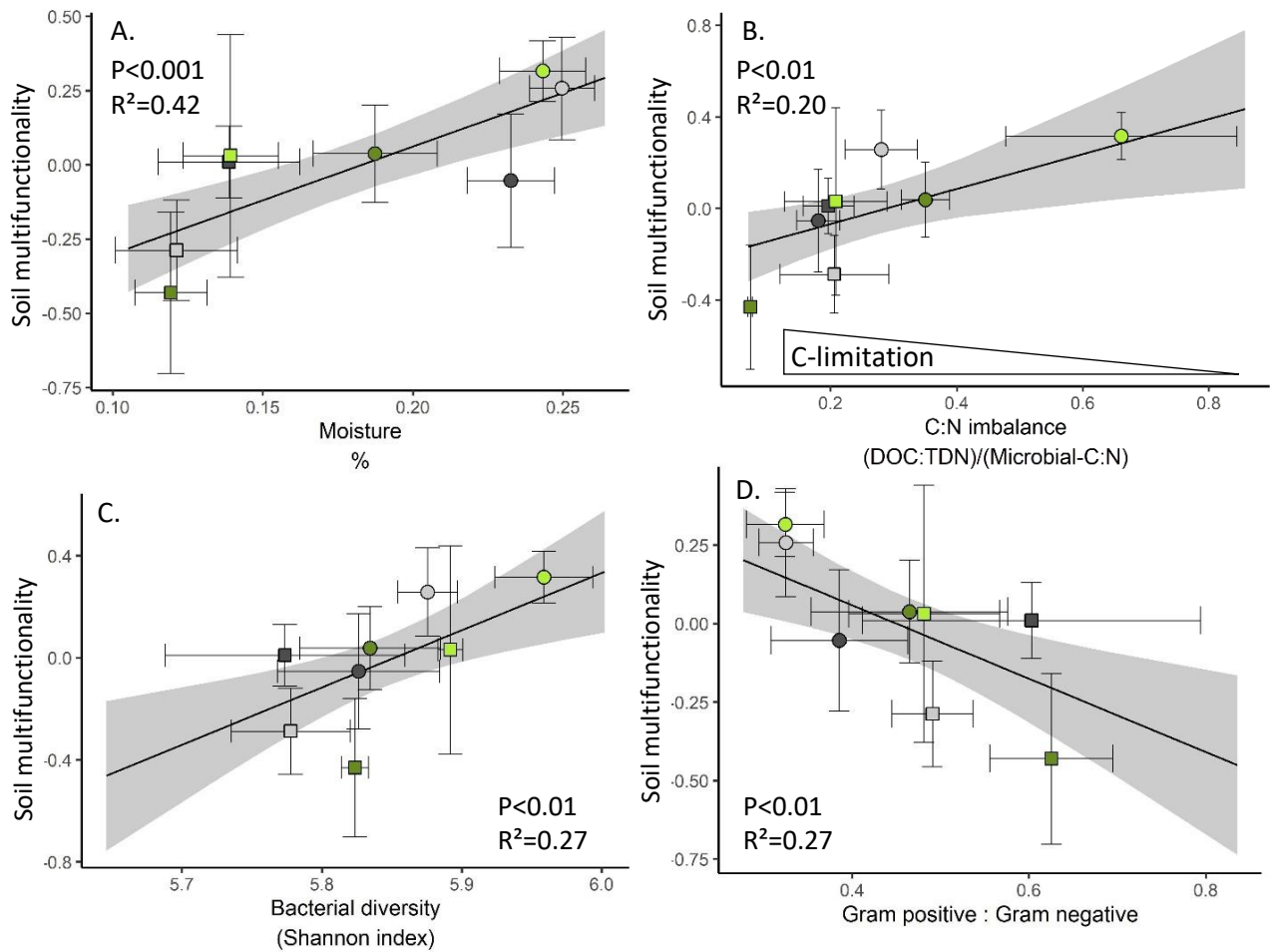
279 *Table 1. P-values and R<sup>2</sup> associated with the effects of Drought, Fertilization, Plant presence and their interactions on*  
 280 *bacterial and fungal community composition. Effects were assessed by PERMANOVA on a Bray-Curtis distance matrix*  
 281 *based on bacterial (16S rRNA) and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

|                 | Drought<br>(D)      | Fertilization<br>(F) | Plant<br>presence<br>(P) | D × F | D × P | F × P       | D × F × P | Total R <sup>2</sup> |
|-----------------|---------------------|----------------------|--------------------------|-------|-------|-------------|-----------|----------------------|
| <b>Bacteria</b> |                     |                      |                          |       |       |             |           |                      |
| p-value         | <b>p &lt; 0.001</b> | <b>p &lt; 0.001</b>  | <b>p &lt; 0.001</b>      | 0.07  | 0.10  | <b>0.03</b> | 0.15      |                      |
| R <sup>2</sup>  | <b>0.10</b>         | <b>0.12</b>          | <b>0.07</b>              | 0.03  | 0.03  | <b>0.04</b> | 0.03      | 0.43                 |
| <b>Fungi</b>    |                     |                      |                          |       |       |             |           |                      |
| p-value         | <b>p &lt; 0.001</b> | <b>0.02</b>          | <b>p &lt; 0.001</b>      | 0.60  | 0.33  | 0.38        | 0.30      |                      |
| R <sup>2</sup>  | <b>0.10</b>         | <b>0.05</b>          | <b>0.13</b>              | 0.02  | 0.03  | 0.03        | 0.03      | 0.39                 |

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285 While SMF showed contrasting response to drought and fertilization depending on plants presence, SMF  
 286 showed consistent relationships with some soil and microbial properties across planting treatments (Figure S5  
 287 and S6). In both planted and unplanted soils, SMF was positively correlated with soil moisture, C:N imbalance  
 288 and bacterial diversity, and negatively correlated with the bacterial gram positive : gram negative ratio (Figure  
 289 3). These 4 variables were selected for their consistent relationships with SMF, and variance partitioning showed  
 290 that 25% was commonly explained by them (SI Figure 5). Microbial properties explained 24 additional %, with  
 291 8 % explained by both microbial properties and 16% explained only by the GP:GN ratio.

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

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## 300 **4 DISCUSSION**

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

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

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

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

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

342 negative bacteria, such as *Proteobacteria*, are known to decrease with C limitation (Fierer et al. 2007, Fanin et  
343 al. 2018), and water stress (Naylor and Coleman-Derr 2018), as observed in our mesocosm study. Although  
344 such aggregation of bacteria at high phylogenetic level is robust only for deeply conserved traits and could thus  
345 miss some difference between taxa that exist at lower level (Martiny et al. 2015, Ho et al. 2017), high level  
346 classification show some ecological coherence and have been key in the developpemnt of generic theory in  
347 microbial ecology (Philippot et al. 2010). With this consideration in mind, Gram negative bacteria was  
348 associated with copiotrophic (r strategy) bacteria that use labile C to sustain fast growth (De Vries and Shade  
349 2013, Fanin et al. 2018). Such growth strategy can promote microbial biomass and enzymatic processes (Piton  
350 et al. 2020b), and eventually the high SMF that we observed. Altogether, a large proportion (25%) of SMF was  
351 explained by these four properties (soil moisture, C:N imbalance, bacterial diversity and GPGN) stressing their  
352 coupling in the soil system. However, about same proportion (24%) was additionally explained only by the  
353 microbial properties (bacterial diversity and GPGN), suggesting that high SMF not only emerged from favorable  
354 stoichiometric and moisture conditions for microbial growth, but also relied on microbial diversity.

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

## 368 **1 CONCLUSION**

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

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## COMPETING INTERESTS

386

The authors declare no competing interests.

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- 570

## SUPPLEMENTARY MATERIALS

### SUPPLEMENTARY FIGURES

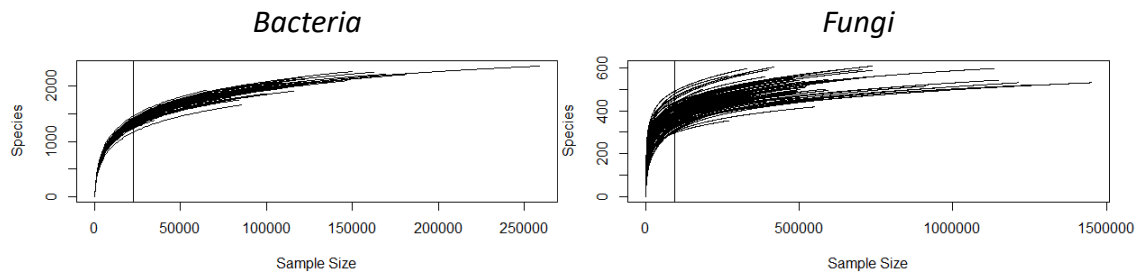


Figure S1. Rarefaction curves for Bacteria and Fungi. Vertical lines represent resampling size used for data rarefaction of each sample to have similar sequencing depth equal to the number of reads of the sample with the lowest reads numbers (Bacteria: 22 571 sequences/sample, Eukaryota : 93 742 sequences/sample).

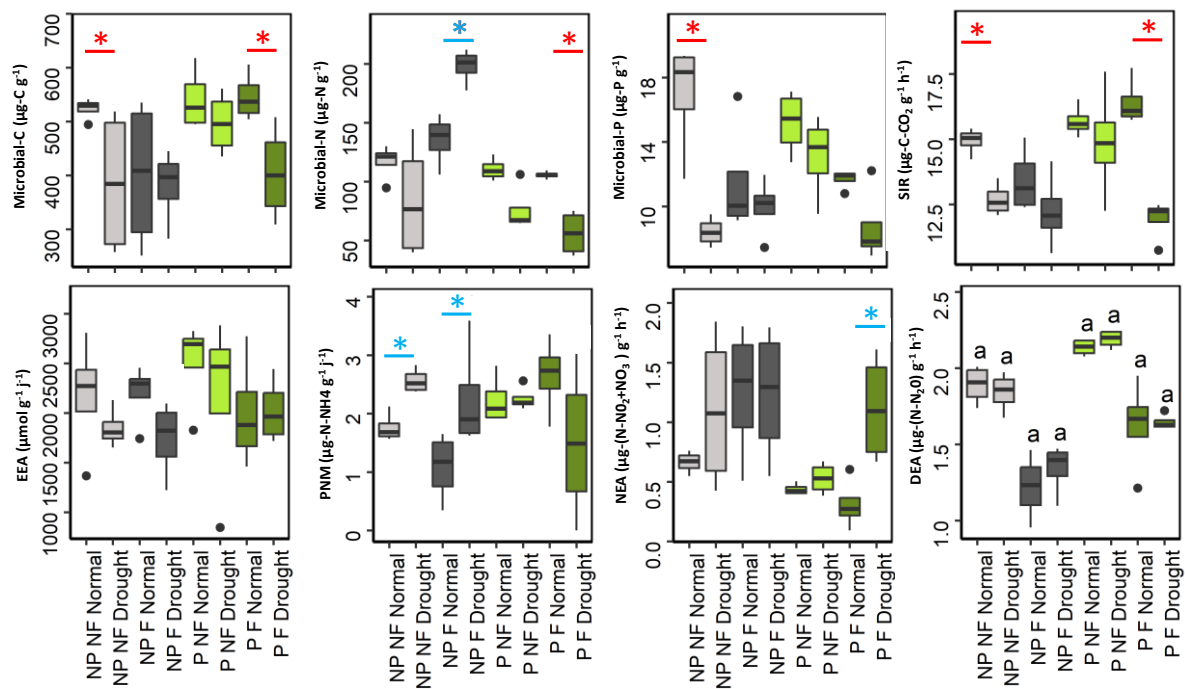


Figure S2. Effect of drought, fertilization and plant presence on soil microbial functions. Star indicate significant difference between drought and normal climate treatment. SIR=Substrate-induced-respiration. PNM=Potential N mineralization. NEA=Potential nitrification activity. DEA=Potential denitrification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

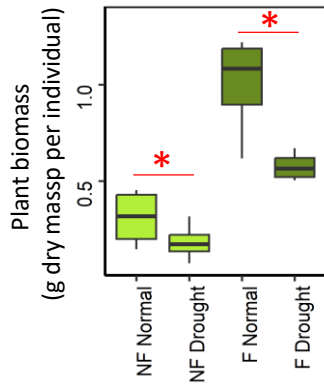


Figure S3. Plant biomass response to drought with or without fertilisation. Star indicate significant difference between drought and normal climate treatment.

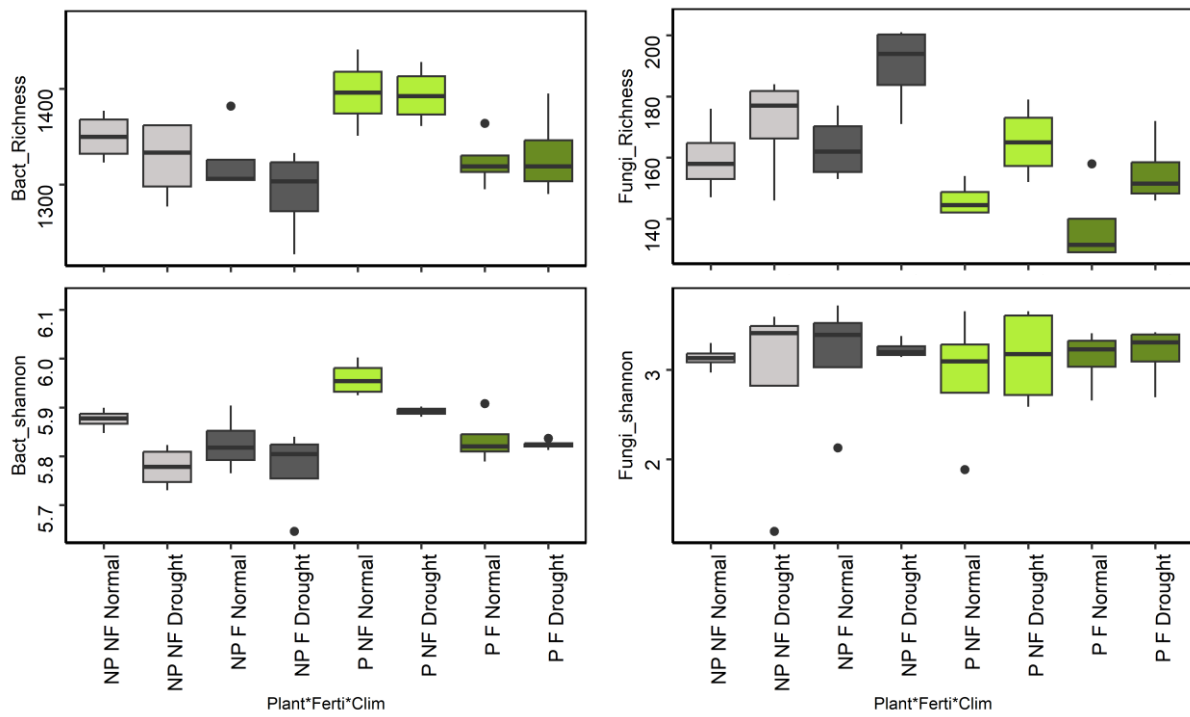


Figure S4. Soil microbial diversity response to drought, fertilization and plant presence. Model statistics are provided in Table S2. NP=No plant, P=Plant presence, F=Fertilized, NF=No fertilization

## A. Without plant

## B. With plants

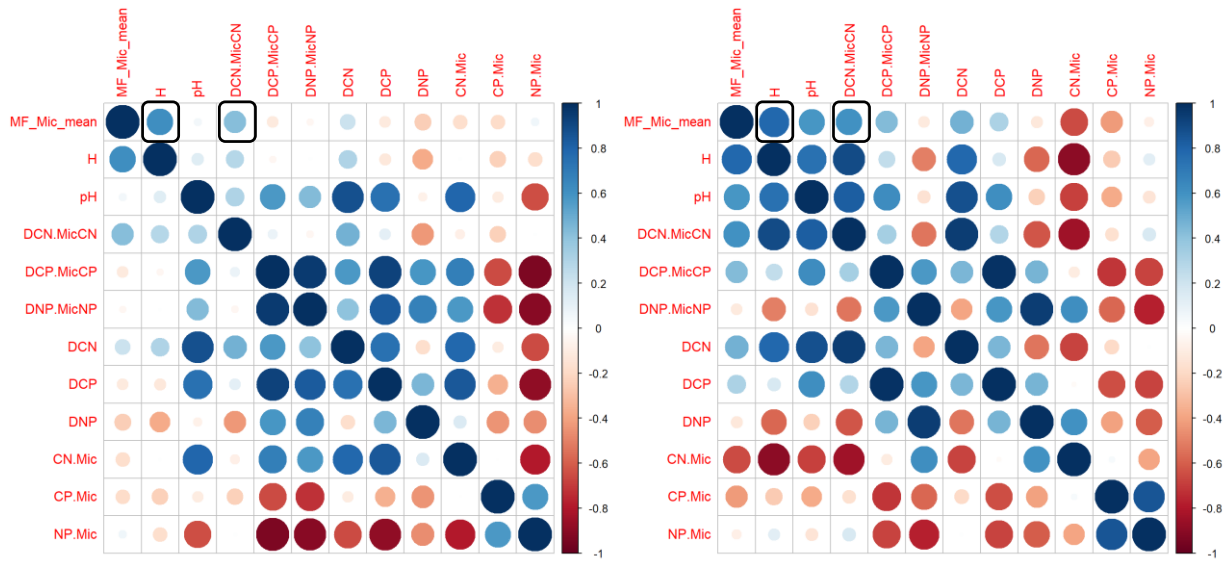


Figure S5. Spearman correlation matrix between soil multifunctionality (MF\_Mic\_mean), soil moisture, pH and stoichiometry (DCN=DOC:TDN, DCP=DOC:PO<sub>4</sub>, DNP=TDN:PO<sub>4</sub>) in soil without plants (A) or with plants (B). Circle color represent correlation direction and circle size and color intensity represent correlation coefficient.

## A. Without plant

## B. With plants

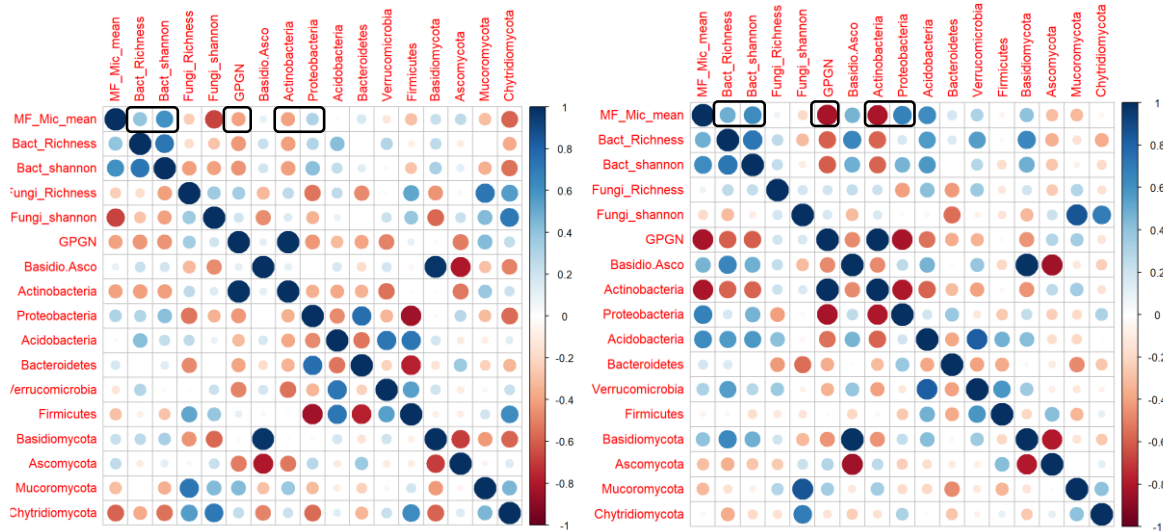
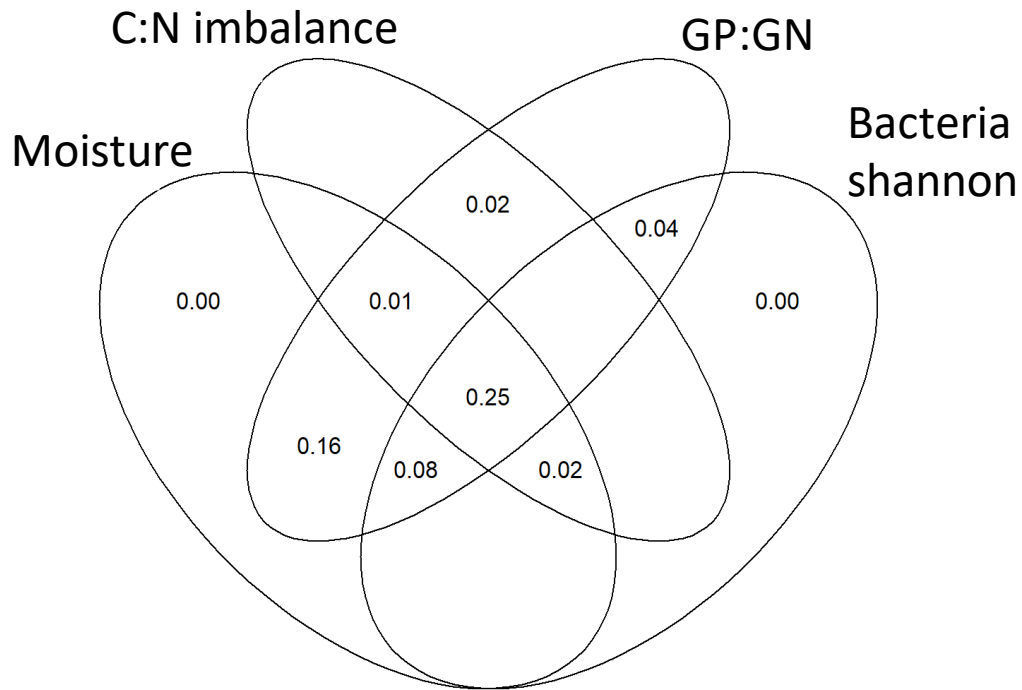


Figure S6. Spearman correlation matrix between soil multifunctionality (MF\_Mic\_mean), soil microbial community diversity, phylum relative abundances and gram positive : gram negative ratio (GPGN) in soil without plant (A) or with plants (B). Circle color represent correlation direction and circle size 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

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

<sup>†</sup> (Taberlet et al. 2018), <sup>‡</sup> (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, stoichiometry and microbial diversity.

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