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1 Research Paper

2 **Title**

3 Environmental drivers of microbial functioning in Mediterranean forest soils

4 **Authors**

5 Caroline Brunel<sup>1,2</sup>, Anne-Marie Farnet Da Silva<sup>1</sup> and Raphael Gros<sup>1</sup>

6 <sup>1</sup> Mediterranean Institute of marine and continental Biodiversity and Ecology, IMBE, Aix  
7 Marseille Université, UMR CNRS 7263, IRD, Avignon Université, Campus l'Etoile, Av.  
8 Escadrille Normandie Niemen, 13397, Marseille, Cedex 20, France

9 <sup>2</sup> IRD, IPME; 911 avenue Agropolis, BP 64501, 34394, Montpellier, France

10 Corresponding Author: Caroline Brunel (caroline.brunel@ird.fr, tel: +33 658 483 326)

11 **ORCID IDs:**

12 Caroline BRUNEL : 0000-0002-0705-5165

13 Anne-Marie FARNET DA SILVA : 0000-0001-6603-1174

14 Raphael GROS : 0000-0001-6296-0558

15 **Abstract**

16 Mediterranean forests own distinct characteristics resulting from climate, soil, and vegetation that  
17 affect soil microbial communities' assembly and their associated functions. We initiated a multi-  
18 scalar analysis of environmental drivers of soil functioning to (1) identify pertinent factorial scales  
19 and (2) determine the relative importance of soil, vegetation, and geoclimate relative influences in  
20 shaping soil microbial functions across the French Mediterranean forests. Soil samples (0-15 cm)  
21 were collected from 60 forest sites and soil physico-chemical and microbiological properties were  
22 assessed across different factorial scales i.e., bioclimates, slope exposures, and forest stands.  
23 Patterns in microbial catabolic potential (i.e., extracellular enzymes and microbial respiration) and  
24 carbon (C) source utilisation (i.e., catabolic-level physiological profiling) were partitioned  
25 between vegetation cover, soil characteristics and geoclimate components. Our results reveal that  
26 the catabolic potential of soil microbes was strongly influenced by the forest stands, and mainly  
27 relied on ammonium and nitrate contents. In contrast, variation in C-source utilisation was mainly  
28 explained by vegetation cover. Soil metabolic capacities of microorganisms and resulting C  
29 dynamics were largely constrained by climate parameters, which suggests potentially important  
30 consequences for soil C storage. Our study revealed diverse structuration patterns between the  
31 catabolic potential and the carbon-source utilisation of soil microbial communities, and gives  
32 insights into the underlying mechanisms of soil microbial functioning in Mediterranean forests.

33 **Key words**

34 Ecological filters; Mediterranean forest stands; Microbial metabolism; Scale-dependent  
35 structuration; Soil microbial communities; Soil physico-chemical properties

36

37 **Introduction**

38 Studies of the microbial processes involved in the decomposition of soil organic carbon (C) have  
39 considerably increased in recent years owing to the major role soil C storage plays in the context  
40 of climate change [1]. In the C sink that forest soils represent [2], soil organic C stocks result  
41 largely from the balance between (i) inputs of C determined by the vegetation cover, (ii) processes  
42 of soil C humification by microbial communities and stabilization and (iii) outputs *via* soil erosion,  
43 CO<sub>2</sub> production from roots and microorganisms [3]. Thus, C dynamics in soils depend on complex  
44 interactions between climate conditions (e.g. long term mean annual precipitation and temperature)  
45 that shape forest structure and tree productivity, soil physico-chemical properties (such as calcium  
46 carbonate content and pH) influenced by the geological substratum and their combined effect on  
47 soil microbial communities and their metabolism [4].

48 Microbes are the primary decomposers of plant material due to their unique ability to produce  
49 enzymes to break down both simple molecules such as cellulose and more complex plant derived  
50 compounds such as lignin [5]. They mobilize and transform a broad range of soil C substrates,  
51 assimilating C in their biomass, emitting CO<sub>2</sub> or stabilizing C in soil [6]. There is a lot of debate  
52 on the potential drivers controlling soil microbiome assembly and the mechanisms maintaining  
53 community structure and composition, and we argue that these rules may be better understood if  
54 put in perspective (adapted to the needs and contexts). Soil pH, the chemical composition of  
55 organic matter (OM), and climate conditions have been pointed out as key driver of soil  
56 functioning at local to continental scales [7–11]. In a recent study from different geographical and  
57 climatic zones across Europe, variations in bacterial community diversity and structure were  
58 mainly driven by parameters corresponding to similar edaphic and climatic properties [12].

59 Although these findings provide essential information on soil communities structuration at large  
60 scale, the results cannot be transferred to more specific contexts.

61 In the Mediterranean context particularly, where forests are subjected to specific constrains such  
62 as drought stress, heat waves, low nutrient availability and recalcitrant organic compounds [13]  
63 we still lack a clear understanding of factors controlling microbial C metabolism and driving soil  
64 C dynamics. Experimental determination of drivers has helped to develop which drivers may be  
65 of importance on Mediterranean soils (i.e., soil OM quality and water availability [14, 15]), but a  
66 more accurate determination of drivers from measurements done *in situ* will give greater insight  
67 to the mechanisms maintaining microbial communities' structures and functions in these soils.  
68 Determining pertinent spatial scales to observe variation in the functioning of such specific soils  
69 is now required to clarify how this ecosystem compartment is driven and to assess microbial  
70 functioning and C cycling vulnerability to environmental changes.

71 Here we report on a study designed to (1) determine pertinent factorial scales for unraveling  
72 microbial functional patterns and (2) estimate the relative importance of environmental  
73 components (vegetation, geoclimate and soil properties) in shaping of soil microbial functioning  
74 and (3) identify specific drivers. To disentangle the patterns and drivers of soil microbial  
75 functioning in Mediterranean forest soils, we sampled 60 sites covering an area of about 10.000  
76 km<sup>2</sup> in south-eastern France. We hypothesized that soil microbial metabolic capabilities and C  
77 substrate affinities varied according to bioclimates at a regional scale, side slope exposure at a  
78 landscape scale and forest stand at a local scale (Fig. 1) due to environmental variations specific  
79 to these scales.

## 80 **Methods**

## 81 *Study sites and sampling design*

82           The sampling was conducted in April 2013 in South-Eastern France (Provence-Alpes-Côte  
83 d'Azur), an area characterized by frequent and intense droughts and heat waves typical of the  
84 Mediterranean climate. Mean annual temperature and precipitation respectively reach 11.3°C and  
85 799mm (from WorldClim [16], Appendix 2, Fig. S1). Soils are characterized by carbonatic  
86 pedofeatures (i.e., fine calcareous silt clay loam). We conducted our study in sub-humid and humid  
87 bioclimates [17] corresponding to meso-Mediterranean and supra-Mediterranean altitudinal  
88 arrangement of vegetation respectively covered with *Pinus halepensis* and *Quercus ilex*, and *Pinus*  
89 *sylvestris* and *Quercus pubescens*. Three forest stands were selected for each of the two  
90 bioclimates, and were a mono-specific stand of *Pinus* and a mono-specific stand of *Quercus*, and  
91 a mixed stand 60/40% of *Pinus/Quercus*. All forest stands were about 60 ±10 years-old and were  
92 without forest management for 35 years. For each of the six types of forests, five 20mX20m plots  
93 were selected on both south- and north-facing slopes. These plots were considered to be true  
94 replicates as the distance between them exceeded 2km. All sites were located at altitudes from 500  
95 to 900m for meso-Mediterranean and from 900 to 1100m for supra-Mediterranean bioclimates.  
96 Plots were first randomly localized by crossing information from the French Geological Survey  
97 (for pedofeature), the Forest Property Regional Center (for forest stands informations) and the  
98 National Institute of Geography (for altitude and exposition). The sixty forest plots were visited  
99 for cross-validation and were then integrated to the survey (Fig. 1b).

100           Within each plot, bulk soil samples were collected after removing the litter layer and  
101 digging ten small pits over an approximately 20 cm × 20 cm area (the depth was ranging between  
102 10 and 15 cm and was defined by the A horizon limits). The ten sub-samples were sieved (2 mm),  
103 pooled and mixed to form a composite sample and placed into a polyethylene bag. In the lab, one

104 aliquot of each composite sample was stored at 4°C for microbial analyses. A second aliquot was  
105 air dried in a warm room (48 hours at 30 degrees) for chemical analyses.

106 *Soil chemical characteristics*

107 Soil water holding capacity (WHC) was determined by the amount of water held in the soil  
108 sample vs. the dry weight of the sample. Soil C and N content were measured using a C/N  
109 elementary analyzer (Flash EA 1112 ThermoScientific series, Waltham, Massachusetts, USA).  
110 Determination of CaCO<sub>3</sub> in 10 g soils (dry weight equivalent) was based on the release of CO<sub>2</sub>  
111 after addition of HCl 4N [18]. The percentage of inorganic C (C in CaCO<sub>3</sub>) was calculated as  
112 follows: %C-CaCO<sub>3</sub> = 11.991 / 100 x %CaCO<sub>3</sub>. Organic C was calculated as the difference  
113 between total C and inorganic C contents. Soil pH was determined in distilled water (5 g dry weight  
114 in 12.5 mL) after a 45 min equilibration [19]. A subsample of 10 g (dry weight equivalent) was  
115 analyzed after extraction in 100 ml of 1 M KCl with electrodes to determine inorganic NH<sub>4</sub><sup>+</sup> and  
116 NO<sub>3</sub><sup>-</sup> concentration in soils (expressed per gram of dried soil).

117 Soils C biochemical forms were characterized with solid-state <sup>13</sup>C-NMR spectroscopy  
118 using cross-polarization and magic angle spinning (CP-MAS). Spectra were obtained on a Bruker  
119 Avance 400MHz NMR spectrometer (Rheinstetten, Germany) at a <sup>13</sup>C resonance frequency of 106  
120 MHz and a Bruker double-bearing probe (further detailed in Appendix 1). The relative distribution  
121 of C groups with different structures was determined by integrating the signal intensities over  
122 defined chemical shift windows using Dmfit software [20]. Spectra were divided into four  
123 chemical regions (Mathers et al., 2003): alkyl C (0-45 ppm), O-alkyl C (45-112 ppm), aromatic C  
124 (112-160 ppm) and carbonyl C (160-185 ppm). To describe OM quality, the following ratios of

125 humification ( $HR_1$  and  $HR_2$ ) and aromaticity (AR) were calculated [21]:  $HR_1 = \text{alkyl C} / \text{carboxyl C}$   
126 ;  $HR_2 = \text{alkyl C} / \text{O-alkyl C}$  ;  $AR = \text{aromatic C} / (\text{alkyl C} + \text{O-alkyl C} + \text{aromatic C})$

### 127 *Soil microbial functioning*

128 A full description of the methods used for microbial analyses is provided in the Appendix  
129 1. Briefly, the determination of C source utilisation (i.e., catabolic-level physiological profiling,  
130 CLPP) was performed using BIOLOG® Eco plate (BIOLOG Inc., Hayward, CA). Plates with an  
131 average well colour development (AWCD) of 0.4 were used in the statistical calculations. Shannon  
132 index and the mean growth on carbohydrates, carboxylic and ketonic acids, amines/amides,  
133 polymers and amino-acids substrates were then determined. Microbial biomass (MB) was  
134 estimated using Substrate-Induced Respiration (SIR). Ten grams (dry weight equivalent) of  
135 standardized samples at 60% of WHC were placed in 117 ml flushed-air glass jars and amended  
136 with a powder of talc and glucose (1000  $\mu\text{g C g}^{-1}$  soil). After ninety minutes, 1 ml of air was  
137 sampled with a syringe and injected into a gas chromatograph (Chrompack CHROM 3 – CP 9001,  
138 Middelburg, The Netherlands) to determine  $\text{CO}_2$  production. The gas chromatograph was equipped  
139 with a thermal conductivity detector and a packed column (Porapak). The carrier gas helium flow  
140 was regulated at 60  $\text{ml h}^{-1}$ . The  $\text{CO}_2$  concentration of flushed air was subtracted from the  $\text{CO}_2$   
141 concentration of each sample and resulting values were adjusted to 22°C according to Ideal Gas  
142 Laws using  $Q_{10} = 2$ . Substrate-induced respiration rates were converted into MB using equations  
143 given by [22]. Basal respiration was determined without adding glucose and talc powder and was  
144 estimated to calculate the metabolic quotient  $q\text{CO}_2$  (the ratio of basal respiration to microbial  
145 biomass), which is a sensitive ecophysiological indicator of soil stress induced by environmental  
146 conditions [23]. The activity of five extracellular enzymes (EEA) involved in soil C and N cycles  
147 were assessed ( $n=3$ ) for each soil sample to determine the catabolic potential of microbial



148 communities. Tyrosinase activity was assessed according to the modified method of Saiya-Cork  
149 et al. [24]. Two ml of 25 mM L-DOPA solution (L-3,4-dihydroxyphenylalanine) in potassium  
150 phosphate buffer (50 mM, pH 6.5) were added to 0.4 g of soil (fresh weight), mixed and incubated  
151 for 30 min, in darkness at 25°C. The mixture was centrifuged for 3 minutes at 12 000 g before  
152 absorption was measured at 590 nm. Transesterase activity of lipase was assessed according to the  
153 method of Goujard et al. [25]. Two ml of distilled water and 4 ml of 10 mM *p*-nitrophényl-  
154 caprylate in heptane were added to 1 g of soil (fresh weight) and incubated for 12 h at 30 °C. The  
155 reaction was stopped and colour revealed by adding 200µL of the mixture to 4 mL of 0.1 M NaOH,  
156 which was immediately centrifuged for 2 min at 12 000 g. The amount of *p*-nitrophenol released  
157 was measured at 412 nm. Cellulase activity was assayed using CarboxyMethylCellulose (CMC)  
158 1% in 2 mL of sodium acetate buffer (50 mM, pH 6) added to 0.5g of soil (fresh weight) incubated  
159 for 4h at 50°C. Glucose was quantified according to the Somogyi-Nelson method [26] and  
160 absorption was read at 870 nm [27]. Protease activity was measured using 5 g of soil (fresh weight)  
161 in 5 mL of casein at 2% in Tris HCl buffer (50mM, pH 8.1). The mixture was incubated for 3 hours  
162 at 50°C and then the reaction was stopped with 5 mL of Trichloroacetic acid solution (at 15%) and  
163 the mixture centrifuged (2min, 12 000g). Aromatic amino acids were detected using Folin reagent  
164 (33%) at 700nm. Tyrosine was used as standard. Urease activity was assessed using 0.5g of soil  
165 (fresh weight) in 2mL of urea solution (80mM) in a sodium acetate buffer (50mM, pH 6). The  
166 mixture was incubated for 2h at 37°C and then centrifuged (2min, 12 000g). Ammonium was  
167 revealed in microplates using an adapted Mulvaney method (1996) [28]: to 30µL of the supernatant  
168 were added 15µL of EDTA solution, 60µL of Na-salicylate solution and 30µL of hypochlorite  
169 solution. After stabilization (45 min), mixture absorption was measured at 667 nm. Enzyme  
170 activities were expressed as µmoles of reaction products released per minute (U) per gram of dry

171 soil ( $\text{U.g}^{-1}\text{DS}$ ). Another 10 g of soil was incubated for 30 days at 25 °C and maintained at initial  
172 moisture and the selective electrodes (Fisher BioBlock Scientific, Hampton, United-States) were  
173 used to determine net ammonification and nitrification rates. Ammonification and nitrification  
174 rates are here defined as the difference in concentrations of ammonium and nitrate respectively  
175 before and after incubation.

### 176 *Geoclimate and vegetation variables*

177 Mediterranean forests grow on a wide variety of sites defined by different geoclimatic  
178 properties that include climate (e.g., precipitation and temperatures) and physiographic  
179 characteristics (e.g., slope, distance to the sea, and elevation), here referred as geoclimate. Climatic  
180 data were collected from the WorldClim BioClim dataset (0.93km x 0.93km). The 19 collected  
181 variables (listed in supplementary Methods, Appendix 1, Table S1) represent temperature and  
182 precipitation annual trends, seasonality, and extreme or limiting environmental factors and results  
183 from interpolations of observed data representative of 1950-2000 [16]. Geographic data (Altitude,  
184 Exposure, Slope, Distance to the sea, Latitude and Longitude) were obtained from the National  
185 Institute of Geography BD ALTI® database (1mx1m). Values were extracted from each site  
186 locations using QGIS software (QGIS Development Team, 2.8 ‘Wien’ 2015).

187 Vegetation cover surveys were based on species identification and overlapped among the  
188 different layers that were further analysed using an abundance community matrix (Braun-Blanquet  
189 approach [29]). The species composition was determined at each site for tree, shrub and herb  
190 layers. Vegetation measurements included the plant species richness, the angiosperm and  
191 gymnosperm abundances and their relative abundance, the vegetation evenness, the Shannon  
192 diversity indexes of the whole community, and of the herb, shrub and non-tree layers.

193 *Statistical analyses*

194           The different variables and matrix considered were summarized in Table S1 (Appendix 1).  
195 Two sets of microbiological data were obtained, and were a matrix of the catabolic potential based  
196 on the EEA and SIR analyses and a matrix of C source utilisation based on CLPP. Three  
197 environmental datasets (i.e., soil, geoclimate [hereafter geoclimate variables] and vegetation were  
198 constituted respectively from 12 soil variables, 25 geoclimate variables, and from the vegetation  
199 cover. Dissimilarity matrix were calculated based on Euclidian distances for geoclimate, soil,  
200 CLPP and EEA datasets and Manhattan distance for the vegetation cover.

201           Permutational analysis of variance (PERMANOVA), implemented in the ‘adonis’ function  
202 (‘vegan’ package [30]), were used to test for significant effects of the factorial scales and their  
203 interaction on the environmental components (distance matrixes).

204           The effects of factorial scales and their interaction on each microbial and soil variable were  
205 also addressed using analysis of variance (ANOVA) on linear models. Shapiro and Levene tests  
206 were respectively used to assess the normality and equality of variances. Variables that did not fit  
207 linear model requirements were transformed using the ‘bestNormalize’ function (‘bestNormalize’  
208 package, Peterson, 2018). Used transformations are listed in the Table S1 (Appendix 1).

209           Variation partitioning was completed to rank the influence of environmental components  
210 on microbial functioning, their variations were examined as follows. First, non-metric  
211 multidimensional scaling (NMDS), which were best designed to represent the ordering  
212 relationships among objects in a small and specified number of axes [31], were used for variable  
213 reduction [33] of each distance matrixes and the 5 first axis were subsequently used. Then,  
214 variations in microbial functioning were partitioned into the pure effects of geo-climatic, soil and

215 vegetation components (i.e., variation of the model explained independently by each factor) and  
216 into their interactions (i.e., variation of the model explained simultaneously by two or three factors)  
217 using constrained and un-constrained distance-based redundancy analysis (db-RDA, function  
218 ‘varpart’, vegan package [30]). The significance of the testable fractions was determined by  
219 ANOVA of the partial db-RDA test using 999 permutations and results were represented by Venn  
220 diagrams.

221 Unconstrained RDA were then performed on models that considered a subset of the  
222 environmental variables. We first reduced the number of explanatory variables based on a variance  
223 inflation factor (VIF) analysis to account for multicollinearity among covariates [34]. Variance  
224 inflation factor values were calculated for a full model then for a reduced model obtained by  
225 sequentially deleting each variable for which the VIF was the highest until all remaining VIFs  
226 were below 2 [35]. Significance of covariates was tested by ANOVA using 999 permutations.

227 Finally, we observed relationships linking microbial variables to environmental variables  
228 by determining the Spearman correlation coefficient ( $\rho$ ).

229 All statistical analyses were performed using R version 3.5.0. To account for multiple  
230 comparisons Benjamini and Hochberg false discovery rate corrections were applied when  
231 required [36].

## 232 **Results**

### 233 *Relevance of bioclimate, slope exposure and forest stand in shaping soil functioning patterns*

234 First, PERMANOVA were performed to test the effects of the different factorial scales and their  
235 interaction on both the microbiological and environmental components (Fig. 2 and Table S1 in

236 Appendix 2). Bioclimates explained a low but significant part of variation in both C source  
237 utilisation ( $F=2.28$ ,  $r^2=0.04$ ,  $p=0.006$ ) and in catabolic potential ( $F=4.15$ ,  $r^2=0.06$ ,  $p=0.005$ ).  
238 PERMANOVA also revealed modification in enzymatic rates induced by forest stands ( $F=2.503$ ,  
239  $r^2=0.08$ ,  $p=0.011$ ). As expected, variation in geoclimate and vegetation cover were well described  
240 both across bioclimates ( $F=124.88$ ,  $r^2=0.66$ ,  $p=0.001$  and  $F=42.831$ ,  $r^2=0.37$ ,  $p=0.001$ ,  
241 respectively) and the interaction between bioclimate and forest stands ( $F=3.75$ ,  $r^2=0.19$ ,  $p=0.020$   
242 and  $F=4.11$ ,  $r^2=0.07$ ,  $p=0.001$ , respectively). Both bioclimate and forest stand defined pertinent  
243 scales that could be used to disentangle variations in soil microbial functioning. Bioclimate was  
244 the main factor driving microbial functioning. In contrast, variation due to slope exposure was  
245 imperceptible on soil, vegetation cover or microbial patterns. It is worth mentioning here that we  
246 observed differences in both mean annual temperatures and annual precipitation between north-  
247 and south-exposed sites in both meso- and supra-Mediterranean contexts (Appendix 2, Fig. S1),  
248 but did not capture the expected lower temperatures on north-facing slopes in the supra-  
249 Mediterranean context. Additionally, PERMANOVA revealed that soil properties were not  
250 structured through any of the considered scales, suggesting that soil physico-chemical  
251 characteristics were likely to be driven at larger or smaller scales.

252 Bioclimates had a substantial effect on both soil physico-chemical and microbial characteristics  
253 (Fig. 3 and Table S2 in Appendix 2). Total N ( $F=21.893$ ,  $p_{\text{adj}}<0.001$ ) and organic C content  
254 ( $F=27.761$ ,  $p_{\text{adj}}<0.001$ , Fig. 3h) indicated a greater quantity of OM in soils from meso- than from  
255 supra-Mediterranean forests together with a higher water holding capacity of the soils ( $F=11.513$ ,  
256  $p_{\text{adj}}=0.008$ , Fig. 3g). On the other hand, certain characteristics of soil linked with the inorganic  
257 fraction, such as calcium carbonate and pH, did not vary between bioclimates. No differences in  
258 OM aromaticity (AR) were observed between the two bioclimates, while a higher amount of

259 organic acids with long alkyl-chains ( $HR_1$ ,  $F=8.725$ ,  $p_{adj}=0.0019$ , Fig. 3i) was found in supra-  
260 Mediterranean forest soils. This suggested a higher humification rate associated with production  
261 of organic acids with short-alkyl chains ( $F=16.104$ ,  $p_{adj}=0.001$ , Fig. 3j) in meso-Mediterranean  
262 soils.

263 None of the enzyme potential or C source preferences was differently expressed between the two  
264 bioclimates although the  $H'$  index calculated from CLPP indicated slightly but not significantly  
265 higher functional diversity in meso-Mediterranean soils ( $F=5.160$ ,  $p=0.028$ ,  $p_{adj}=ns$ ). Relating  
266 microbial-C to organic-C underlined a higher biomass content per unit of OM in supra-  
267 Mediterranean soils ( $F=23.517$ ,  $p_{adj}<0.001$ , Fig. 3l). The catabolic quotient  $qCO_2$  was also higher  
268 in the meso-Mediterranean context ( $F=20.499$ ,  $p_{adj}<0.001$ , Fig. 3k).

269 Both slope exposure and forest stand had substantial influences on microbial parameters and soil  
270 characteristics. Cellulase activity was higher on south-facing slopes ( $F=14.132$ ,  $p_{adj}=0.011$ , Fig.  
271 3f), where we observed slightly higher carboxyl C and WHC (respectively,  $F=7.002$ ,  $p=0.011$ ,  
272  $p_{adj}=ns$  and  $F=5.061$ ,  $p=0.029$ ,  $p_{adj}=ns$ ). The influence of forest stand was expressed on  
273 measurements of OM recalcitrance, i.e N content and C/N ratio, aromatic C and AR ratio, and  
274 tyrosinase activity. Total N content was higher in soils of oak than pinewoods and mixed stands  
275 ( $F=20.872$ ,  $p_{adj}=0.006$ , Fig. 3a), which resulted in the opposite C/N trend ( $F=20.368$ ,  $p_{adj}<0.001$ ,  
276 Fig. 3c). Aromatic content and Ar ratio ( $F=8.061$ ,  $p_{adj}=0.006$ , Fig. 3d) were higher in pinewoods  
277 ( $F=9.557$ ,  $p_{adj}=0.004$ ). *Pinus* forests soils, characterized by lower N and higher pH ( $F=6.333$ ,  
278  $p_{adj}=0.020$ , Fig. 3e), were observed together with a weaker tyrosinase activity ( $F=8.141$ ,  
279  $p_{adj}=0.006$ , Fig. 3b).

## 280 *Variation partitioning of soil microbial functioning*

281 Because microbial patterns varied between the two bioclimates, variance partitioning analyses  
282 were carried out at both regional and sub-regional scales to determine the effects of geoclimate,  
283 soil and vegetation on both C source utilisation and catabolic potential patterns (Fig. 4 and Table  
284 S3, Appendix 2).

285 Overall explanation by environmental components reached *ca.* 30% and 45% of the C source  
286 utilisation (Fig. 4a) and of catabolic potential respectively (Fig. 4d) at the regional scale, and  
287 increased to *ca.* 50% (Fig.4b, 4c) and 65% (Fig. 4e, 4f) at landscape and local scales respectively.  
288 Carbon source utilisation patterns were mainly and significantly influenced by vegetation cover  
289 ( $F=1.352$ ,  $r^2=0.11$ ,  $p=0.030$ , Fig. 4a) at the regional scale, but no significant influences of the three  
290 components were observed at sub-regional meso- and supra-Mediterranean scales (Fig. 4b, 4c).  
291 Soil physico-chemical prints explained a large part of variation in microbial catabolic potential at  
292 both regional (shared effect:  $F=5.111$ ,  $r^2=0.32$ ,  $p=0.001$ ; pure effect:  $F=3.929$ ,  $r^2=0.243$ ,  $p=0.001$ ,  
293 Fig. 4d) and sub-regional scales. In meso-Mediterranean forest, variation in catabolic potential was  
294 due to the shared effect of soil properties ( $F=3.288$ ,  $r^2=0.406$ ,  $p=0.001$ , Fig. 4e) encompassing 16%  
295 of vegetation-soil common explanation. In supra-Mediterranean forests, both soil (shared effect:  
296  $F=2.747$ ,  $r^2=0.364$ ,  $p=0.003$ ; pure effect:  $F=2.376$ ,  $r^2=0.0255$ ,  $p=0.004$ , Fig. 4f) and vegetation  
297 (pure effect:  $F=1.902$ ,  $r^2=0.204$ ,  $p=0.029$ , Fig. 4f) explained variation in catabolic potential.

### 298 *Driving forces of soil microbial functioning*

299 To disentangle effects of specific drivers amongst the edaphic, geoclimate and vegetation  
300 components, we tested the influence of the main environmental covariates on soil microbiological  
301 patterns. Collinear factors were first removed sequentially based on their VIF scores. Remaining  
302 covariates included site exposition, slope, longitude, mean temperature of the wettest quarter,

303 CaCO<sub>3</sub>, total N, carboxyl C, O-alkyl C, nitrate, ammonium, bulk density, and vegetation richness  
304 (Table S4, Appendix 2). Only edaphic parameters stand out as driving forces of microbial  
305 functioning patterns. Carbon source utilisation was related to total N content at regional scale  
306 (F=1.825, p=0.019), and to soil bulk density in supra-Mediterranean forests (F=1.698, p=0.035).  
307 Catabolic potential was structured by CaCO<sub>3</sub> (F=9.49, p=0.001), total N (F=7.389, p=0.001),  
308 nitrate (F=5.320, p=0.002) and ammonium (F=3.157, p=0.009) content at regional scale, and by  
309 CaCO<sub>3</sub> content (F=5.780, p=0.001) in meso-Mediterranean context.

310 To further describe underlying mechanisms of microbial functioning, we examined relationships  
311 linking microbial assemblages to environmental parameters (Fig. 5 and supplementary Fig. 2 in  
312 Appendix 2). Carbon source preferences and associated functional diversity index were unrelated  
313 to environmental variables. Five edaphic parameters were favoring soil microbial properties: the  
314 BR, *q*CO<sub>2</sub>, urease, tyrosinase, and cellulase increased with soil WHC; BR, MB and urease activity  
315 increased with the soil alkyl C fraction; BR and *q*CO<sub>2</sub> increased with soil organic C content, MB  
316 and MB per unit of organic C increased with HR1. Soil pH and CaCO<sub>3</sub> both negatively affected  
317 BR, MB and tyrosinase activity. The metabolic quotient (*q*CO<sub>2</sub>) was particularly sensitive to  
318 several climate parameters as it was upregulated by temperature indexes (MTWaQ, min TCM,  
319 MTDQ, MTCQ, annual MT, max MTWaM) and downregulated by precipitation indexes (annual  
320 P, PWaQ, PDQ and PDM). Interestingly, tyrosinase activity was restrained by gymnosperm total  
321 and relative abundances together with plant species richness. Although vegetation has been  
322 previously shown to influence overall soil microbial patterns, vegetation diversity indexes were  
323 not directly related to any specific microbial markers, suggesting that forest community  
324 composition rather than forest community structure plays an important role in microbial  
325 functioning.



## 326 **Discussion**

### 327 *Multiple scaling of soil microbial functioning*

328           Comparison between C utilisation and microbial catabolic potential patterns highlighted  
329 different structuration scales (i.e., a strong influence of bioclimates on C source utilization vs. a  
330 finer effect of forest stands on catabolic potentials), and testified to scale-dependent variations in  
331 microbial functioning. While limitations in the BIOLOG methodology for the characterization of  
332 whole communities are well known, CLPP remain a useful tool to detect culturable copiotrophic  
333 bacteria living in forest soil [37]. Fungi have broader enzymatic capabilities [38] and potentially  
334 greater C use efficiency [39] than bacteria. Shift in the respective functional capabilities of fungi  
335 and bacteria could partly explain the observed patterns (Fig. 2d and 2e), with substantial  
336 implications relevant to C and N cycling as these taxa own distinct C use efficiencies [40].

337           Variation in C source utilisation was detected between the two bioclimates considered, and  
338 was mainly explained by vegetation cover (Fig. 4). However, C use patterns did not differ between  
339 forest stands and no substrate preference has been related with descriptor of vegetation (Fig. 5).  
340 This suggested that some plant species associated with bioclimates influenced C-substrate affinity  
341 over the dominant tree species considered here. Previous studies in grasslands showed  
342 modification in soil microbial communities in response to plant community composition [41, 42]  
343 but fewer considered treed ecosystems [43, 44], and to our knowledge, only one considered CLPP  
344 profiles. In this study [45], both tree diversity and identity affected CLPP. Tree species and even  
345 tree genotypes have been previously shown to influence litter chemistry and to control C and  
346 nutrient dynamics [46, 47]. Although not further tested here, it is likely that the observed  
347 modifications in CLPP reveal a shift in community composition of copiotrophic bacteria [37], that  
348 preferentially consume the labile pool of organic C (e.g., freshly fallen litter) [48]. The main driver

349 of C use patterns was the total N content, which has previously been revealed as a driver of  
350 microbial functional and taxonomic diversity. For instance, Fierer et al. [10] observed shifts in C  
351 use utilisation across N gradients together with bacterial phylogenetic and metagenomic responses.  
352 Moreover, in our study, functional diversity decreased with soil pH, which has often been pointed  
353 out as an important driver of bacterial diversity [49] and of functional diversity approached *via*  
354 CLPP [50].

355         Unlike C source utilisation, catabolic potential patterns were shaped by forest stands  
356 beyond the influence of bioclimates, and more strongly relied on N-related markers (i.e., nitrate,  
357 ammonium and total N content). This is likely because decomposer N-requirements are not  
358 completely fulfilled by N plant litter concentration [51]. Nitrogen has to be immobilized by  
359 decomposers from their resources until the elements reach a critical threshold that allows N  
360 conversion into microbial biomass or production of EEA [52]. Nitrogen content significantly  
361 changed across the six forest stands considered in our study with particularly exacerbated  
362 differences between oak and pine forests. Such differences in OM quality between these  
363 Mediterranean tree species have been previously shown together with significant consequences in  
364 the resulting soil organic C stocks [53]. These results together indicate that the functional  
365 composition of microbial communities (estimated through the C uses) is shaped at larger scales  
366 than their actual catabolic potential, which relies on marker of OM quality defined at the forest  
367 stand scale.

### 368 *Differential microbial functioning across bioclimates*

369 It is a central principle of biogeography that climate exerts a dominant control over the natural  
370 distribution of species [54]. In our study, we revealed that the vegetation stage was not a suitable

371 predictor of soil chemical properties. According to Pons and Quézel (1998) [55], the meso-  
372 Mediterranean bioclimate corresponds to a warm bioclimate with drought waves, while supra-  
373 Mediterranean winters are harsher and summer more temperate. In the Mediterranean context,  
374 vegetation has acquired morphological traits to face the deficit of water, e.g. slow growth, thick  
375 cuticle, allelopathic compounds and low nutrient content [56]. These particular climate conditions,  
376 exacerbated in meso-Mediterranean forests, select sclerophyllous vegetal species (such as *Quercus*  
377 *ilex*, *Quercus coccifera* etc.) that we expected to detect in soil physico-chemical prints. We did not  
378 detect such general pattern. However, a higher carboxyl C contents in meso-Mediterranean soils  
379 could relate to a larger sclerophyllous plant community in meso-Mediterranean forests (as  
380 carboxyl C reflects the cutin polymer of leaves cuticle [57]).

381 Meso-Mediterranean soils were characterized by higher organic C and WHC. Water  
382 holding capacity in soil relies on its OM content [58]. Differences in C content can be driven by  
383 higher C inputs (i.e., primary production) and/or by lower microbial mineralization rates. Calcium  
384 carbonate content had a strong influence in shaping microbial catabolic potential, even more  
385 pronounced in meso-Mediterranean soils. Exchangeable calcium positively correlates with SOC  
386 concentration and its resistance to microbial degradation [59]. This is in line with our results that  
387 point out higher organic C in meso-Mediterranean soils where the CaCO<sub>3</sub> content had higher  
388 influence on the microbial catabolic potential. As water molecules prevent the direct approach or  
389 sorption of an organic substance to a mineral's surface [59], lower precipitation in meso-  
390 Mediterranean forests might have increase an inhibitor effect of CaCO<sub>3</sub> on microbial catabolic  
391 potential.

392 Slightly higher functional microbial diversity under recalcitrant vegetation and more arid  
393 conditions from meso-Mediterranean could suggest a shift of functional communities [60] since

394 harsher conditions tend to select resistant and specialist populations corresponding to an  
395 oligotrophic lifestyle [61]. Tardy et al. [62] showed that a higher microbial diversity improved the  
396 functional stability of microbial communities after heat stresses, which are common in the meso-  
397 Mediterranean bioclimate. We also found respectively a small and a significant increase in  
398 microbial basal respiration and in  $q\text{CO}_2$  of meso-Mediterranean forest soils that suggests a better  
399 potential for mineralization in meso-Mediterranean when water is available (60 % WHC). Thus,  
400 in meso-Mediterranean forest soils, when hydric conditions are suitable, the mineralization  
401 potential is likely to be high and to lead to  $\text{CO}_2$  production (for instance in spring and fall), while  
402 during summer drought, OM decomposition is probably drastically inhibited. This suggests that  
403 the observed higher C contents in the meso-Mediterranean context then result from climate  
404 constraints.

#### 405 *Detection of variations in microbial functioning at smaller scales*

406 We found that soil chemical and microbial characteristics (total N, aromatic C and  
407 tyrosinase activity) related to OM recalcitrance varied among forest stands (Fig. 3 a-e). In a  
408 previous study, Brunel et al. [53] investigated the effect of relative oak/pine tree composition using  
409 the same tree species and bioclimates and determined that relative aromatic content increased  
410 linearly with pine abundance. Recalcitrant OM, mainly aromatic compounds, limits microbial  
411 growth by toxic effects on microbial cells (for instance on membranes), limiting bioavailability of  
412 nutrients, and can also bind to microbial enzymes and inhibit their activities [63, 64]. As soil N is  
413 tightly bound to recalcitrant humic acids, a limited availability and/or quantity of N under  
414 pinewood is likely to enhance aromatic C accumulation in pine stands [65].

415 Side slope exposure has previously been reported to affect forest soils directly through its  
416 influence on radiation, temperature and moisture [66], and indirectly by affecting vegetation cover  
417 and structures [67]. Variations in geoclimate were not perceptible between south- and north facing  
418 slopes. This can point out two mutually non-exclusive scenarios: (i) that the used climate data are  
419 unsuitable to unravel differences at the slope scale (pixel of 0.86 km<sup>2</sup> for the BioClim modeled  
420 variables [16]), and/or (ii) that microclimatic variations between sites were consistently larger than  
421 the variations caused by differences induced by slope exposure. We detected higher cellulase  
422 activities in soils of south-exposed forests, and slight modifications in carboxyl C content. In  
423 contrast with our results, differences in vegetation cover between north- and south-facing slopes  
424 have been previously reported in Mediterranean areas; overall vegetation cover and species  
425 richness were shown to be reduced on north-facing slopes and a greater number of evergreen  
426 species have been observed on south slopes [68, 69]. In their study on forest soils, Måren et al [70]  
427 showed that, in arid environments, slope aspect is less important in driving soil properties than in  
428 moister environments where more microclimatic contrasts are observed.

#### 429 *Environmental drivers and their potential implications in the context of climate change*

430 While Mediterranean forests have the potential to strongly contribute to global C sink [71],  
431 their SOC stocks are known to be lower than other forest types. This pattern is commonly  
432 explained by factors such as landuse legacy (abandoned pastures) and fire frequency, and also by  
433 the low productivity of Mediterranean tree species [72]. Here we also observed that soil microbial  
434 functioning is largely explained by climate parameters. We reported above that C stocks in meso-  
435 Mediterranean forest soils resulted from climate constraints. Two other observations tend to reveal  
436 that soil microbial functioning in Mediterranean forest soils is controlled by climate variation.  
437 First, qCO<sub>2</sub>, which indicates the efficiency by which soil microorganisms use C-resources in soil

438 [73], was strongly associated to climate variation (up-regulated by 6 indexes of temperature and  
439 down-regulated by 5 indexes of precipitation). Then, 5 microbial markers (i.e., basal respiration,  
440 metabolic quotient, urease, tyrosinase, cellulase activities and ammonium mineralization rate)  
441 were strongly related to the water holding capacity, which reflects the water-balance fluctuations  
442 in soils. As in the Mediterranean region, average annual temperatures are well above current global  
443 warming trends [74], our results suggest that soil microbial functioning and resulting C dynamics  
444 will be drastically affected in the coming years. Our results support recent findings of Diamond et  
445 al. [75] who reveal that climate change can have a direct impact on the relative abundance and  
446 metabolic capacities of microorganisms in Mediterranean soil ecosystems, with potentially  
447 important impacts for both soil C storage and gas release.

#### 448 **Conclusion**

449 The use of a multi-scalar sampling design allowed us to show the prevailing influence of  
450 bioclimates and forest stands in controlling soil functioning over slope exposure and provide  
451 insights into the underlying mechanisms. Because climate is responsible for vegetation type, which  
452 in turn provides different soil C substrates that are metabolized under different conditions (e.g.,  
453 temperature or moisture), the bioclimate effect *per se* is hard to disentangle. However, by  
454 describing variations associated with the bioclimate in soil properties, climate and vegetation  
455 cover, this study provides clues on which facet of the bioclimatic context (i.e., geo-climate, soil or  
456 vegetation) are the most structuring. We found that vegetation cover better explained variations in  
457 C source utilization, whereas edaphic characteristics better explained variations in catabolic  
458 potential of microbial communities. These results, therefore, contribute to a better understanding  
459 of the environmental drivers of microbial functioning across the French Mediterranean landscape.  
460 Further studies focusing on soil metabarcoding and transcriptomic would be required to shed light

461 on the environmental filters of microbial composition in relation to their expressed metabolism,  
462 and on the drivers of bacterial and fungal communities respectively.

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469

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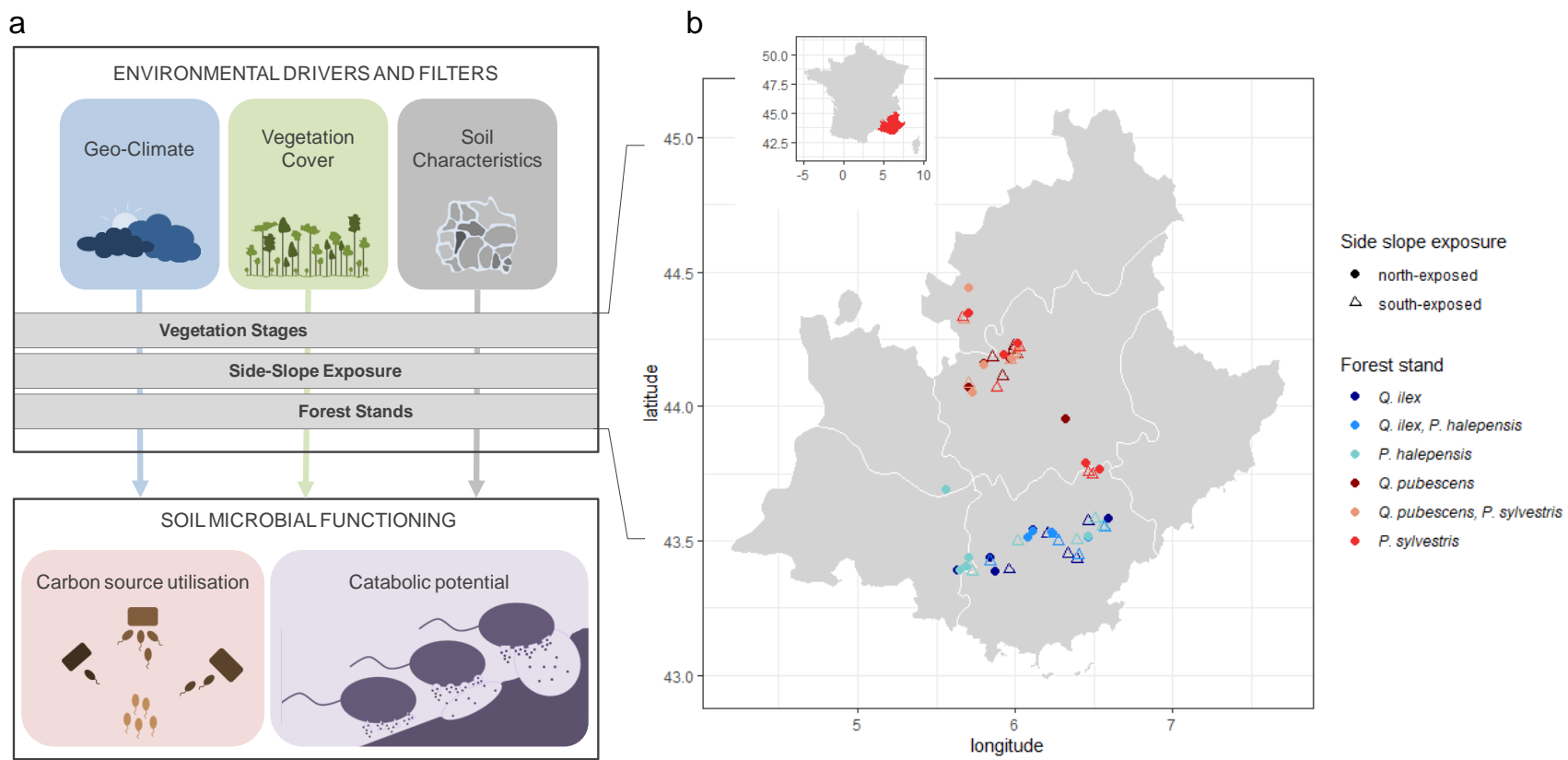
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668 **Figures**

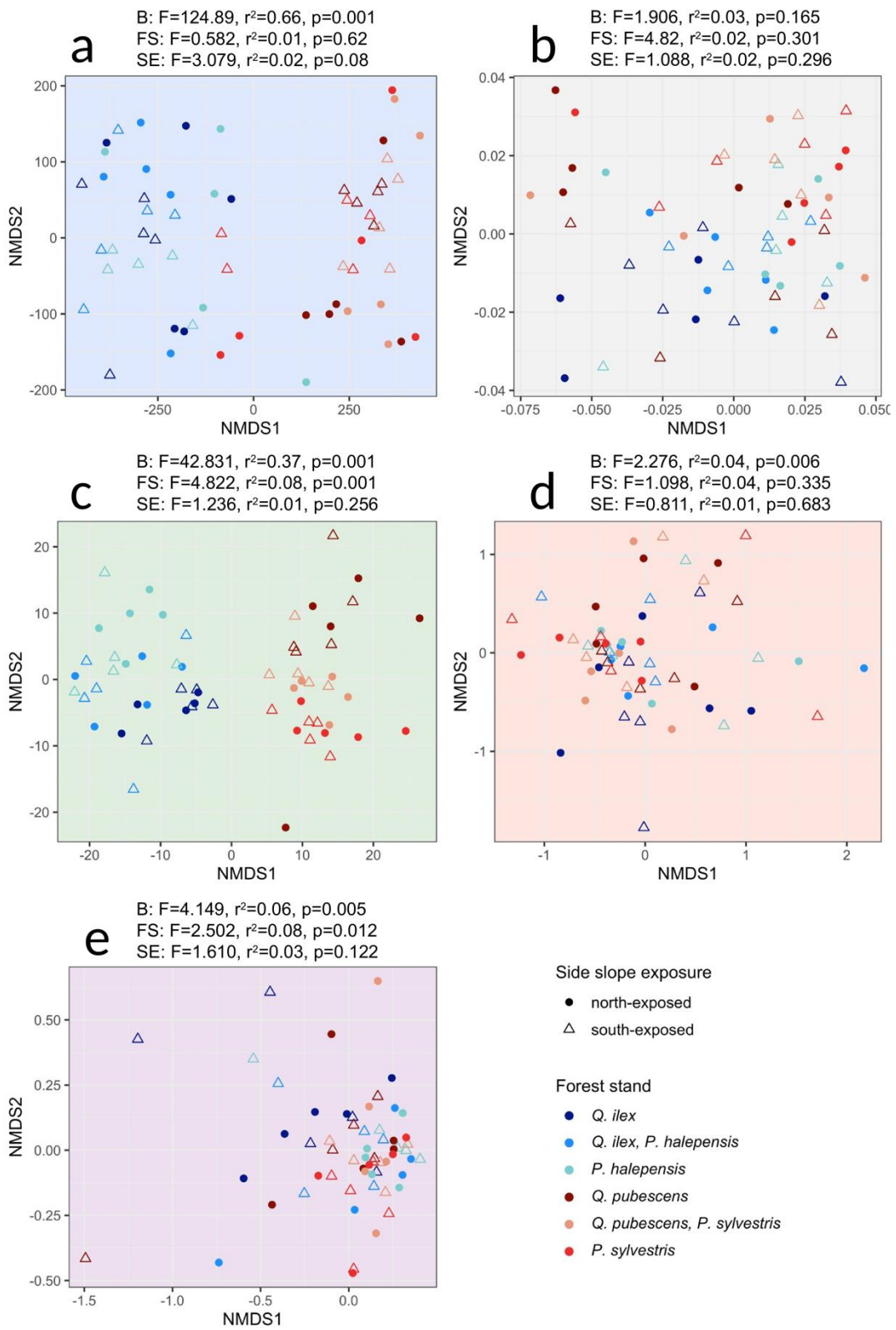
669 **Fig. 1** | The overview of experimental design; (a) the schematic representation of the study design. The variables assessed and matrix used to describe  
670 geoclimate, vegetation cover, soil characteristics, carbon source utilisation and catabolic potential are fully detailed in the supplementary methods  
671 (Appendix 1, Table S1) ; (b) Sampling sites map. The colors of the symbols ( $n=60$ ) refer to the different forest stands: red and blue tones correspond  
672 to meso- and supra-Mediterranean bioclimates, respectively; filled and open symbols correspond to north- and south-exposed plots, respectively.

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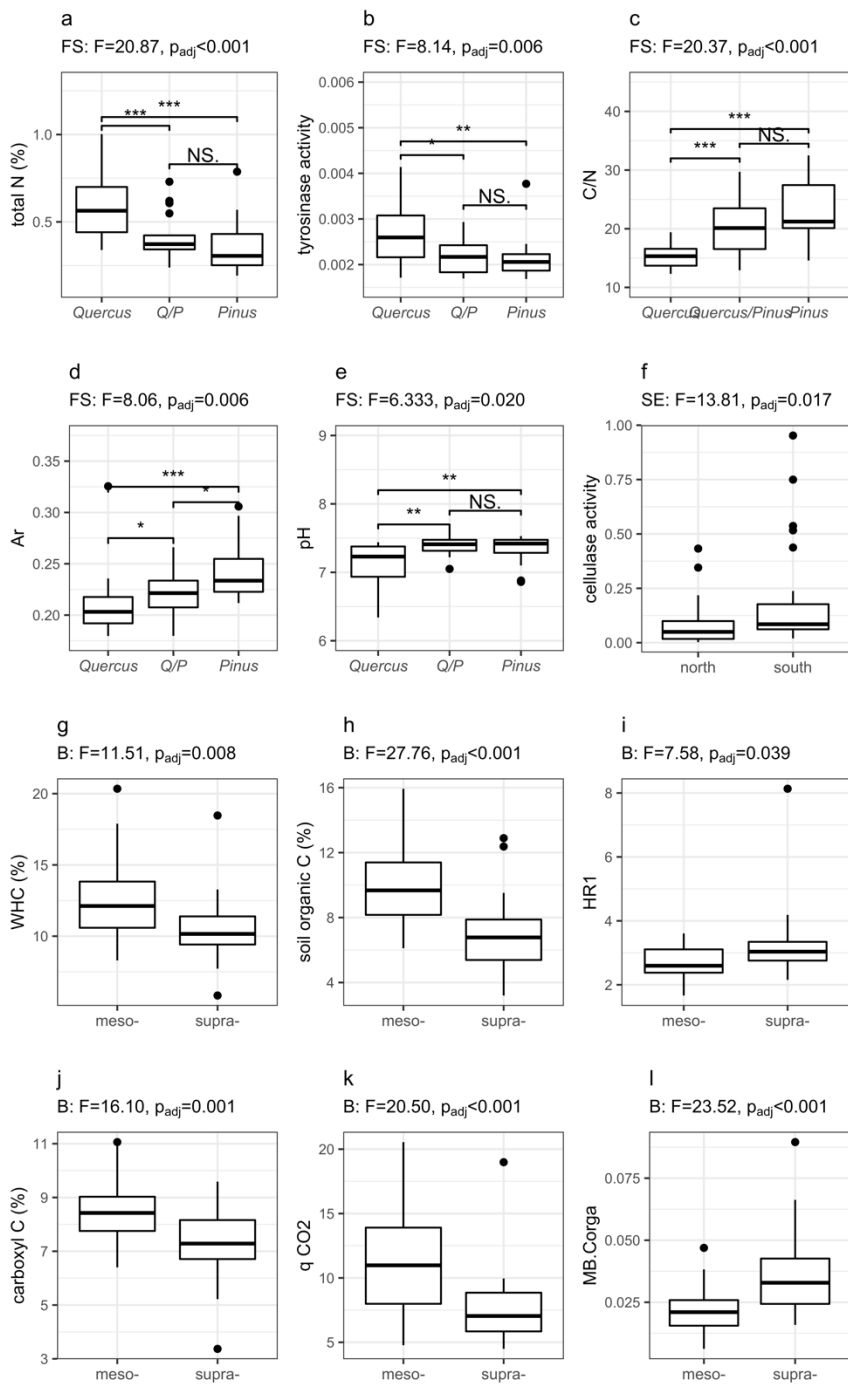
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675 **Fig. 2** | Nonmetric multi-dimensional scaling (NMDS) of (a) geoclimate, (b) soil, (c) vegetation, (d) carbon source  
676 utilisation, and (e) microbial catabolic potential patterns based on Euclidian (a, b, d, e) and Manhattan (c)  
677 dissimilarity distances. The colors of the symbols refer to the different forest stands: red and blue tones correspond  
678 to meso- and supra-Mediterranean bioclimates, respectively; filled and open symbols correspond to north- and  
679 south-exposed plots, respectively. PERMANOVA results of main factors (B for bioclimate, FS for forest stand  
680 and SE for slope exposure) are provided above each panel. Interaction effects (all not significant) are provided in  
681 Appendix 2, Table S1.



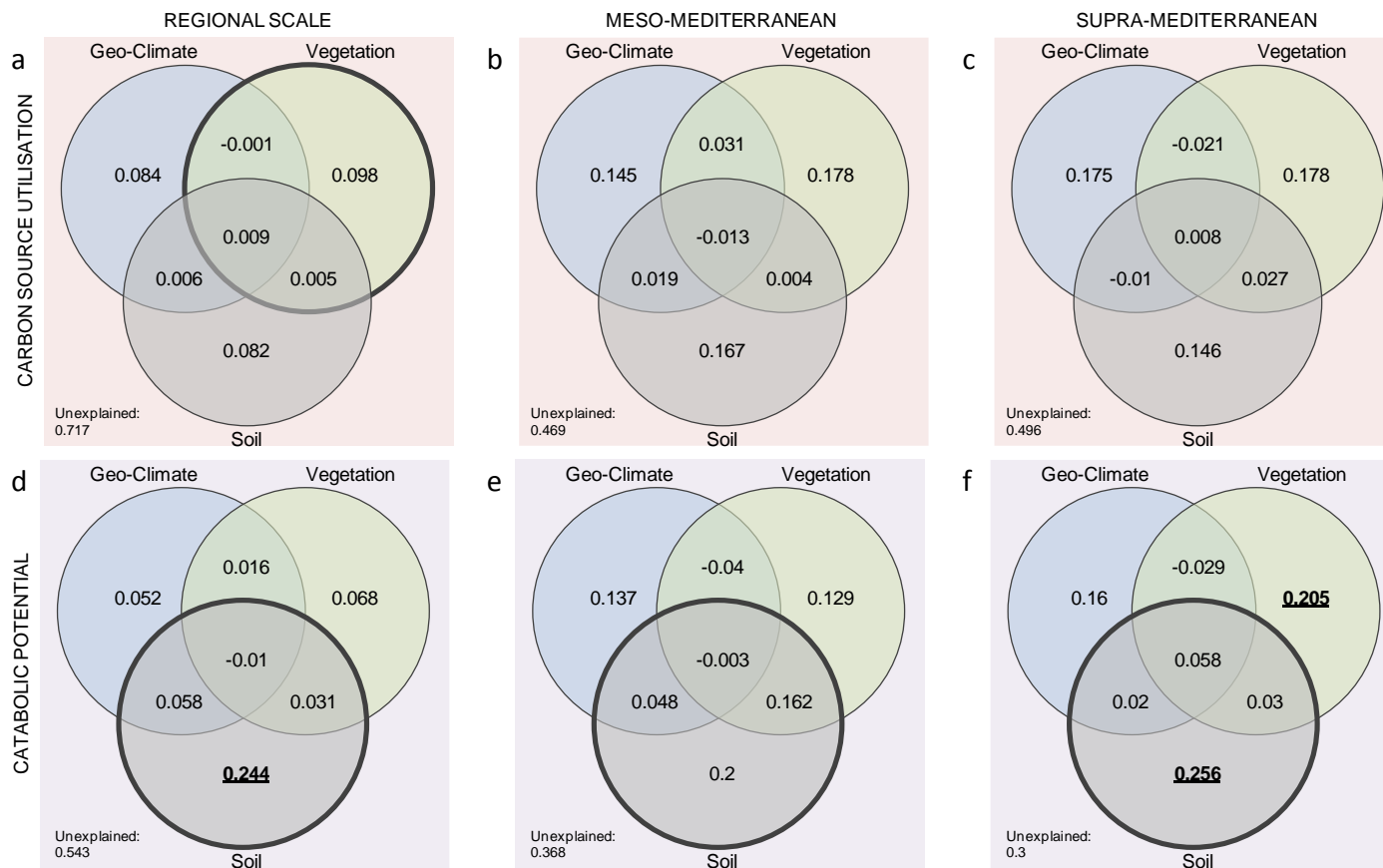


683 **Fig. 3** | Shaping of soil biological and chemical characteristics across the considered factorial scales (B for  
 684 bioclimate, FS for forest stand and SE for slope exposure). Panel a, b, c, d and e respectively stand for the total  
 685 N, the microbial tyrosinase activities, the C/N ratio, the aromaticity ratio and pH as affected by forest stands  
 686 (Q/P stands for mixed Quercus/Pinus stands). Panel f stands for the microbial the slope exposure effect on  
 687 cellulase activity. Panels g to l respectively stand for the water holding capacity, the soil organic carbon, the  
 688 HR1 ratio, the Carboxyl C fraction, the microbial metabolic quotient and the microbial biomass per unit of  
 689 organic C respectively, as affected by the meso- or supra- mediterranean bioclimates. Only significant effects,  
 690 i.e.,  $p \leq 0.05$  after Benjamini and Hochberg (1995) post-hoc correction, are shown.



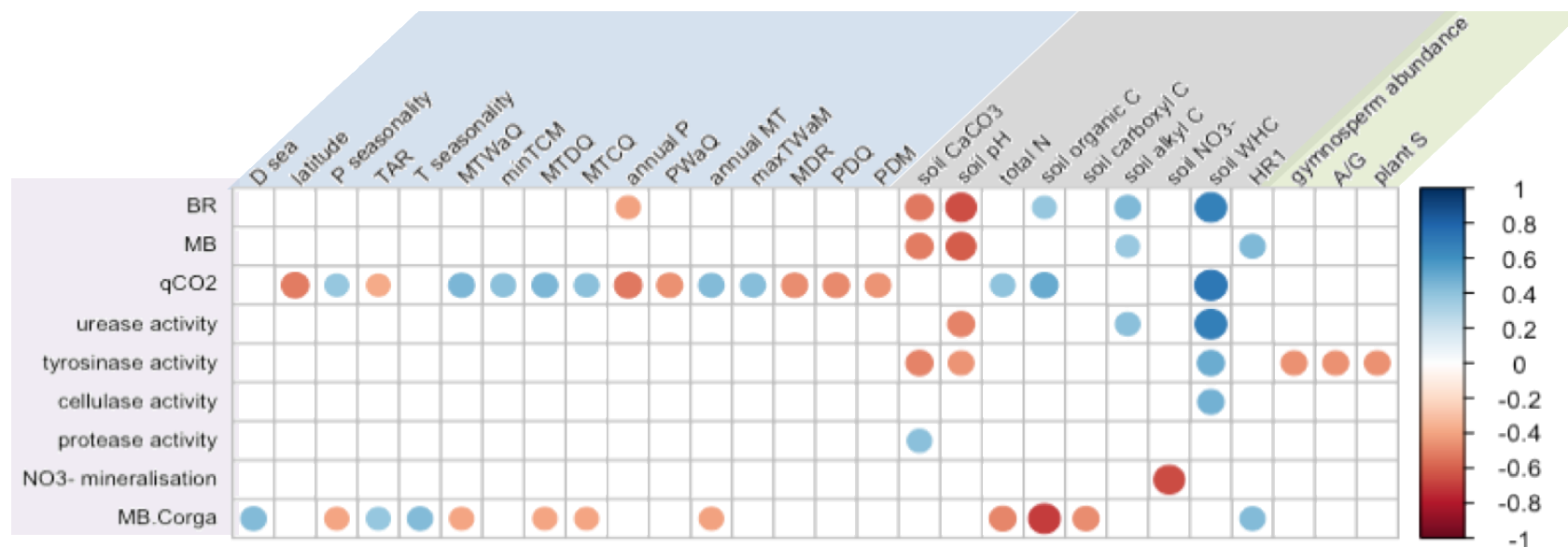
691

692 **Fig. 4** | Variation partitioning of soil microbial carbon source utilisation (a, b and c) and catabolic potential (d, e and f) into soil, vegetation and  
 693 geoclimate components at different spatial scales (a and d at the regional-scale ( $n=60$ ); b and e at the sub-regional meso-Mediterranean scale ( $n=30$ );  
 694 and c and f at the sub-regional supra-Mediterranean scale ( $n=30$ )). Each Venn diagram represents a given biological variation ( $r^2$ ) partitioned into the  
 695 relative effects of each component or combination of components. Pure and shared effects of the different fractions were analyzed by constrained and  
 696 un-constrained db-rda tests respectively, and are reported in the Table S3. Significant component are underlined when implying a pure effect and are  
 697 enclosed by a bold line when implying a whole component.



698

699 **Fig. 5** | Heat map of Spearman correlation coefficient ( $\rho$ ) between microbial and environmental variables. The color ramp refers to  
 700 the Spearman correlation coefficient, blue tones stand for positive relations and red tones for negative relations, and the point size  
 701 refers to the correlation strength ( $|\rho|$ ). Top annotations refer to environmental variables and left annotations to microbial  
 702 variables ( $D_{sea}$ : distance to the sea; P seasonality: precipitation seasonality; TAR: temperature annual range; T seasonality :  
 703 temperature seasonality; MTWaQ: maximum temperature of the warmest quarter; minTCM: minimum temperature of the coldest  
 704 month; MTDQ: mean temperature of the driest quarter ; MTCQ: mean temperature of the coldest quarter; annual P: annual  
 705 precipitation; PWaQ: precipitation of the warmest quarter; annual MT: annual mean temperature; max TWaM: maximum temperature  
 706 of the warmest month; MDR: mean diurnal range; PDQ: precipitation of the driest quarter; PDM: precipitation of the driest month;  
 707 WHC: water holding capacity; HR1: humification ratio 1; A/G: angiosperm to gymnosperm ratio; BR: basal respiration; MB:  
 708 microbial biomass;  $qCO_2$  : metabolic quotient; MB.Corga: microbial biomass per unit of organic carbon). Only variables that show  
 709 significant correlations are shown, i.e.,  $p \leq 0.05$  after Benjamini and Hochberg (1995) post-hoc correction.



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