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1 Research Paper

2 **Title**

3 Environmental drivers of microbial functioning in Mediterranean forest soils

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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

Introduction

Studies of the microbial processes involved in the decomposition of soil organic carbon (C) have considerably increased in recent years owing to the major role soil C storage plays in the context of climate change [1]. In the C sink that forest soils represent [2], soil organic C stocks result largely from the balance between (i) inputs of C determined by the vegetation cover, (ii) processes of soil C humification by microbial communities and stabilization and (iii) outputs *via* soil erosion, CO₂ production from roots and microorganisms [3]. Thus, C dynamics in soils depend on complex interactions between climate conditions (e.g. long term mean annual precipitation and temperature) that shape forest structure and tree productivity, soil physico-chemical properties (such as calcium carbonate content and pH) influenced by the geological substratum and their combined effect on soil microbial communities and their metabolism [4].

Microbes are the primary decomposers of plant material due to their unique ability to produce enzymes to break down both simple molecules such as cellulose and more complex plant derived compounds such as lignin [5]. They mobilize and transform a broad range of soil C substrates, assimilating C in their biomass, emitting CO₂ or stabilizing C in soil [6]. There is a lot of debate on the potential drivers controlling soil microbiome assembly and the mechanisms maintaining community structure and composition, and we argue that these rules may be better understood if put in perspective (adapted to the needs and contexts). Soil pH, the chemical composition of organic matter (OM), and climate conditions have been pointed out as key driver of soil functioning at local to continental scales [7–11]. In a recent study from different geographical and climatic zones across Europe, variations in bacterial community diversity and structure were mainly driven by parameters corresponding to similar edaphic and climatic properties [12].

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

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

Here we report on a study designed to (1) determine pertinent factorial scales for unraveling microbial functional patterns and (2) estimate the relative importance of environmental components (vegetation, geoclimate and soil properties) in shaping of soil microbial functioning and (3) identify specific drivers. To disentangle the patterns and drivers of soil microbial functioning in Mediterranean forest soils, we sampled 60 sites covering an area of about 10.000 km² in south-eastern France. We hypothesized that soil microbial metabolic capabilities and C substrate affinities varied according to bioclimates at a regional scale, side slope exposure at a landscape scale and forest stand at a local scale (Fig. 1) due to environmental variations specific to these scales.

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 CaCO₃ in 10 g soils (dry weight equivalent) was based on the release of CO₂
111 after addition of HCl 4N [18]. The percentage of inorganic C (C in CaCO₃) was calculated as
112 follows: %C-CaCO₃ = 11.991 / 100 x %CaCO₃. 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 NH₄⁺ and
116 NO₃⁻ concentration in soils (expressed per gram of dried soil).

117 Soils C biochemical forms were characterized with solid-state ¹³C-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 ¹³C 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

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

Soil microbial functioning

A full description of the methods used for microbial analyses is provided in the Appendix 1. Briefly, the determination of C source utilisation (i.e., catabolic-level physiological profiling, CLPP) was performed using BIOLOG® Eco plate (BIOLOG Inc., Hayward, CA). Plates with an average well colour development (AWCD) of 0.4 were used in the statistical calculations. Shannon index and the mean growth on carbohydrates, carboxylic and ketonic acids, amines/amides, polymers and amino-acids substrates were then determined. Microbial biomass (MB) was estimated using Substrate-Induced Respiration (SIR). Ten grams (dry weight equivalent) of standardized samples at 60% of WHC were placed in 117 ml flushed-air glass jars and amended with a powder of talc and glucose (1000 $\mu\text{g C g}^{-1}$ soil). After ninety minutes, 1 ml of air was sampled with a syringe and injected into a gas chromatograph (Chrompack CHROM 3 – CP 9001, Middelburg, The Netherlands) to determine CO_2 production. The gas chromatograph was equipped with a thermal conductivity detector and a packed column (Porapak). The carrier gas helium flow was regulated at 60 ml h^{-1} . The CO_2 concentration of flushed air was subtracted from the CO_2 concentration of each sample and resulting values were adjusted to 22°C according to Ideal Gas Laws using $Q_{10} = 2$. Substrate-induced respiration rates were converted into MB using equations given by [22]. Basal respiration was determined without adding glucose and talc powder and was estimated to calculate the metabolic quotient $q\text{CO}_2$ (the ratio of basal respiration to microbial biomass), which is a sensitive ecophysiological indicator of soil stress induced by environmental conditions [23]. The activity of five extracellular enzymes (EEA) involved in soil C and N cycles 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

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

Geoclimate and vegetation variables

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

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

Statistical analyses

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

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

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

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

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

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

Finally, we observed relationships linking microbial variables to environmental variables by determining the Spearman correlation coefficient (ρ).

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

Results

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

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

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

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

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

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

Driving forces of soil microbial functioning

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

303 CaCO₃, 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 CaCO₃ (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 CaCO₃ 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*CO₂, urease, tyrosinase, and cellulase increased with soil WHC; BR, MB and urease activity
315 increased with the soil alkyl C fraction; BR and *q*CO₂ increased with soil organic C content, MB
316 and MB per unit of organic C increased with HR1. Soil pH and CaCO₃ both negatively affected
317 BR, MB and tyrosinase activity. The metabolic quotient (*q*CO₂) 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.

Discussion

Multiple scaling of soil microbial functioning

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

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

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

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

Differential microbial functioning across bioclimate

It is a central principle of biogeography that climate exerts a dominant control over the natural 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_3 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_3 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

harsher conditions tend to select resistant and specialist populations corresponding to an oligotrophic lifestyle [61]. Tardy et al. [62] showed that a higher microbial diversity improved the functional stability of microbial communities after heat stresses, which are common in the meso-Mediterranean bioclimate. We also found respectively a small and a significant increase in microbial basal respiration and in $q\text{CO}_2$ of meso-Mediterranean forest soils that suggests a better potential for mineralization in meso-Mediterranean when water is available (60 % WHC). Thus, in meso-Mediterranean forest soils, when hydric conditions are suitable, the mineralization potential is likely to be high and to lead to CO_2 production (for instance in spring and fall), while during summer drought, OM decomposition is probably drastically inhibited. This suggests that the observed higher C contents in the meso-Mediterranean context then result from climate constraints.

Detection of variations in microbial functioning at smaller scales

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

Side slope exposure has previously been reported to affect forest soils directly through its influence on radiation, temperature and moisture [66], and indirectly by affecting vegetation cover and structures [67]. Variations in geoclimate were not perceptible between south- and north facing slopes. This can point out two mutually non-exclusive scenarios: (i) that the used climate data are unsuitable to unravel differences at the slope scale (pixel of 0.86 km² for the BioClim modeled variables [16]), and/or (ii) that microclimatic variations between sites were consistently larger than the variations caused by differences induced by slope exposure. We detected higher cellulase activities in soils of south-exposed forests, and slight modifications in carboxyl C content. In contrast with our results, differences in vegetation cover between north- and south-facing slopes have been previously reported in Mediterranean areas; overall vegetation cover and species richness were shown to be reduced on north-facing slopes and a greater number of evergreen species have been observed on south slopes [68, 69]. In their study on forest soils, Måren et al [70] showed that, in arid environments, slope aspect is less important in driving soil properties than in moister environments where more microclimatic contrasts are observed.

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

While Mediterranean forests have the potential to strongly contribute to global C sink [71], their SOC stocks are known to be lower than other forest types. This pattern is commonly explained by factors such as landuse legacy (abandoned pastures) and fire frequency, and also by the low productivity of Mediterranean tree species [72]. Here we also observed that soil microbial functioning is largely explained by climate parameters. We reported above that C stocks in meso-Mediterranean forest soils resulted from climate constraints. Two other observations tend to reveal that soil microbial functioning in Mediterranean forest soils is controlled by climate variation. First, qCO₂, which indicates the efficiency by which soil microorganisms use C-resources in soil

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

Conclusion

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

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

Acknowledgments

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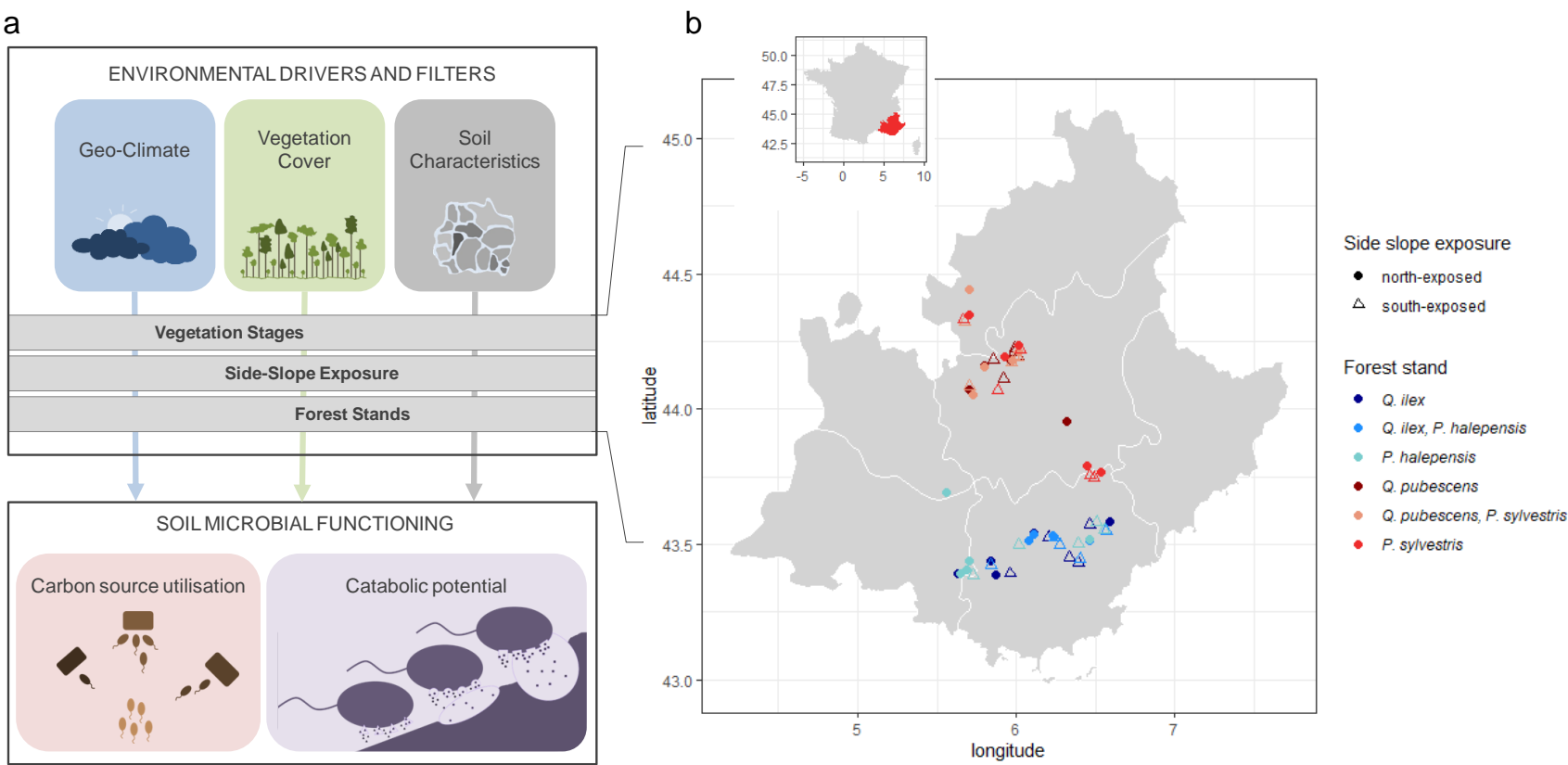
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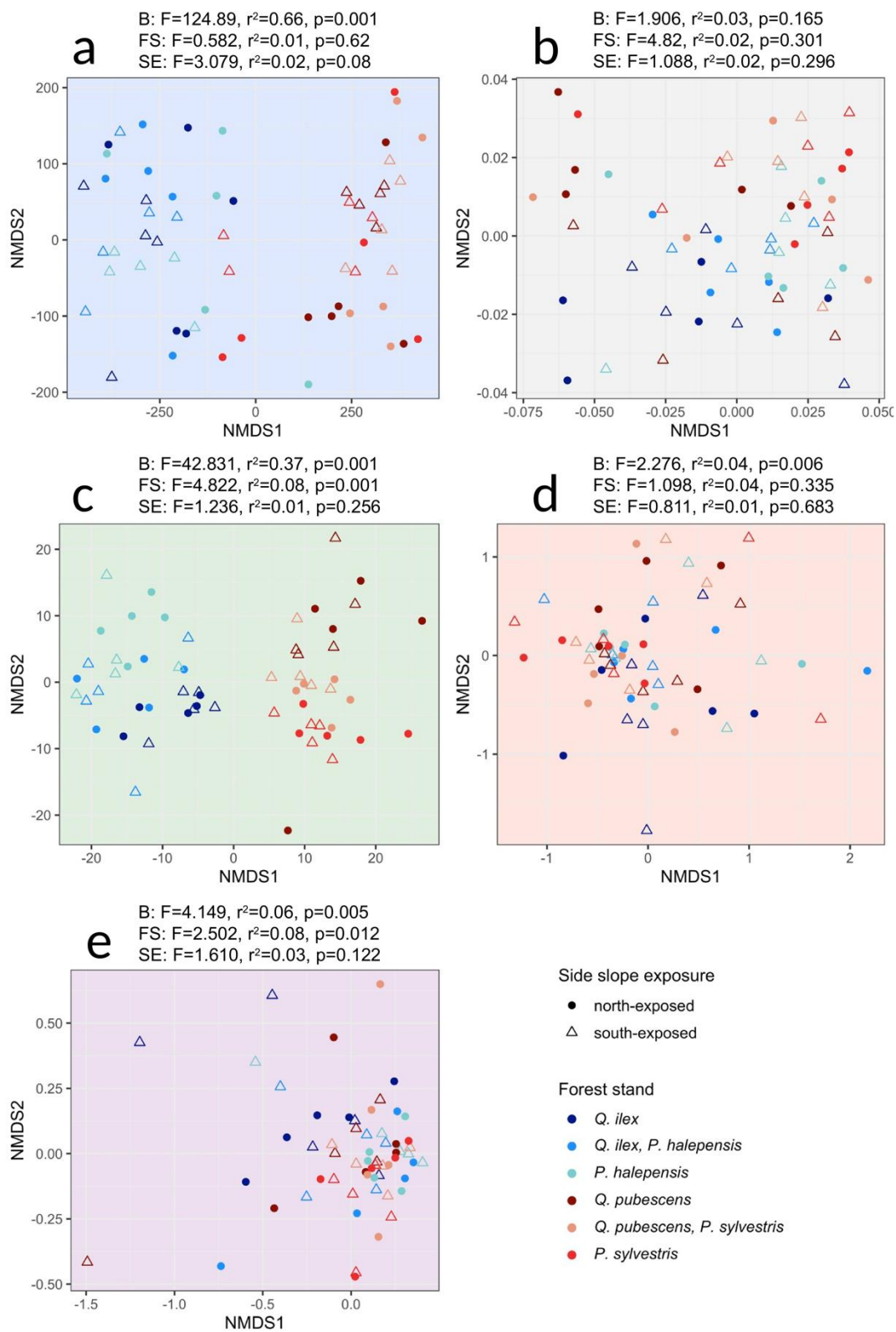
668 **Figures**
 669 **Fig. 1 |** The overview of experimental design; (a) the schematic representation of the study design. The variables assessed and matrix used to describe
 670 geoclimate, vegetation cover, soil characteristics, carbon source utilisation and catabolic potential are fully detailed 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.

673



674

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.

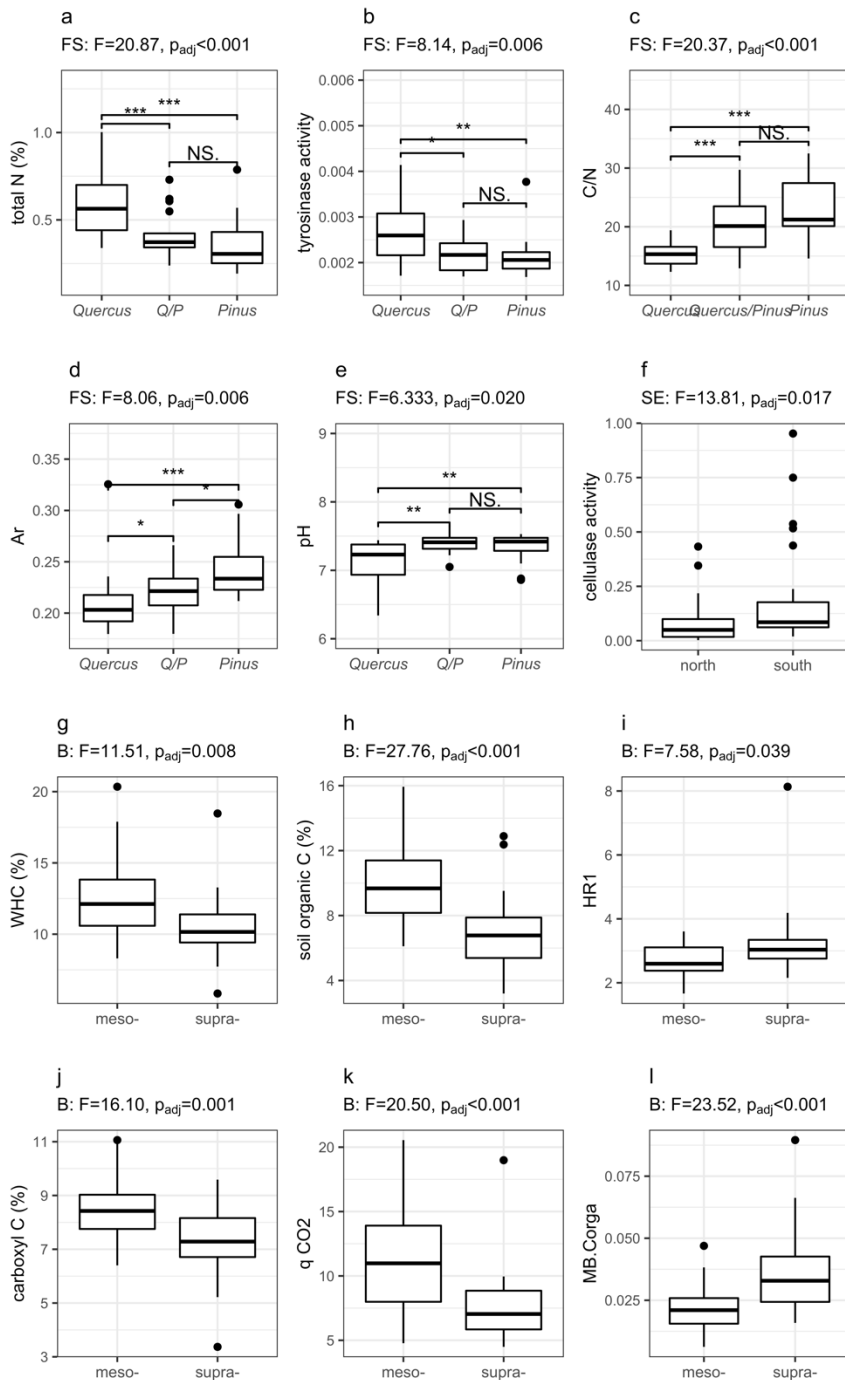


Fig. 4 | Variation partitioning of soil microbial carbon source utilisation (a, b and c) and catabolic potential (d, e and f) into soil, vegetation and geoclimate components at different spatial scales (a and d at the regional-scale ($n=60$); b and e at the sub-regional meso-Mediterranean scale ($n=30$);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

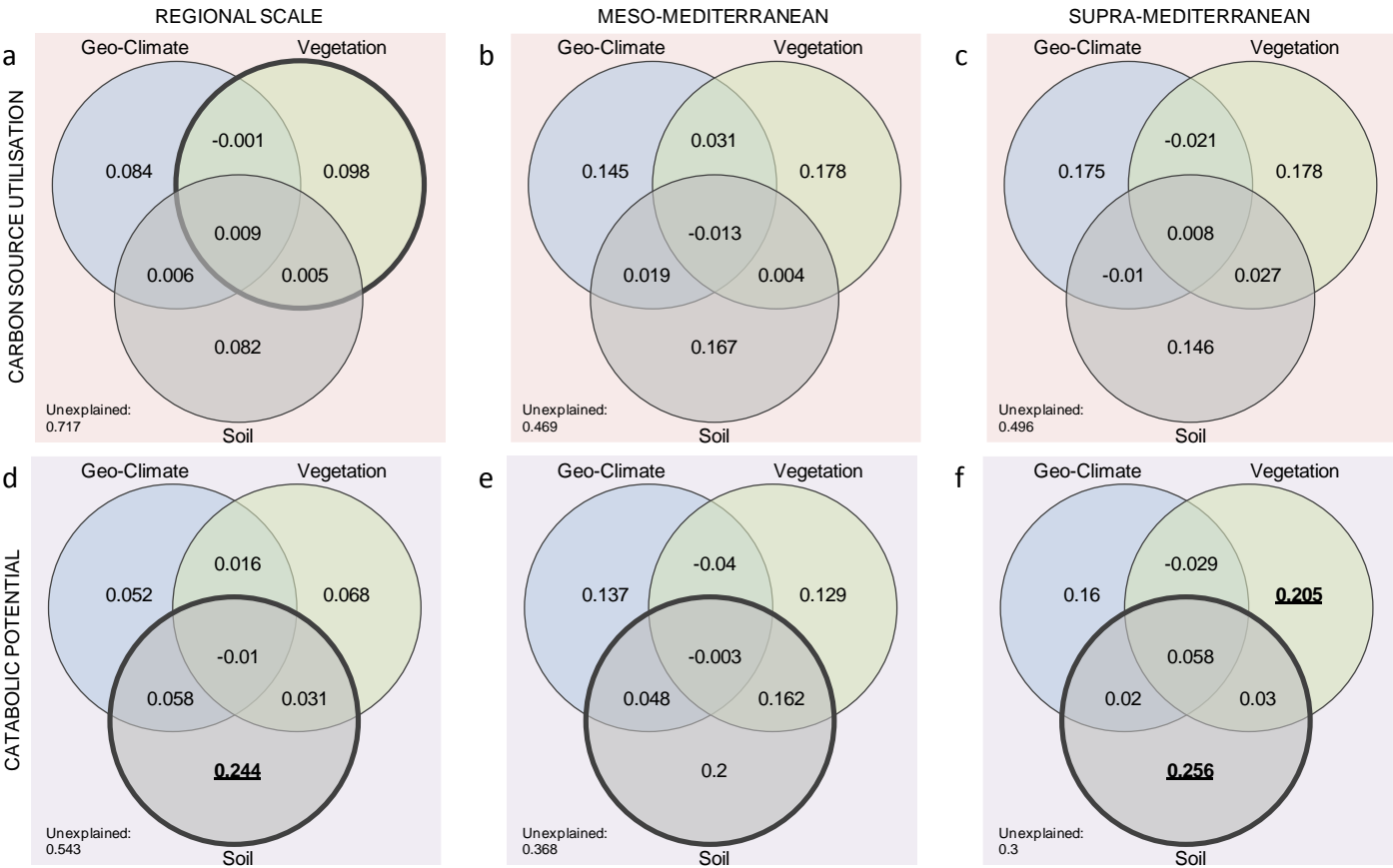
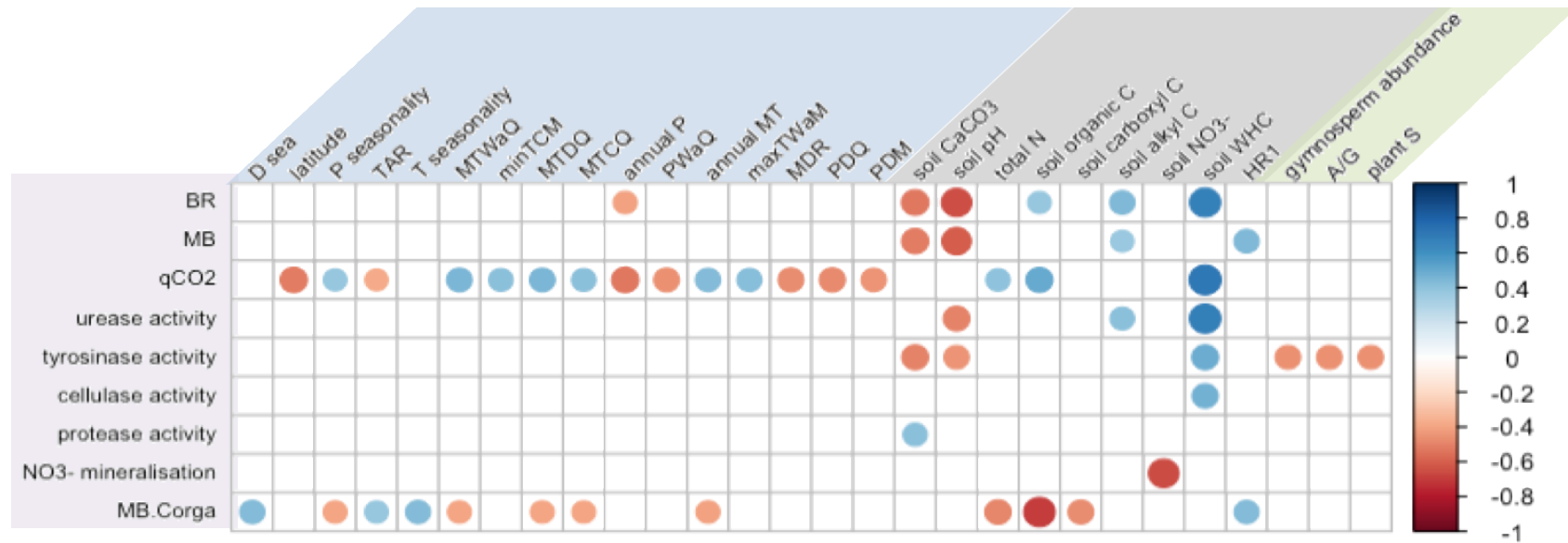


Fig. 5 | Heat map of Spearman correlation coefficient (ρ) between microbial and environmental variables. The color ramp refers to the Spearman correlation coefficient, blue tones stand for positive relations and red tones for negative relations, and the point size refers to the correlation strength ($|\rho|$). Top annotations refer to environmental variables and left annotations to microbial variables (D_{sea} : distance to the sea; P seasonality: precipitation seasonality; TAR: temperature annual range; T seasonality : temperature seasonality; MTWaQ: maximum temperature of the warmest quarter; minTCM: minimum temperature of the coldest month; MTDQ: mean temperature of the driest quarter ; MTCQ: mean temperature of the coldest quarter; annual P: annual precipitation; PWaQ: precipitation of the warmest quarter; annual MT: annual mean temperature; max TWaM: maximum temperature of the warmest month; MDR: mean diurnal range; PDQ: precipitation of the driest quarter; PDM: precipitation of the driest month; WHC: water holding capacity; HR1: humification ratio 1; A/G: angiosperm to gymnosperm ratio; BR: basal respiration; MB: microbial biomass; $q\text{CO}_2$: metabolic quotient; MB.Corga: microbial biomass per unit of organic carbon). Only variables that show significant correlations are shown, i.e., $p \leq 0.05$ after Benjamini and Hochberg (1995) post-hoc correction.





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